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Decisiones en los macrófagos: proliferar, activarse o morir

Mònica Comalada Vila

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Memoria presentada por

Mònica Comalada Vila

para optar al grado de Doctora en Biología

Director de Tesis

Dr. Antonio Celada Cotarelo

Catedrático de Inmunología

Director de Tesis

Dr. Jordi Xaus Pey

Departamento Inmunología

Puleva Biotech

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Abreviaturas

Listado de Abreviaturas

AML, leucemia mieloide aguda
AMPc, adenosina monofosfato cíclico
AP-1, *activating protein-1*
Apaf-1, *apoptotic protease activating factor 1*
APC, célula presentadora de antígeno
ASK, *apoptosis signal-regulating kinase*
ATF2, *activating transcription factor 2*
BMDM, *bone marrow derived macrophages*
CAD, *caspase-activated deoxyribonuclease*
CAK, *cell cycle-activated kinase*
CaN, calcineurina
CaM, calmodulina
CaMK, *calcium/calmodulina-dependent kinase*
CAPK, proteína-quinasa activada por ceramida
CAPP, proteína-fosfatasa activada por ceramida
CBP, *CREB binding protein*
CD200R, receptor del CD200
Cdk, quinasa dependiente de ciclo celular
Cer, ceramida
CHO, *chinese hamster ovary*
CIITA, *class II transactivator*
CIP1, *CDK-interacting protein-1*
cki, inhibidor de las cdks
c-Myc, *cellular homologue of avian myeloblastosis virus oncogene*
CRE, elemento de respuesta al AMPc
CREB, *CRE binding protein*
CsA, ciclosporina A
CS, condroitin sulfate
Curc, curcumina
Cyc, ciclina
DAG, diacilglicerol
DAPI, 4, 6-diamidino-2-phenylindole
DD, *death domain*
Dec, decorina

DED, *death effector domain*
DMEM, *Dulbecco's modified Eagle's medium*
DNA-PK, *DNA-activated protein kinase*
DP-1, miembro de la familia de factores de transcripción E2F
dsRNA, *double-stranded RNA*
E2F, familia de factores de transcripción que incluyen subunidades E2F y DP
ECM, *extracellular matrix*
ECSIT, *evolutionary conserved signaling intermediate*
EDA, *extradomain A*
EGF, factor de crecimiento epidérmico
EGFR, receptor del EGF
elF, factor de iniciación eucariótico
Elk-1, *ETS-domain protein*
ERK, *extracellular signal-regulated kinase*
FAD, *Fas-associated death domain*
FADD, *Fas-associated protein with death domain*
FAK, *focal adhesion kinase*
FBS, suero fetal bovino
FGF, factor de crecimiento para fibroblastos
FITC, fluoresceína (isotiocianato)
FKBP, *FK506-binding protein*
FN, fibronectina
GADD45, *growth arrest and DNA damage protein 45*
GAF, *gamma-interferon activated factor*
GAG, glicosaminoglicanos
GAS, *gamma-interferon activated sequence*
GF, GF109203X, inhibidor de PKC
GM-CFU, unidad formadora de colonias granulocito/macrófago
GM-CSF, factor estimulador de colonias granulocito/macrófago
GMPc, guanina monofosfato cíclico
GPI, glicofosfatidilinositol
GSK, *glycogen synthase kinase*
HA, ácido hialurónico
HS, sulfato de heparán
HSP, *heat shock protein*

IAP, inhibitor of apoptosis

IFN-α/β, interferón-alfa/beta

IFN-γ, interferón-gamma

IFN-γR, receptor del interferón-gamma

IGF-1, *insuline growth factor-1*

IκB, inhibidor de NF-κB

IKK, *I-kappa-b kinase*

IL, interleucina

IL-1R, receptor de la IL-1

iNOS, sintasa de óxido nítrico inducible

IP_3, inositol trifosfato

ISRE, elemento de respuesta a la estimulación con IFN

IRF-1, factor regulado por interferón-1

JAK, *Janus kinase*

JM, región juxta-membrana

JNK, *c-Jun N-terminal kinase*

KI, *kinase insert*

KO, *knock-out*

LBP, *LPS-binding protein*

LFA-1, *lymphocite function-assossiated antigen-1*

LPS, lipopolisacárido

LRR, *leucine rich repeat*

MAP, proteína asociada a microtúbulos

MAPK, *mitogen-activated protein kinase*

MAPKAPK2, *MAPK-activated protein kinase 2*

MAPKK, *MAPK kinase*

MAPKKK, *MAPKK kinase*

MBP, proteína básica de la mielina

M-CFU, unidad formadora de colonias de macrófagos

MCMT, *DNA-(cystosine-5) methyltransferase*

M-CSF, factor estimulador de colonias de macrófagos

M-CSFR, receptor del M-CSF

MDA-6, *melanoma differentiation-associated protein*

MDM2, *murine double minute 2, a p53-associated oncogene*

MDR, *multridrug resistance protein*

MEK, *MAPK/ERK Kinase*

MEKK, *MAPK/ERK kinase kinase*

MHC, complejo principal de histocompatibilidad

MIP-1α, *macrophage inflamatory protein-1 alpha*

MKK, *MAPK kinase*

MKP-1, *MAPK phosphatase-1*

MLK, *mixed-lineage protein kinase*

MNK, *MAP kinase-interacting kinase*

MSK, *mitogen- and stress- activated protein kinase*

MyOD, *miogenic determination factor*

NF-AT, factor nuclear activador de linfocitos T

NF-κB, factor nuclear kappa B

NGF, factor de crecimiento neuronal

NGFR, receptor del NGF

NK, *natural killer*

NLS, *nuclear localization signal*

NO, óxido nítrico

p300, *histone acetyltransferase*

p53, proteína supresora de tumores

PAF, factor activador de plaquetas

PAK-2, *p21-activated kinase-2*

PCNA, *DNA polymerase c processivity factor*

PC, fosfatidilcolina

PC-PLC, fosfolipasa C especifica de fosfatidilcolina

PD, PD98059, inhibidor de MEK

PDGF, factor de crecimiento derivado de plaquetas

PDK, quinasas dependientes de fosfatidilinositoles

PGE_2, prostaglandina E_2

PH, dominio de homología a plecstrina

PHM, *Poly(2-Hidroxyethil Methacrylate)*

PI3-K, fosfatidilinositol 3-quinasa

PIP_2, fosfatidilinositol bifosfato

PI_3P, fosfatidilinositol-3-fosfato

PIP3, fosfatidilinositol, 3,4,5-trifosfato

PI-PLC, fosfolipasa C específica por fosfatidilinositol

PKA, proteína-quinasa A

PKC, proteína-quinasa C

PKG, proteína-quinasa G

PKR, *double-stranded RNA (dsRNA)- activated protein kinase*

PLA_2, fosfolipasa A_2

PLC, fosfolipasa C

PLCγ2, fosfolipasa C gamma 2

PPIASE, *peptidil-prolyl cis-trans isomerasa*

PRAK, *p38-regulated/activated kinase*

PtdIns, fosfatidilinositol

PTEN, *phosphatase and tensin homologue deleted on chromomosome 10*

PS, fosfatidilserina

PtdSer, fosfatidilserina

PTP1C, proteína tirosina-fosfatasa 1C

RAIDD, *RIP-associated ICH/CED-3-homologous protein with a death domain*

rasGAP, *ras GTPase activating protein*

pRb, retinoblastoma

RK, *reactivating kinase*

RSK, *ribosomal S6 kinase*

RSV, *respiratory syncitial virus*

RTK, receptores de tirosina quinasa

SAPK, *stress-activated protein kinase*

SB, SB203580, inhibidor de p38

SCF, *Stem Cell Factor*

SDI1, *senescent cell-derived inhibitor*

SEK, *SAPK/ERK kinase*

SGK, *serum/glucocorticoid-regulated kinase*

SH2, región de homología a Src de tipo 2

SLRP, *small leucine rich repeats*

SMAD, c*ontraction of Sma and Mad* (*Mothers against decapentaplegic*)

Smasa, esfingomielinasa

SMT, S-m*ethylisothiourea sulfate*

SNAP, (+*)-S-Nitroso-N-acetylpenicillamine*

Sos, intercanviador de nucleósidos de guanina

SRC, *receptor steroids coactivator*

SRE, elemento de respuesta al suero

SRF, factor de respuesta al suero

STAT, *signal transducer and activator of transcription*

TAK, *TGF*-β-*activated kinase*

TCA, *trichloroacetic acid*

TCF, *ternary complex factor*

TCR, receptor de linfocitos T

TGF-β, factor de crecimiento transformante beta

TIR, *Toll/IL-R homology domain*

TK, tirosina-quinasa

TLR, *Toll-like receptor*

TNF-α, factor de necrosis tumoral alfa

TNF-αR, receptor del factor de necrosis tumoral alfa

TPA, 12-O-tetradecanoilforbol 13-acetato

Tpl-2, *tumor progression locus 2*

TRADD, *TNF receptor-associated death domain*

TRAF, *TNF-receptor-activated factor*

TRE, elemento de respuesta al TPA

WAF1, *wild-type p53-activated factor*

Inhibidores

Relación de productos utilizados para la activación/inhibición de proteínas implicadas en la transducción de las señales

PRODUCTO	PROPIEDADE	CASA COMERCIAL
BAPTA/AM	Agente quelante de calcio	Calbiochem, San Diego, CA
Ciclosporina A (CsA)	Inhibidor específico de la Calcineurina	Sandoz (Basel, Suiza)
Curcumin	Inhibidor de la actividad JNK	Calbiochem, San Diego, CA
FK506 (Tacrolimus)	Inhibidor de la Calcineurina	Calbiochem, San Diego, CA
Go6976	Inhibidor específico de las isoformas de PKC convencionales α y βI	Calbiochem, San Diego, CA
GF109203X (GF)	Inhibidor de PKC convencionales (α, βI, βII y γ) y nuevas (δ y ε)	Calbiochem, San Diego, CA
PD98059 (PD)	Inhibidor selectivo de MEK-1/2	Calbiochem, San Diego, CA New England Biolabs Inc. Beberly, MA
Rapamicina	Inhibidor de la activación de la $p70^{S6}$ quinasa	ICN Pharm. Inc., Costa Mesa, CA
SB203580	Inhibidor específico de p38 MAPK	Calbiochem, San Diego, CA
SMT	Inhibidor de la produccion de NO	Calbiochem, San Diego, CA
SNAP	Donador de Óxido Nítrico (NO)	Calbiochem, San Diego, CA
W-13	Antagonista de la Calmodulina	Calbiochem, San Diego, CA
Wortmannina	Inhibidor de PI-3K	Sigma Chem. Co., St. Louis, MO
Verapamil	Inhibidor de las MDR	Calbiochem, San Diego, CA

Introducción

1. LOS MACRÓFAGOS

Los macrófagos forman parte del sistema fagocítico mononuclear, el cual engloba a un conjunto de células (monoblastos, promonocitos, monocitos y macrófagos) derivadas de un precursor común y una de cuyas funciones principales es la fagocitosis. Junto con los neutrófilos, el sistema fagocítico mononuclear constituye el principal mecanismo de defensa del organismo frente agentes extraños como los microorganismos (Abbas y col., 1991). A diferencia de otras células del sistema inmunológico, tales como los linfocitos B y T, los cuales en condiciones basales se encuentran en quiescencia o reposo y cuya activación siempre conlleva un proceso de expansión clonal caracterizado por una elevada tasa proliferativa, los macrófagos, o bien se encuentran proliferando, o bien se activan, dejan de proliferar y pasan a ejercer sus funciones específicas. En ausencia de estímulos que los induzca a proliferar o a activarse, los macrófagos sólo pueden permanecer en estado de reposo durante un corto periodo de tiempo y posteriormente mueren por procesos de apoptosis (Celada, 1994).

1.1. Origen de los macrófagos

Todas las células del sistema inmunitario tienen un origen común en la médula ósea. Allí se hallan las células madre pluripotentes (*stem cells*) con capacidad autoregeneradora y con el potencial de diferenciarse hacia los distintos tipos celulares de la sangre. En respuesta a factores de crecimiento y citocinas como las interleucinas IL-1, IL-3 e IL-6, la célula madre pluripotente experimenta una división celular desigual o heteromitosis dando lugar a una nueva célula madre y a una célula pluripotencial mieloide, GEMM-CFU (*granulocyte/ erythrocyte/ megakaryocyte/ macrophage- colony forming unit*). En presencia de IL-1 e IL-3, este precursor genérico experimenta un proceso de diferenciación denominado determinación (*commitment*) y pasa a convertirse en un progenitor común a las líneas monocítica y granulocítica, GM-CFU (*granulocyte/ macrophage colony forming unit*)

(Fig. 1). En este punto, la interleucina IL-3 y el factor de crecimiento GM-CSF (*granulocyte/ macrophage- colony stimulating factor*) inducen la proliferación de los GM-CFUs. Sin embargo, el factor de crecimiento M-CSF (*macrophage-colony stimulating factor*) no sólo induce la proliferación de estas células, sino también su diferenciación hacia precursores propiamente monocíticos denominados M-CFUs (*macrophage- colony forming units*). La diferenciación terminal de los M-CFUs, proceso conocido como maduración, y la consiguiente generación de monocitos, también requiere la presencia de M-CSF (Celada, 1994; Valledor y col., 1998).

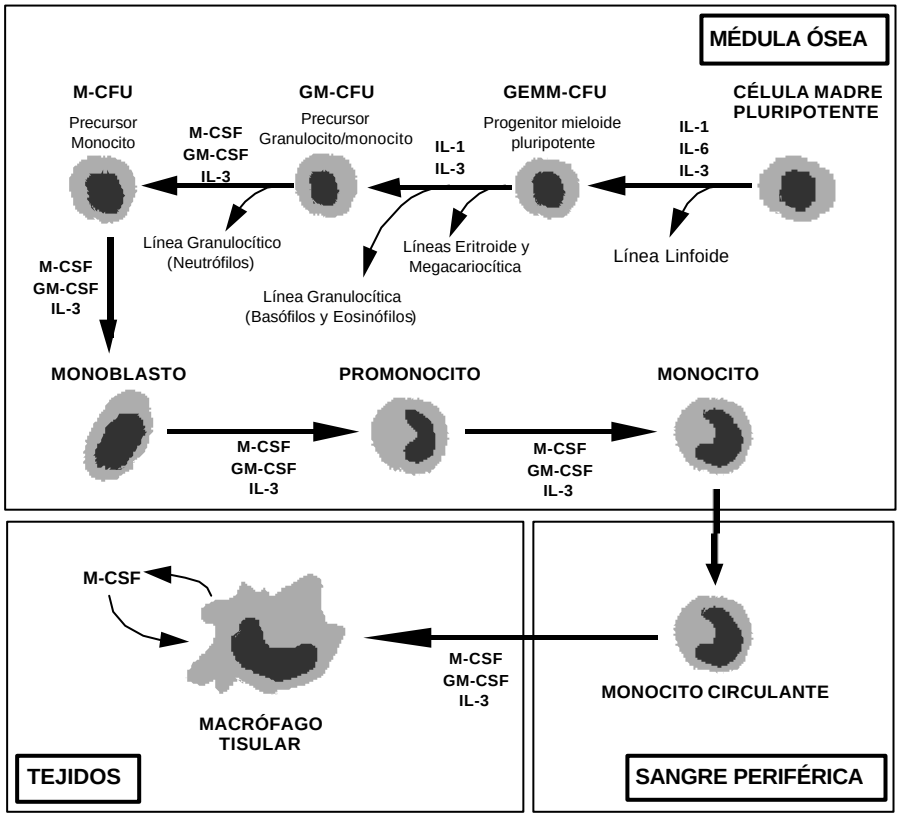


Figura 1. Proceso de diferenciación del macrófago. IL, interleucinas; M-CSF, factor estimulador de colonias de macrófagos; GM-CSF, factor estimulador de colonias de granulocitos y macrófagos; GEMM-CFU, unidad formadora de colonias de granulocitos, eritrocitos, megacariocitos y macrófagos; GM-CFU, unidad formadora de colonias de granulocitos y macrófagos; M-CFU, unidad formadora de colonias de macrófagos (Valledor, 1998)

El monocito es más pequeño que su progenitor inmediato, el promonocito, pero tiene una capacidad fagocítica y un sistema lisosomal más desarrollados. Normalmente, el monocito pierde su capacidad proliferativa, aunque en ciertas condiciones, puede llegar a dividirse. Así, la generación basal de monocitos en la médula ósea es un proceso regulado por el balance entre los factores de crecimiento (M-CSF, GM-CSF e IL-3), producidos por las propias células durante su diferenciación y por las células del estroma medular; así como por una serie de agentes inhibidores, tales como la prostaglandina E_2, los interferones α y β y la lactoferrina (Celada, 1994; Valledor y col., 1998). El monocito es capaz de dejar la médula ósea, pasar al torrente circulatorio y, desde allí, a los tejidos, gracias a la interacción entre moléculas de adhesión (adhesinas e integrinas) existentes en la superficie de los monocitos y de las células del endotelio vascular. Además, este proceso puede estar regulado por las necesidades del organismo (inflamación, isquemia, necrosis, apoptosis, etc) produciendo una disminución del flujo sanguíneo y, por tanto, asegurando que los monocitos y las células del endotelio interaccionen más fácilmente, favoreciendo así la extravasación de los monocitos hacia los tejidos.

Una vez en los tejidos, el monocito culmina su maduración y se convierte en un macrófago. Este proceso conlleva un aumento del tamaño celular, un mayor desarrollo del sistema lisosomal y del contenido en enzimas hidrolíticos y un incremento del número y tamaño de las mitocondrias, así como de su metabolismo energético. La vida media de los macrófagos en los tejidos puede ser muy larga (meses-años), sin embargo, muchos de los macrófagos que llegan a los tejidos acabarán desapareciendo por procesos de muerte celular. En los tejidos, un pequeño numero de macrófagos se diferenciarán bajo la influencia de citocinas y, dependiendo del tipo de tejido, se convertirán en células de la microglía en el sistema nervioso central, en osteoclastos del hueso, en histiocitos del tejido conectivo, en células de Kupffer del hígado, en células de clase A del tejido sinovial, en células de Langerhans de la dermis o en células dendríticas. Todas estas células pertenecen a la línea macrofágica. Estos macrófagos tisulares son capaces de proliferar en dichos tejidos en condiciones basales (no activados) gracias a la presencia o a la producción autocrina de M-CSF, así como a la presencia de citocinas y de otros factores de crecimiento no específicos como el GM-CSF y la IL-3 (Celada y Maki, 1992) (Fig. 2).

Cuando estas células son estimuladas por mediadores producidos en los focos de inflamación, dejan de proliferar y pasan a activarse, aumentando así la capacidad para desarrollar sus funciones especializadas (ver apartado 1.2). En condiciones fisiológicas, el IFN-γ secretado por los linfocitos T_H1 activados, es el principal agente activador de los macrófagos. Sin embargo, en condiciones patológicas tales como una infección bacteriana por ejemplo, el lipopolisacárido (LPS), principal constituyente de la pared de las bacterias Gram-negativas, también puede activar muchas de las funciones de los macrófagos. Por el contrario, en ausencia de estímulos (cuando en los tejidos no hay ni IFN-γ ni M-CSF), los macrófagos morirán por apoptosis (Fig.2). De esta forma se establece un balance entre la producción de precursores celulares en la médula ósea y la eliminación de la mayoría a nivel tisular (Xaus y col., 2001a).

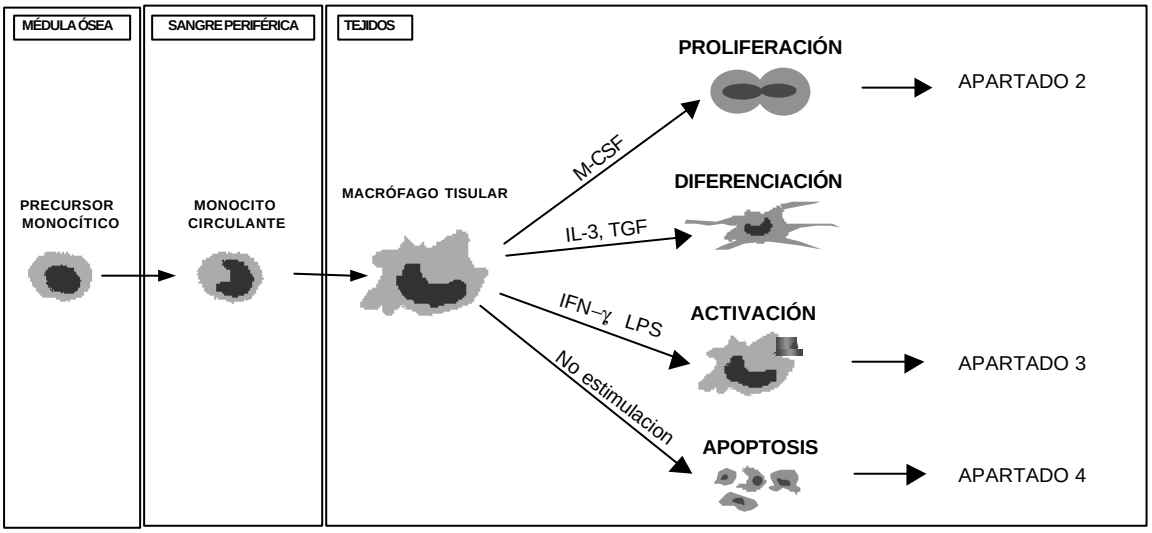


Figura 2. La estimulación de los macrófagos a nivel tisular determina su actividad. Los precursores de la línea macrofágica se diferencian hacia monocitos en el seno de la médula ósea. Los monocitos abandonan la médula ósea y, a través del torrente circulatorio, acceden a los distintos tejidos del organismo, donde se diferencian hacia macrófagos tisulares. En función de los estímulos a los que se ven expuestos, los macrófagos adquieren diferentes actividades: proliferación, diferenciación hacia formas macrofágicas especializadas de determinados tejidos, activación y realización de sus funciones características en el seno de la respuesta inmunológica, o bien muerte por apoptosis. IFN-γ, interferón-gamma; IL-3, interleucina-3; M-CSF, factor estimulador de colonias macrofágicas; TGF, factor de crecimiento transformante.

1.2. Función de los macrófagos

Una vez en los tejidos requeridos, los macrófagos juegan un papel crítico en el desarrollo de la respuesta inmunológica. Estas células actúan como mediadoras de innumerables mecanismos diferentes dentro de la respuesta inmunológica, no sólo participan en la inmunidad natural, sino que además, están adaptados para desempeñar funciones esenciales en la inmunidad específica adquirida, tanto humoral como mediada por células. De forma basal, los macrófagos se encuentran en disposición de realizar una serie de funciones sin necesidad de ningún tipo de estímulo, aunque muchas de éstas pueden realizarse con mayor eficiencia tras la activación de la célula. Sin embargo, para otras funciones se requiere que la célula haya sido previamente activada para poder llevarlas a cabo. Los macrófagos realizan algunas de sus funciones directamente, a través de interacciones célula-célula, o indirectamente, mediante la liberación de sustancias capaces de afectar a otras células. A este nivel, los macrófagos disponen de un repertorio muy extenso de factores autocrinos, paracrinos y endocrinos que engloban desde agentes mitogénicos hasta agentes quimiotácticos o inductores de apoptosis (Tabla I) (Nathan, 1987).

En el contexto de la inmunidad natural, los macrófagos fagocitan y eliminan partículas extrañas, tales como bacterias, virus, parásitos, macromoléculas, e incluso células propias dañadas o muertas, como es el caso de los eritrocitos viejos y de las células apoptóticas, impidiendo así que estas puedan verter su contenido potencialmente tóxico al medio extracelular y evitando, por tanto, el posible desencadenamiento de procesos inflamatorios.

Además, los macrófagos pueden matar células tumorales mediante la secreción de ciertos enzimas (por ejemplo, perforina y granzima), citocinas (TNF-α) e intermediarios reactivos del nitrógeno y del oxígeno (Tabla I), así como a través de fenómenos de interacción célula-célula. El mecanismo de citotoxicidad de estos efectores consiste en la inducción de apoptosis de las células diana (Aliprantis y col., 1996) (ver apartado 4.4.1. de la Introducción). Una vez estas células se convierten en

cuerpos apoptóticos, el macrófago puede fagocitarlos y degradarlos eficazmente. Del mismo modo, los macrófagos pueden provocar la muerte extracelular de microorganismos y controlar la dispersión de una infección mediante la secreción de enzimas (por ejemplo, lisozima), metabolitos del oxígeno y del nitrógeno e intermediarios del metabolismo lipídico (por ejemplo, prostaglandinas) (Tabla I).

TABLA I. Productos secretados por los macrófagos (Nathan, 1987)

TIPO	PRODUCTO
Hormonas polipeptídicas	IL-1, IL-6, TNFα (Factor de necrosis tumoral α), IFNs α/β (Interferones α/β), FGF (Factor de crecimiento de fibroblastos), PDGF (Factor de crecimiento derivado de plaquetas), TGF (Factor de crecimiento transformante), GM-CSF, M-CSF, Factor activador de neutrófilos, Hormonas adenocorticotrópicas, Eritropoyetina
Hormonas esteroideas	25-dihidroxivitamina
Componentes del sistema del complemento	C1, C2, C3, C3a, C3b, C4, C5, C5a, Factor B, Factor D, etc.
Factores de coagulación	V, VII, IX, X, Protrombina, Factor tisular, Protrombinasa, Activador del plasminógeno, Inhibidor del activador del plasminógeno, Inhibidores de la plasmina
Enzimas hidrolíticos	Hidrolasas, ácidas lisosómicas, Proteasas, Lipasas, Lisozima, Colagenasa, Elastasa, Perforina, Granzima, etc.
Inhibidores de enzimas y citocinas	Inhibidores de proteasas, Lipomodulina, Inhibidor de IL-1
Proteínas de la matriz extracelular	Fibronectina, Decorina, Proteoglicanos tipo condriotín sulfato, etc.
Proteínas ligadoras	Transferrina, Apolipoproteína E, Avidina, etc.
Oligopéptidos con función biológica	Glutatión
Intermediarios del metabolismo lipídico	Prostaglandinas, Tromboxano, Leucotrienos, PAF (Factor activador de plaquetas), etc.
Intermediarios del metabolismo de ácidos nucleicos	Timidina, Uracilo, Ácido úrico
Intermediarios reactivos del oxígeno	Superóxido, Peróxido de hidrógeno, Radicales hidroxilo
Intermediarios reactivos del nitrógeno	Óxido nítrico (NO), Nitratos, Nitritos

Algunas de las substancias secretadas por los macrófagos, en especial citocinas como el TNF-α, IL-1-β e IL-6, promueven el reclutamiento de otras células inflamatorias, principalmente neutrófilos en los puntos de inflamación y son responsables de la mayor parte de los efectos sistémicos de la inflamación (como por ejemplo, la fiebre). Finalmente, los macrófagos también producen factores de crecimiento para fibroblastos (FGF, TGF-β) y para el endotelio vascular, de manera que se favorece el riego sanguíneo y la reparación de tejidos dañados. No obstante, estos factores de crecimiento también han sido implicados en la vascularización de los tejidos sólidos y de los tumores.

Durante la respuesta inmunológica adquirida mediada por células, los macrófagos funcionan como células presentadoras de antígeno (APC, *antigen-presenting cell*), es decir, procesan antígenos extraños y los presentan en su superficie para que puedan ser reconocidos por los linfocitos T. Además, producen una serie de proteínas que, una vez secretadas o expresadas en la membrana celular, promueven la activación de los linfocitos T. Recíprocamente, ciertas citocinas secretadas por los linfocitos T activados, principalmente el Interferón gamma (IFN-γ), activan a su vez a los macrófagos, aumentando la eficiencia de éstos en las funciones de presentación de antígeno, fagocitosis y lisis celular (ver apartado 3.2 de la Introducción).

Finalmente, los macrófagos también participan en la eliminación de antígenos extraños durante la respuesta inmunológica adquirida humoral. Por un lado, estas células liberan proteínas del complemento que, junto con los anticuerpos, recubren la superficie de un gran número de antígenos, proceso conocido como opsonización. Dado que los macrófagos expresan en su superficie receptores específicos tanto para la porción constante de las inmunoglobulinas como para las proteínas del complemento, el proceso de reconocimiento y fagocitosis de antígenos extraños opsonizados por parte de estas células se ve ampliamente potenciado (Abbas y col., 1991; Celada, 1994).

2. PROLIFERACIÓN DE LOS MACRÓFAGOS

Los procesos de proliferación celular son el conjunto de fenómenos que conducen a una célula a dividirse en dos, manteniendo cada una de las células hijas toda la información contenida en su predecesora. Los procesos de división celular están finamente regulados tanto por factores intrínsecos (ciclo celular) como extrínsecos (factores de crecimiento o señales mitogénicas). En el caso de los monocitos, macrófagos y sus progenitores inmediatos, los procesos de proliferación, diferenciación y supervivencia están regulados por el factor de crecimiento específico de macrófagos, el M-CSF. Este factor inicia una respuesta mitogénica mediante la interacción con su receptor específico induciendo la actividad tirosina quinasa intrínseca del receptor y, en consecuencia, el inicio de múltiples vías de transmisión de la señal, las cuales conducirán a la expresión de genes inmediatos (como *c-fos*, *c-jun* y *c-myc*) y genes de expresión tardía (como ciclina D) necesarios para la correcta regulación y entrada de la célula en el ciclo celular (Roussel, 1997)

2.1. Factor estimulador de colonias de macrófagos (*Macrophage-colony stimulating factor*, M-CSF)

2.1.1. Características bioquímicas y funcionales del M-CSF

El M-CSF, también conocido como CSF-1, es producido principalmente por células del sistema fagocítico mononuclear, células endoteliales y fibroblastos. El RNA mensajero (mRNA) del M-CSF puede experimentar dos formas de procesamiento (*splicing*) alternativos, dando lugar a dos tipos de precursores del factor de crecimiento con dos destinos independientes. Una serie de procesos de glicosilación posteriores permiten la carga de diversos azúcares, así como de una molécula de condriotín sulfato en ambos precursores. Uno de los precursores experimenta un procesamiento proteolítico asociado a su incorporación dentro de vesículas secretoras. Dependiendo del tipo de proteolisis, el M-CSF puede dar como resultado

una glicoproteína o bien un proteoglicano, presentando una molécula de condriotín sulfato asociada (ver apartado 5 de la Introducción). Ambas formas son liberadas al exterior como homodímeros y pasan a tener un efecto autocrino y/o paracrino. La forma proteoglicano, puede ser retenida en la matriz extracelular del tejido y actuar como reservorio de M-CSF. El otro precursor, más pequeño de tamaño, no es dirigido por proteolisis a las vesículas secretoras, por lo que permanece anclado a la membrana y pasa a ser expresado en forma de glicoproteína en la superficie de las células productoras. Esta tercera forma del factor de crecimiento puede tener un papel importante en interacciones célula-célula o bien puede ser liberado lentamente a nivel local por proteolisis del M-CSF de las células que lo sintetizan (Stanley y col.,1997).

Pese a que el M-CSF se encuentra en concentraciones biológicamente activas en la circulación, la mayor parte de los efectos de este factor de crecimiento se producen a nivel local. La falta de este factor de crecimiento provoca la muerte de los macrófagos por apoptosis. El papel clave del M-CSF en el desarrollo monocítico se ha mostrado en los ratones *op/op* que carecen de M-CSF debido a una mutación en el gen que codifica para este factor (Wiktor-Jedrzejczak y col., 1990; Yoshida y col., 1990). Estos ratones desarrollan osteopetrosis debido a la ausencia de osteoclastos, miembros del linaje macrofágico. Además, presentan una severa deficiencia de fagocitos mononucleares en la mayoría de los tejidos. El estudio de estos ratones ha permitido el descubrimiento de ciertas poblaciones de macrófagos independientes de M-CSF, como por ejemplo, las células de Langerhans en la piel y los macrófagos de los tejidos linfoides (Cecchini y col., 1994).

Cabe destacar que fuera del sistema hematopoiético, el M-CSF también juega un papel en el desarrollo de la placenta, glándulas mamarias y el cerebro (Michaelson y col., 1996; Pollard y Hennighausen, 1994; Sapi y Kacinski, 1999). Finalmente ha sido descrito el papel potencial del M-CSF y su receptor específico en diferentes leucemias (Dubreuil y col., 1988) y en carcinomas de ovario, endometrio y mama (Kacinski, 1997; Scholl y col., 1994).

2.1.2. Receptor del M-CSF (M-CSFR)

Todos los efectos biológicos producidos por el M-CSF son mediados por un único receptor (M-CSFR), que corresponde al producto del protooncogén *c-fms* cuya sobrexpresión es responsable de algunos tipos de leucemia mieloide aguda (AML).

El M-CSFR es una glicoproteína transmembranaria que pertenece a la familia de receptores tirosina quinasa (RTK) de tipo III, la cual incluye los receptores α y β del factor de crecimiento derivado de plaquetas (PDGF), el receptor del factor de las células madre (*stem cell factor*, SCF), codificado por el protooncogén *c-kit*, y el receptor Flt3/FLK2. Todos estos receptores presentan una estructura en común a la del receptor del M-CSF. La secuencia extracelular del M-CSFR murino codifica para cinco dominios emparentados con los dominios de las inmunoglobulinas. Los tres primeros dominios tienen alta afinidad por el M-CSF (Wang y col., 1993), mientras que los dos dominios cercanos a la membrana regulan el cambio de conformación tras la interacción con el ligando y/o interacciones con otras proteínas de la superficie celular (Rohrschenider y Woolford, 1991).

La unión del M-CSF induce la dimerización del receptor de forma no covalente, seguida de la estimulación de la actividad tirosina-quinasa situada en la región citoplasmática del mismo. Dicha actividad es responsable de la autofosforilación del receptor en diferentes residuos tirosina y la consiguiente transmisión de la señal hacia el interior de la célula, así como de la posterior internalización y degradación del receptor y su ligando en el interior de lisosomas (Hamilton, 1997a).

La región tirosina-quinasa citoplasmática puede subdividirse desde un punto de vista estructura/función en cinco subdominios que comprenden: (1) una región cercana a la membrana; (2) el sitio de unión de ATP en el dominio quinasa; (3) una región de aminoácidos hidrofílicos insertados, designada como KI (*kinase insert*); (4) la propia región catalítica principal del dominio tirosina-quinasa; y (5) una región C-terminal (*C-tail*) (Fig. 3).

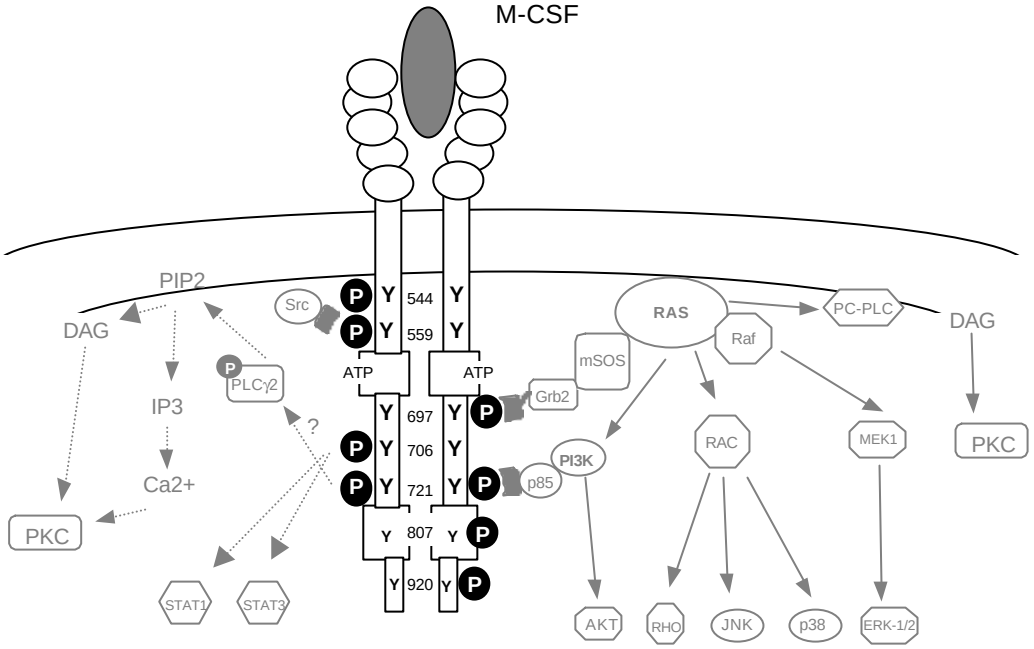


Figura 3. El receptor y las vías de transmisión de la señal del M-CSF. DAG, diacilglicerol; ERK, *extracellular-regulated kinase*; IP3, inositol trifosfato; JNK, *c-jun* N-terminal quinasa; M-CSF, factor estimulador de colonias macrofágicas; P; punto de fosforilación; PC-PLC, fosfolipasa C específica de fosfatidilcolina; PIP2, fosfatidil inositol bifosfato; PI-3K, fosfatidil inositol–3 quinasa; PKC, proteína quinasa C; Src, proteína quinasa de la familia Src; STAT, *signal transducer and activator of transcription*; Y, residuo de tirosina.

De los veinte residuos de tirosina citoplasmáticos presentes en el receptor, sólo se han descrito siete que sean fosforilados y, por tanto, que puedan mediar la señalización a través de la interacción con proteínas citoplasmáticas. En la región cercana a la membrana, la tirosina presente en el motivo Y(559)TFI es fosforilada por la activación del receptor y se asocia con miembros de la familia Src a través de su dominio de unión SH2 (Alonso y col., 1995). En la misma región, la tirosina presente en el motivo Y(544)QVR es fosforilada en la forma vírica (*v-fms*) del M-CSFR e interactúa con una proteína de 55 KDa no identificada (Joos y col., 1996). No se ha descrito ninguna tirosina fosforilada en el sitio de unión del ATP en el dominio quinasa (Fig. 3).

En la región KI están localizados tres lugares de fosforilación en tirosina, Y697, Y706 y Y721. El motivo Y(697)KNI interacciona con el dominio SH2 de la molécula adaptadora Grb2, la cual se encuentra constitutivamente asociada al intercambiador de nucleótidos mSOS. La translocación del complejo Grb2-mSOS es suficiente para la activación de Ras (Lioubin y col.,1996; van der Geer y Hunter, 1993) y la Y(721)VEM interacciona con el dominio SH2 de la proteína adaptadora p85 la cual, posteriormente, se une formando un complejo proteico con la p110 de la fosfatidilinositol-3 quinasa (PI-3K) (Hamilton, 1997a; Reedijk y col., 1992; Roussel, 1997; van der Geer y Hunter, 1993). Por último, aún no se ha detectado la proteína que interacciona con el motivo Y(706)VRR fosforilado del dominio KI del M-CSFR. Recientemente se ha relacionado esta fosforilación con la activación de STAT1 y STAT3 pero no se ha podido demostrar la interacción directa de estas proteínas con M-CSFR (Novack y col., 1995, 1996)

En la región catalítica principal la tirosina Y807 es fosforilada, aunque no se ha descrito todavía ninguna interacción con proteínas adaptadoras, se cree que la fosforilación de esta tirosina puede modular el cambio conformacional del M-CSFR tras el reconocimiento del ligando y, por tanto, controlar la interacción diferencial de las proteínas de señalización (ver apartado 2.1.3. de la Introducción) (Tapley y col., 1990). Finalmente, la región C-terminal del receptor, presenta una única tirosina fosforilada, Y(920), que es utilizada como un sitio de unión a Grb2. Se cree que a esta región se pueden unir otras proteínas y regular así la atenuación de la respuesta del M-CSF (Mancini y col., 1997).

2.1.3. Transducción de la señal del M-CSF

En definitiva, la fosforilación de estas tirosinas conducirá a la activación de distintas vías de señalización. Muchas de estas vías tendrán un denominador común que corresponde a la activación de una pequeña proteína G codificada por el proto-oncogén *ras*, la cual juega un papel crítico en el control del crecimiento celular como componente central de la respuesta mitogénica (White y col., 1995). La activación de

Ras inicia una compleja red de señales de transducción, activando a diferentes miembros de la cascada de las MAPK quinasas: la vía de Raf/MAPK (ERK), involucrada en la señalización de procesos de proliferación (Lavoie y col., 1996; Seger and Krebs, 1995,); y las vías de Rac/JNK y Rac/p38, las dos mediadoras de la respuesta al estrés celular, inhibición de la proliferación y apoptosis (Coso y col., 1995; Minden y col., 1995; Xia y col., 1995) (Fig. 3). También se ha descrito que Ras participa en la activación de la vía de PI-3K/Akt, relacionada con señales de supervivencia (Kauffmann-Zeh y col., 1997), la vía de Rac/Rho, involucrada en el remodelamiento del citoesqueleto (Lamarche y col., 1996), y en la activación de la fosfolipasa C específica de fosfatidilcolina (PC-PLC) (Xu y col., 1993). PC-PLC hidroliza, a fosfatidilcolina (PC), uno de los fosfolípidos constituyentes de la membrana plasmática generando el 1,2-diacilglicerol (DAG) (Fig. 3). La activación de PC-PLC por el M-CSF desempeña un papel importante tanto a nivel de la cascada de señalización de procesos tempranos (generación de DAG), así como también se ha implicado en la regulación de procesos tardíos, como es el paso a través de la fase G_1 del ciclo celular (Leach y col., 1991).

El DAG, así producido, es un segundo mensajero importante en muchas vías de señalización intracelular y es uno de los activadores naturales de ciertas isoformas de la proteína quinasa C (PKC). De hecho, la activación de PKC es uno de los fenómenos esenciales durante la señal mitogénica del M-CSF (Katayama y col., 1989). Curiosamente los macrófagos peritoneales residentes, a diferencia de los macrófagos derivados de médula ósea, no producen DAG (Veis y Hamilton, 1991) y, por tanto, apenas proliferan en respuesta al M-CSF (Celada y Maki,1992).

Además de la interacción de p85/PI-3K con la Y721 del M-CSFR activado, la fosfolipasa Cγ2 (PLCγ2) constituye un segundo candidato a unirse a esta tirosina fosforilada del receptor (Bourette y col., 1997). Bourette y colaboradores, han establecido un modelo que permite explicar como una misma tirosina fosforilada del M-CSFR puede interaccionar con los dominios SH2 de dos proteínas distintas, p85/PI-3K y PLCγ2. Este modelo se basa en un cambio de conformación que experimentaría el receptor cuando es fosforilada la posición Y(807). La fosforilación de Y(807) permite

la interacción de algunas proteínas adaptadoras con determinadas tirosinas fosforiladas del receptor, entre ellas la PLCγ2 y miembros de la familia Src, ya que se ha visto que una mutación en Y(807) del receptor reduce fuertemente la asociación de estas proteínas con el receptor (Bourette y col., 1995; Courtneidge y col., 1993). En contraposición, la proteína adaptadora Grb2 y p85/PI-3K, las cuales interaccionan en los sitios Y697 y Y721 del receptor, aumentan su unión al receptor cuando la posición Y807 no puede ser fosforilada (Bishayee y col., 1999; Bourette y col., 1997). Además, este modelo sugiere que el estado no fosforilado de Y(807) es suficiente para las señales de proliferación, mientras que ambos estados son necesarios en las señales de diferenciación celular (Bourette y col., 1995).

Por tanto, la fosforilación de Y807 determina la existencia de un balance entre la unión de PI-3K o PLCγ2 al receptor del M-CSF y como resultado se produce un balance en la producción o degradación de fosfatidilinositoles. Mientras que PI-3K fosforila fosfatidilinositoles 4,5-P2 (PtdIns(4,5)P2 o PIP2) generando PtdIns-(3,4,5)P3, requeridos para la fosforilación y activación de la serina/treonina quinasa PKB/Akt por PDK1 (ver apartado 2.1.3.3.); la PLCγ2 degrada los mismos PtdIns(4,5)P2 produciendo dos nuevos productos; DAG y el inositol 1,3,4 P3 (IP3). El IP3, actúa como un segundo mensajero, capaz de estimular la movilización de iones de calcio, contribuyendo a la activación de las isoformas de PKC dependientes de calcio (Fig. 3).

2.1.3.1. Activación de PKC

PKC es una proteína-quinasa que fosforila específicamente residuos de serina o treonina. El término PKC hace referencia a una familia compuesta por múltiples isoformas de este enzima. Tales isoformas han sido clasificadas en tres grandes grupos (convencionales, nuevas y atípicas) dependiendo de su estructura primaria y de los requerimientos necesarios para su activación (Dekker y Parker, 1994; Hug y Sarre, 1993).

Las PKC convencionales, α, βI, βII y γ, requieren la presencia de iones calcio (Ca^{2+}) y DAG o ésteres de forbol para su activación. También necesitan fosfatidilserina (PtdSer) como cofactor. PtdSer es un fosfolípido que se encuentra presente de forma constitutiva en las membranas celulares. Por este motivo, para ejercer su función, las PKC convencionales (y otras) necesitan ser translocadas a la membrana plasmática o al citoesqueleto (Dekker y Parker, 1994; Hug y Sarre, 1993). Las PKC nuevas también requieren DAG/ésteres de forbol y PtdSer, pero no dependen de la presencia de Ca^{2+} para su activación. A este grupo pertenecen los isozimas δ, ε, θ, y η. Las PKC atípicas, representadas por las isoformas ι /λ y ζ no se activan por Ca^{2+}, ni por DAG/ésteres de forbol. En su lugar pueden ser activadas por fosfatidilinositol (3,4,5)-trifosfato (PIP_3), ceramida y ácido fosfatídico (Akimoto y col., 1996; Domínguez y col., 1992). Una nueva serina-treonina quinasa regulada por DAG y ésteres de forbol fue posteriormente identificada, y originalmente se asignó como un miembro de una nueva familia de PKC. Denominada PKCμ /PKD, se diferencia de los otros isoenzimas PKC en su regulación y selectividad por el sustrato (Newton, 1997).

Todas estas isoformas de PKC no sólo presentan diferencias de estructura y regulación importantes, sino que, además, pueden fosforilar a substratos específicos y controlar diferentes vías de transducción de señal en una misma célula (Dekker y Parker, 1994; Hug y Sarre, 1993; Newton, 1997). Además, distintos miembros de la familia de PKCs exhiben un patrón de expresión diferencial dependiendo del tipo celular. Cuando se analizan diferentes líneas celulares, diferentes poblaciones monocítico/macrofágicas primarias o incluso diferentes estados de diferenciación o activación de un mismo tipo celular se aprecian variaciones significativas en la expresión de las isoformas de PKC (Lin y Chen, 1998; Meldrum y col, 1998; Monick y col, 1998; Valledor y col, 1999; 2000a).

En trabajos anteriores de nuestro grupo hemos descrito que nuestro modelo celular de macrófagos (macrófagos derivados de médula ósea), sólo expresan tres isoformas de PKC; PKC βI (convencional), PKCε (nueva) y PKCξ (atípica) (Fig. 4). Entre ellas, hemos observado que PKCε se encuentra involucrada en la regulación

de diversos aspectos muy importantes en la biología del macrófago, en particular en la regulación de la proliferación y activación (Valledor y col., 1999; 2000a).

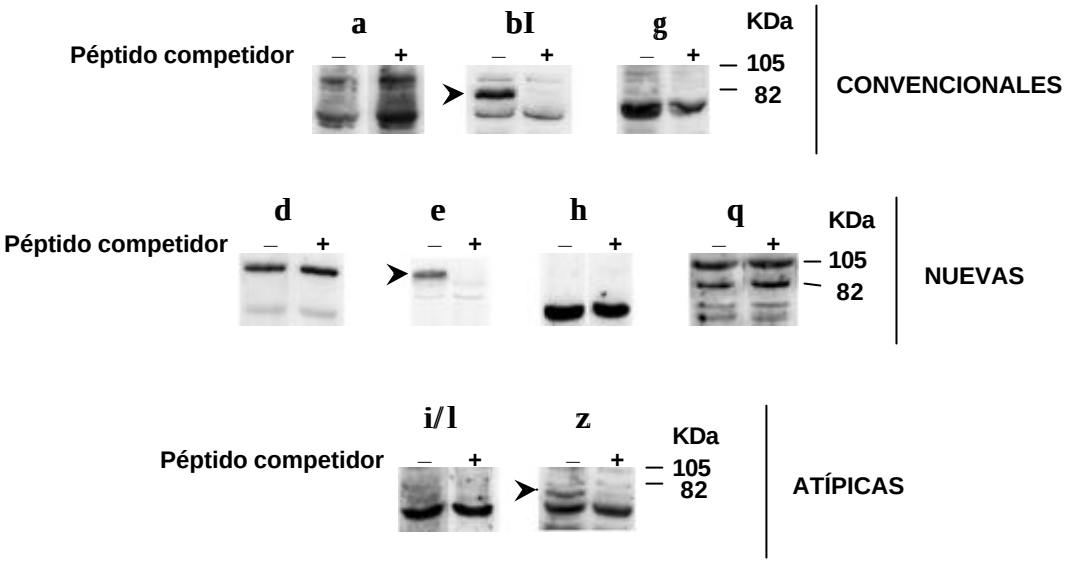


Figura 4. Los macrófagos derivados de la médula ósea sólo expresan tres isoformas de PKC. La expresión de las diferentes isoformas de PKC en BMDM fue analizada por Western blot con anticuerpos específicos para cada una de las isoformas, en presencia o ausencia del péptido contra el cual se generó dicho anticuerpo (péptido competidor) (Valledor y col., 1999).

Entre los potenciales substratos fisiológicos de PKC encontramos una serie de proteínas con funciones diversas como son las proteínas mediadoras de la transducción de señales (Ras, Raf, S6 quinasa, etc.), las moléculas que intervienen en diferentes procesos metabólicos (tales como canales y bombas de transporte), las proteínas nucleares que intervienen en la síntesis de DNA (DNA topoisomerasa y laminina B), MARCKS, una molécula responsable de la redistribución de los filamentos de actina desde la membrana hacia el citoplasma y factores de transcripción y traducción (Hug y Sarre, 1993).

En nuestro modelo celular, hemos descrito que la activación de PKCε por parte del M-CSF (y también el LPS) es la responsable de la expresión de la fosfatasa MKP-1 (*MAPK phosphatase-1*). MKP-1 es una proteína tirosina-fosfatasa con especificidad dual, localizada en el núcleo, donde es capaz de defosforilar e inactivar *in vivo* e *in*

vitro a diferentes miembros de la familia de MAPKs (ERK, JNK/SAPK y p38RK), representando un mecanismo de control negativo de estas vías (Alessi y col., 1993; Sun y Col., 1993, Valledor y col., 1999; 2000) regulando así los procesos de proliferación de los macrófagos entre otros. La expresión constitutiva de MKP-1 inhibe la síntesis de DNA inducida por el suero y por un mutante activo de Ras en los fibroblastos (Brondello y col., 1995; Sun y col., 1994). Hasta el momento se desconoce la vía de transmisión de la señal por la cual PKCε induce la expresión de MKP-1.

2.1.3.2. Cascada de activación de las MAPKs

En mamíferos, el término general MAPK (*mitogen-activated protein Kinase*) comprende a una superfamilia de proteínas quinasas con especificidad dual serina/treonina-quinasa dirigida por residuos prolina, que pueden ser activadas por una gran variedad de estímulos. Su activación depende de la señal iniciada por receptores de factores de crecimiento fosforilados en tirosina (como PDGF, EGF, FGF) o receptores de hormonas (como la hormona del crecimiento), de receptores asociados a proteínas G (*seven transmembrana receptors*) (como ANGII, endotelina) o de receptores de citocinas inflamatorias como los de la familia del TNF-α. Además, pueden ser activadas por condiciones de estrés en el entorno, como por ejemplo por shoc osmótico, irradiación, lesiones en el DNA o por productos bacterianos como el LPS. La activación de las MAPKs en respuesta a estos estímulos controla la expresión de genes, el metabolismo celular y funciones del citoesqueleto, contribuyendo a la regulación de procesos celulares tan complejos como la migración, la mitogénesis, la diferenciación o la supervivencia celular.

Estas MAPK quinasas han sido divididas en tres grandes grupos en función de los estímulos que inducen su activación. Así por ejemplo, los factores de crecimiento o estímulos mitogénicos promueven principalmente la activación de las quinasas ERK (*extracellular signal-regulated kinase*); mientras que, estímulos de estrés celular inducen la activación de otras dos subfamilias, las JNK/SAPK (*c-jun NH2-terminal*

kinase o *stress-activated MAP kinase*) y un tercer grupo caracterizado por p38RK (*Reactivated kinase*) (ver apartado 3.1.3.2.)

La activación de cada familia de MAPK se produce en forma de cascada. Dicha cascada está formada por tres módulos con actividad quinasa, donde los miembros del módulo superior fosforilan y activan a los miembros del módulo inferior. La especificidad de la respuesta de la MAPK viene dada por la activación específica de determinados miembros de cada uno de estos módulos. Estos módulos se esquematizan como MAPKKK---MAPKK—MAPK. De esta manera, el tercer módulo, correspondiente a las familias ERK, JNK y p38 (MAPK) serán fosforiladas y activadas por un segundo módulo de MAPK quinasas (MAPKKs). Estas MAPKKs son quinasas con especificidad dual y catalizan una doble fosforilación en tirosina y en treonina sobre las MAPKs necesaria para que éstas sean completamente activas. Por otro lado, las MAPKKs serán fosforiladas y activadas por serina/treonina quinasas que funcionan como MAPKK quinasas (MAPKKKs), el primer módulo. La regulación de estas quinasas podría parecer complicada por el gran número de combinaciones que existen, pero no es así. Primero, las MAPKKs representan un número menor de miembros que el módulo de las MAPK. Segundo, las MAPKKs presentan una alta especificidad por sus substratos MAPK, conduciendo a un mínimo de combinaciones en los módulos MAPKK-MAPK. Tercero, los MAPKKKs presentan diferentes motivos de regulación que los que presentan las MAPKK o MAPK. Por tanto, las MAPKKKs pueden ser reguladas por una variedad de proteínas especificas activadas en respuesta a los diferentes estímulos extracelulares. Por último, la regulación de la cascada MAPKKK-MAPKK-MAPK dependerá de su interacción con unas proteínas adaptadoras denominadas "*scaffolding*". El papel de estas proteínas consiste en aumentar la concentración local de las proteínas quinasas y sus substratos específicos (Pearson y col., 2001; Treisman, 1996)

2.1.3.2.1. Activación de ERK (*extracelular signal-regulated kinase*)

La cascada de activación de ERK es la que, hasta el momento, ha sido más extensamente estudiada, ya que se activa bajo el estímulo de numerosos agentes

mitogénicos. ERK-1 y ERK-2, también conocidas como p44- y p42- MAPK, son expresadas de forma ubicua y su abundancia relativa dependerá del tipo de tejido. Por ejemplo, en muchas células del sistema inmunológico ERK-2 se encuentra predominantemente expresada, mientras que en células de origen neuroendocrino, la expresión de ambas quinasas es similar (Lewis y col, 1998).

El segundo módulo de activación (MAPKK) está compuesto por los miembros de la familia MEK; MEK-1 y MEK-2. No existen evidencias de que ERK-1/2 puedan ser fosforiladas por otros efectores, aunque no es posible descartar esta posibilidad (Crews y col.,1992) (Fig. 5)

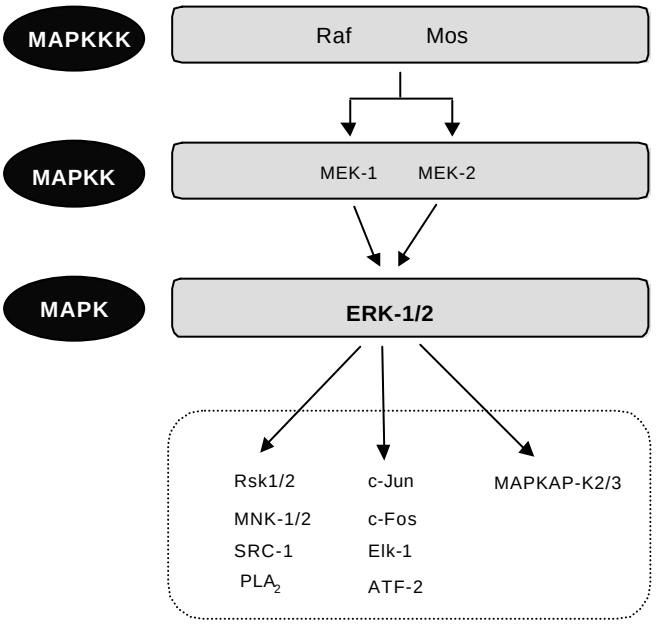


Figura 5. La cascada de activación de la familia ERK. ERK, *extracellular- related kinase*; MAPK, *mitogen-activated protein kinase*; MAPKAPK, *MAPK activated protein kinase*; MAPKK, *MAPK Kinase*; MAPKKK, *MAPKK Kinase;* MEK, *MAPK/ERK kinase*; PLA_2, fosfolipasa A_2.

De todos los posibles estimuladores de MEK que se conocen, son quizás, las isoformas de Raf y Mos las únicas capaces de fosforilar a este segundo módulo en una cascada sencilla (Kyriakis y col., 1992). La familia de proteínas quinasas de Raf está compuesta por A-Raf, B-Raf y Raf-1 (o c-Raf). De estas isoformas, Raf-1 es la que está presente de forma ubicua en todos los tejidos. En tejidos neuronales y testículos está presente de forma predominante B-Raf, mientras que A-Raf se localiza en tejido urogenital. La regulación de Raf-1 es muy compleja, ya que Raf-1 forma

parte de un complejo multiproteico y su activación depende de interacciones proteína-proteína, fosforilaciones de residuos tirosina, treonina y serina, y de su localización celular (Morrison y Cutler, 1997). Por tanto, el estado de fosforilación y activación de Raf-1 dependerá de múltiples familias de proteínas, incluyendo miembros de la familia Src, de la familia PKC, de PAK (*p21$^{(Rac/cdc42)}$-activated protein*), y de Akt.

Como hemos comentado anteriormente, la activación de la cascada ERK se produce a través de receptores fosforilados en tirosina, tal y como ocurre con el receptor del M-CSF (ver apartado 2.1.2). La fosforilación de estos receptores tiene como resultado la organización y activación de diversos complejos multiproteicos. Uno de estos complejos se produce tras la activación de la proteína G monomérica, Ras. La activación de Ras se inicia con el reclutamiento de proteínas adaptadoras como Shc y Grb2 al receptor a través de la interacción de sus dominios SH2 con los residuos tirosina del receptor. Entonces el intercambiador de nucleótidos, mSOS promueve el intercambio de un GDP por un GTP. El complejo Ras-GTP es capaz de interaccionar directamente con un número elevado de factores, entre ellos, las isoformas de Raf. La interacción de Ras con Raf-1 induce un cambio conformacional de Raf-1, activándolo o simplemente proporcionándole el entorno adecuado para su activación (Moodie y col., 1993; Vojtek y col., 1993).

Una vez activadas, ERK-1 y ERK-2 son capaces de fosforilar y regular, ya sea positiva o negativamente, numerosas proteínas citoplasmáticas, incluyendo proteína-quinasas adicionales y otras proteínas involucradas en la transmisión de señales como la fosfolipasa A_2, así como componentes del citoesqueleto. Además, las ERKs pueden translocarse al núcleo, donde regulan la actividad de factores de transcripción que controlan la expresión de genes de acción inmediata (Treisman, 1996) (Fig. 5).

ERK-1/2 tiene un efecto directo sobre las quinasas Rsk (también conocidas como *pp90 ribosomal S6 quinasa*). Rsk1 y Rsk2 una vez fosforiladas pueden interaccionar con proteínas implicadas en la activación transcripcional, como CREB (*cAMP-response element binding protein*) (Xing y col., 1996), el coactivador CBP (Nakajima y col., 1996), c-Fos (Chen y col., 1993), SRF (*Serum response factor*) (Rivera y col., 1993) o el receptor de estrógenos (Joel y col., 1998). Las

serina/treonina quinasas, Mnk1 y Mnk2 (*MAP Kinase-interacting kinase*) también pueden ser fosforiladas con diferente especificidad tanto por ERK-1/2 como por p38. Aunque Mnk2 es un buen substrato *in vitro* de las dos cascadas de activación, Mnk1 es mejor substrato para las quinasas p38. Una vez activadas por estímulos mitogénicos o factores de estrés, Mnk1 y Mnk2 fosforilan al factor de iniciación eucariótico (eIF-4E) con el consiguiente inicio de la síntesis proteica en los ribosomas (Waskiewicz y col., 1997). Por otro lado, ERK-1/2 (al igual que p38) participa en la activación de otra familia de serina/treonina quinasas, las MAPKAP-K2 y -3 (*MAPK-Activated Protein Kinase*). La acción de estas quinasas sobre la proteína de shoc térmico HSP27 contribuye a la reorganización del citoesqueleto afectando a la motilidad celular (Engel y col., 1995).

Por otro lado, la traslocación de ERK-1/2 al núcleo permite la fosforilación del coactivador SRC-1 (*Receptor steroids coactivator-1*) potenciando la activación de receptores nucleares esteroideos (Rowan y col., 2000). En el núcleo, ERK también puede activar o fosforilar a algunos miembros de la familia de factores de transcripción de AP-1 (*activating protein-1*) incluyendo c-Jun (Hibi y col., 1993), c-Fos (Chen y col., 1993), y ATF2 (*Activating transcription factor*) (Gupta y col., 1995) (Fig. 5). Sin embargo, la primera función atribuida a ERK-1/2 fue la fosforilación del factor de transcripción Elk-1 (*ETS-domain proteins*). Este factor pertenece a un complejo transcripcional de los TCF (*Ternary complex factor*) que median la transcripción de genes que contienen en sus promotores dominios SRE (*Serum response element*) como *c-fos* (Janknecht y col., 1993; Price y col., 1995).

Cabe mencionar que la vía de señalización a través de ERK no sólo es activada por factores mitogénicos, hormonas y citocinas sino también por agentes antiproliferativos, como es el caso del LPS en macrófagos (Reiman y col.,1994; Valledor y col., 2000b).

2.1.3.3. Activación de la vía PI-3K/Akt

La fosforilación de la tirosina Y(721) del M-CSFR es la responsable de la interacción del receptor con el dominio SH2 de la proteína adaptadora p85 y de su unión con la subunidad p110 de la fosfatidilinositol-3 quinasa (PI-3K), formando un complejo proteico reclutado en la parte interna de la membrana plasmática (Rameh y col, 1995; Rameh y Cantley, 1999; Toker y Cantley, 1997). PI-3K también puede ser activada por la interacción directa con el protooncogén Ras (Kauffmann-Zeh y col., 1997; Khwaja y col., 1997; Liu y col., 1998a; Rodríguez-Vicana y col., 1994; 1996). Una vez localizada en la membrana, PI-3K cataliza la transferencia de un fosfato procedente del ATP a la posición D-3 de los fosfolípidos de inositol presentes en la membrana plasmática. Hasta el momento se han descrito nueve isoformas de PI-3K en células de mamífero, las cuales han sido agrupadas en tres clases en función de las moléculas que utilizan como substrato (Rameh y Cantley, 1999). Las isoformas principales de PI-3K que son activadas en respuesta al receptor del M-CSF fosforilado generan fosfatidilinositol 3,4 bifosfato (PI3,4P) y fosfatidilinositol 3,4,5 trifosfato (PI3,4,5P). Estos lípidos fosforilados en la posición D-3 funcionan como intermediarios en la señalización de la vía y son capaces de participar en la activación de un elevado número de substratos celulares. Estos incluyen las GTPasas activadas por proteínas G como $p21^{Ras}$ (Ras) (Busher y col., 1995), las tirosina quinasas, las serina/treonina quinasas como las isoformas atípicas de PKC, la GSK (*Glycogen synthase kinase*), la $pp70^{S6K}$ y Akt (Rameh y Cantley, 1999). Algunos de estos substratos han sido implicados en la supresión de la apoptosis (Diaz-Meco y col., 1996; Nishida y col., 1998). Sin embargo, sólo la activación de Akt ya es suficiente para bloquear la apoptosis inducida por un gran número de diversos estímulos, entre ellos la falta de factores de crecimiento en el medio (Fig. 6).

Las proteínas de la familia de Akt, presentan un dominio quinasa central con especificidad por residuos serina o treonina (Bellacosa y col., 1991; Coffer y Woodgett, 1991). Además, la región aminoterminal de Akt incluye un dominio de homología con la plecstrina (PH), el cual, puede mediar interacciones lípido-proteína y/o proteína-proteína (Datta y col., 1995; Mayer y col., 1993; Musacchio y col., 1993). El dominio PH de Akt se une a los intermediarios lipídicos que se han fosforilado

previamente por PI-3K; como consecuencia, Akt se transloca del citoplasma a la cara interna de la membrana plasmática quedando anclada a ella por el dominio PH (Andjelkovic y col., 1997; Currie y col., 1999; Zhang y Vik, 1997). La relocalización de Akt a la membrana plasmática favorece que Akt pueda ser fosforilada por quinasas próximas. Se han identificado cuatro lugares de fosforilación en Akt (Ser-124, Thr-308, Thr-450 y Ser-473) que pueden ser fosforilados *in vivo*. La Thr-308 y Ser-473 son fosforiladas después del tratamiento con estímulos extracelulares, mientras que Ser-124 y Thr-450 ya se encuentran fosforiladas de forma basal (Alessi y col., 1996). La fosforilación de Akt en estos residuos es necesaria para la actividad de Akt tanto *in vitro* como *in vivo* (Anjelkovic y col., 1996; Bellacosa y col., 1991; Burgering y Coffer, 1995; Coffer y Woodgett, 1991; Kohn y col., 1996).

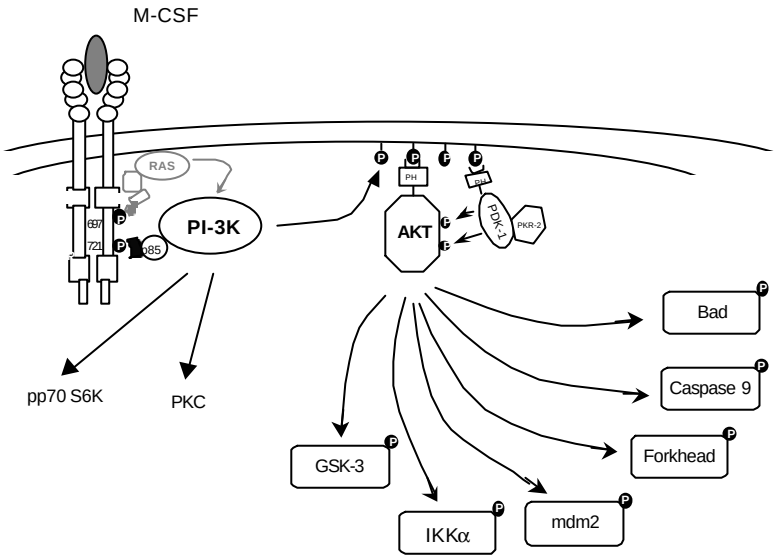


Figura 6. Vía de señalización de la PI-3K. La activación de AKT por PI-3K media la fosforilación de diversos substratos involucrados en la regulación de los procesos de supervivencia celular. IKK, quinasa del Inhibidor del factor κ; M-CSF, factor estimulador de colonias macrofágicas; PKC, proteína quinasa C.

Las quinasas responsables de la fosforilación de Akt en la posición Thr-308 pertenecen a una familia de quinasas dependientes de fosfatidilinositoles (PDKs). El cDNA de PDK-1 codifica por una proteína de 63 KDa que contiene también un dominio PH y un dominio quinasa relacionado con PKA, Akt y PKC (Alessi y col.,

1997). PDK-1, además de Akt, puede también fosforilar *in vitro* a la p70s6k y a miembros de la familia de PKC. En mucho de estos casos la fosforilación es necesaria para su activación pero hace falta además la cooperación de otras quinasas o la interacción directa con los fosfoinositoles de membrana (Rameh y Cantley, 1999). La quinasa PDK-1 se encuentra constitutivamente activa independientemente del estímulo que reciba la célula, pero es sólo tras la estimulación de la célula que PDK-1 es translocada a la membrana donde podrá actuar sobre Akt (Currie y col., 1999). Para la activación de Akt es necesario que PDK-1 forme complejo con otra quinasa, PKR-2 (Balendran y col., 1999). El complejo PDK-1/PKR-2 será así capaz de fosforilar a Akt en ambos residuos, Thr-308 y Ser-473, y por tanto, la regulación de la actividad de Akt dependerá de la unión al dominio PH de los fosfolípidos fosforilados en D-3 por parte de PI-3K. Probablemente, esta interacción con los fosfolípidos generados por PI-3K es responsable de un cambio conformacional de Akt que permite un aumento de la actividad catalítica intrínseca de esta quinasa (Datta y col., 1996; Franke y col., 1997; Klippel y col.,1996) (Fig. 6).

Las observaciones sobre el hecho que Akt sea la principal mediadora de la señal iniciada por PI-3K, junto con las observaciones que PI-3K media la supervivencia inducida por factores de crecimiento, sugiere que Akt debe jugar un papel crítico como regulador de la supervivencia celular. Un gran número de estudios han demostrado que la transfección de alelos de Akt constitutivamente activos bloquean la apoptosis inducida por diferentes estímulos, incluyendo la falta de factores de crecimiento, radiaciones UV, pérdida de la adhesión a la matriz extracelular, alteraciones en el ciclo celular, daño en el DNA y tras el tratamiento de las células con anticuerpos anti-Fas o con TGF-β (revisado en Datta y col, 1999). De forma indirecta, se ha visto que Akt experimenta proteolisis en los primeros estados de apoptosis (Widman y col., 1998).

Además, se ha implicado a Akt en la regulación del citoesqueleto (Carpenter y Cantley, 1996) y en varios modelos de oncogénesis. Así, un número de oncogenes, como Ras, dependen de la activación de Akt para inducir la transformación y el crecimiento tumoral (Skorski y col., 1997). Por otro lado, se ha observado que, la ausencia de la actividad de genes supresores de tumores como PTEN, provoca un

aumento de la actividad Akt y, por tanto, un aumento de la supervivencia de las células tumorales. Es importante matizar que la supresión de apoptosis no es la única función que Akt puede desempeñar en el crecimiento tumoral; en algunos casos, Akt puede inducir directamente la progresión a través del ciclo celular (Ahmed y col., 1997: Brennan y col., 1997).

Sin embargo, el mecanismo específico por el cual Akt promueve la supervivencia celular no esta claro. Se han descrito varios sustratos implicados en los procesos de apoptosis que pueden ser fosforilados por Akt reduciendo así su actividad y, de este modo, inhibiendo la muerte de la célula. Estos sustratos incluyen, por ejemplo, a dos componentes efectores de la maquinaria de apoptosis, BAD y caspasa 9; factores de transcripción como el *forkhead factor* o E2F, implicado en la regulación del ciclo celular; y a la quinasa IKK que regula al factor de transcripción NF-κB, también implicado en procesos de inhibición de la apoptosis a través de los receptores de la familia del TNF-α (*Death receptors*) (Brennan y col., 1997; Brunet y col., 1999; Cardone y col., 1998; Datta y col., 1997; Madrid y col., 2000). Akt también regula la actividad de la serina/treonina quinasa *p21-activated protein kinasa-1* (PAK) (Schurmann y col., 2000), y promueve la entrada nuclear de MDM2 y, disminuyendo los niveles celulares de p53 (Mayo y Donner, 2001), responsables de la muerte celular por diversos estímulos intrínsecos como el daño en el DNA, errores de replicación génica o la falta de factores de crecimiento (revisado en Levine, 1997) (Fig. 6).

2.2. Regulación del ciclo celular

El ciclo celular es el proceso por el cual las células se preparan para su duplicación. Dicho ciclo está dividido en cuatro etapas: G_1, S, G_2 y M. La fase G_1 es el espacio de tiempo que se extiende entre la última división (mitosis) y el inicio de la síntesis de DNA. Durante esta fase, la célula monitoriza la presencia de factores de crecimiento en el entorno. Si las condiciones son favorables, la célula se ocupa básicamente de la replicación del material genético. La fase G_2 representa un espacio de tiempo de seguridad. La célula comprueba que la replicación del DNA haya sido

completada correctamente durante la fase S antes de aventurarse a entrar en la fase mitótica, también conocida como fase M. Durante esta última etapa, la célula divide su contenido en dos y se generan dos células hijas (Alberts y col.,1994)

El ciclo de división completo de los macrófagos tiene una duración aproximada de 24h. El tránsito a través de la fase G_1 dura unas 12 h y durante la mayor parte del mismo, los macrófagos requieren de la presencia constante de M-CSF (Sherr y Stanley, 1990). Una vez el factor de crecimiento determina al macrófago a entrar en la fase S, éste es capaz de continuar a través del resto del ciclo de división celular en ausencia de dicho factor de crecimiento. Los macrófagos permanecen en la fase S, G_2 y M durante 6, 4 y 2 h, respectivamente (Rock y col.,1992).

Después de la mitosis y durante el principio de la fase G_1, la célula puede detener su progreso a través del ciclo de división celular y permanecer en un estado de reposo o quiescencia más o menos prolongado (fase G_0). Esta detención es característica de células en las que la diferenciación terminal conduce a la pérdida de la capacidad proliferativa.

2.2.1. c-Myc

La expresión de *c-myc* es necesaria tanto para la progresión del ciclo celular tanto a nivel del paso de G_0 a G_1 como de G_1 a S. Recientemente, se ha implicado la regulación de *c-myc* a través de la progresión de G_0 a G_1 del ciclo celular con las quinasas de la familia Src, con la consecuente activación de las GTPasas Rho, a pesar que con anterioridad se habían implicado otras vías, como Ras o las MAPK quinasas (Blake y col., 2000; Chiariello y col., 2001). En los últimos años, se ha relacionado también a la vía de Raf/ERK y PI-3K/Akt con la estabilidad de la proteína Myc, mediante la fosforilación de la Ser-62 (Sears y col., 2000).

La expresión de *c-myc*, es estrictamente requerida para la transmisión de señales mitogénicas inducidas por los factores de crecimiento como el M-CSF o PDGF y es suprimida por inhibidores de la proliferación o señales de diferenciación

como por ejemplo por AMPc, el factor de crecimiento transformante (TGF-β), IFN-γ, el inhibidor del ciclo celular, $p21^{Waf1}$, u otros inhibidores de la proliferación celular (He y col., 1998; Henriksson y Lusher, 1996). Varios estudios han demostrado que la regulación de los niveles de Myc son estrictamente esenciales para el correcto funcionamiento de la célula, puesto que la eliminación por delección homocigótica de los genes de *c-myc* resulta letal a nivel embrionario (Charron y col., 1992; Davis y col., 1993), mientras que la sobrexpresión de estas proteínas, ya sea en cultivos celulares como en animales transgénicos, bloquea la diferenciación, e induce la transformación neoplásica y sensibiliza a las células a morir por fenómenos de apoptosis (Coppola y Cole, 1986; Evan y col., 1992). Además en muchos tumores humanos, la expresión de *c-myc* se encuentra potenciada (Nesbit y col., 1999).

A parte de participar en la proliferación, la actividad de Myc puede afectar a la apoptosis y diferenciación de las células. Esta habilidad de afectar a tantos aspectos de la célula puede relacionarse con la participación de esta proteína en una amplia red de interacciones proteicas entre los miembros de la familia Myc (Myc, Max, Mnt/Rox, Mga) y los miembros de la familia Mad (Mad1, Mxi/Mad2, Mad3 y Mad4). Myc, es el miembro mas estudiado de esta familia de factores de transcripción, la cual se caracteriza porque sus miembros presentan dos regiones de unos 20 aminoácidos (cajas Myc 1 y 2) altamente conservadas entre los diferentes miembros de la familia (Sakamuro y Prendergast, 1999). En el extremo carboxi-terminal de Myc se encuentra un motivo básico/ hélice-asa-hélice/ cremallera de leucina (*basic/ helix-loop-helix/ leucine zipper, b/HlH/Z*) que permite la oligomerización con una pequeña proteína de la misma familia denominada Max y el reconocimiento de una secuencia específica llamada motivo E-box, induciendo, de esta manera, la transcripción de genes relacionados con el ciclo celular (Luscher y Larsson, 1999). Por otra parte, la proteína Max puede formar heterodímeros con otra familia de proteínas conocida como Mad. Estas proteínas actúan como represores de la transcripción, interaccionando con el mismo motivo E-box reconocido por Myc/Max (Foley y Eisenman, 1999). Mientras que Max se expresa de forma ubicua y constitutiva, la expresión de Myc y Mad es regulada en relación con el ciclo celular y la estimulación por mitógenos. Los niveles de Myc son elevados en células que se encuentran proliferando, mientras que Mad no se expresa. Sin embargo, en células que han

dejado de proliferar y pasan a diferenciarse la expresión de *c-myc* está totalmente inhibida y pasa a expresarse Mad (Foley y Eisenman, 1999).

Se ha descrito un amplio número de genes que pueden ser inducidos por la actividad transcripcional del complejo Myc/Max, los cuales pueden estar implicados en el ciclo celular como las ciclinas D_1 y D_2, así como *Id2*, el cual codifica para una proteína que inhibe la función del pRb, promoviendo de este modo la progresión a la fase S del ciclo celular y promoviendo la transcripción de la ciclina E. Se ha propuesto que Myc además induce la expresión de la fosfatasa cdc25A estimulando indirectamente la actividad ciclina E/cdk2 (Sears y col., 2000). En resumen, la expresión de c-myc es claramente un estimulador de la progresión a través del ciclo celular y, por tanto, de la proliferación celular.

Además de jugar un papel muy importante en la progresión del ciclo celular, la actividad de Myc induce la expresión de una elevada variedad de otros genes relacionados con la biogénesis ribosómica, síntesis proteica, y en general con el metabolismo celular, así como genes implicados en la apoptosis (revisado en Nasi y col., 2001)

2.2.2. Regulación de la actividad de los complejos ciclina-cdk

La progresión a través del ciclo celular está controlada por la actividad de una familia de serina/treonina quinasas denominadas quinasas dependientes del ciclo celular o cdks (*cell cycle dependent kinases*) (Pines, 1994). La actividad de estas quinasas se encuentra finamente regulada a diversos niveles, puesto que de su correcta activación y desactivación en el momento preciso va a depender todo el proceso de división celular.

Aunque en levaduras este proceso está controlado por una única cdk (*cdc2* en levaduras de fisión o *cdc28* en levaduras de gemación), los eucariotas superiores han evolucionado hacia la utilización de varias cdks para regular las diferentes fases de su ciclo celular. Hasta la fecha se han descrito siete cdks en los mamíferos,

denominadas cdk 1-7 (Fig. 7). Entre ellas presentan una elevada homología (40-70% de identidad) y su dominio catalítico mantiene un elevado grado de conservación con el de otras serina-treonina quinasas (Lees, 1995; Morgan, 1995; Pines, 1994). Existen diversos mecanismos involucrados en la regulación de la actividad de las cdks que garantizan la correcta progresión a través del ciclo celular y su exquisita sensibilidad para detectar las variaciones existentes en el entorno celular.

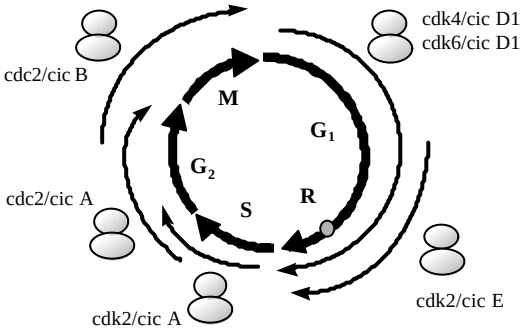


Figura 7. Fases del ciclo celular. La expresión temporal y activación secuencial de los diversos complejos ciclina-cdk determina el paso a través de cada una de las fases del ciclo celular en mamíferos. Cdk, quinasa dependiente del ciclo celular; cic, ciclina; M, mitosis; R, punto de restricción.

En estado monomérico, las cdks prácticamente no presentan actividad alguna. Para su activación, en una etapa inicial, requieren de la unión a unas subunidades reguladoras, denominadas ciclinas. Las ciclinas constituyen una gran familia de proteínas denominadas ciclina A-H, las cuales se unen y activan a diferentes cdks (Sherr, 1993). La unión de las ciclinas a la subunidad cdk provoca un incremento de 40000 veces en su actividad quinasa (Connell-Crowley y col., 1993) debido principalmente a cambios estructurales en la subunidad quinasa (Jeffrey y col., 1995) (Fig. 8).

Durante el ciclo celular, cada ciclina tiene un patrón de expresión único (Fig. 7). Por este motivo, el tiempo de expresión de cada ciclina es crucial para determinar en que fase del ciclo celular será activa la quinasa a la cual se una. De esta forma, la disponibilidad de cada ciclina es un factor limitante para la progresión a través de las diferentes fases del ciclo celular. Las ciclinas de tipo D son sintetizadas al inicio de la

fase G_1 y se unen y activan a cdk-4 y cdk-6 tan pronto como las células abandonan su estado de quiescencia y entran dentro del ciclo celular. Los complejos ciclina E-cdk-2 y ciclina A-cdk-2 se forman posteriormente, durante la fase G_1 tardía, cuando las células se preparan para la síntesis del DNA. La expresión de la ciclina B empieza en la fase S. La cdk-1 (también denominada cdc-2) se une entonces a la ciclina B para formar el complejo quinasa que será activo durante la fase G_2 y la mitosis (Fig. 7).

Los niveles de las diferentes ciclinas están modulados tanto transcripcionalmente como mediante el recambio proteico. Por ejemplo, las ciclinas D están inducidas por citocinas y factores de crecimiento responsables de iniciar la progresión a través del ciclo celular (Matsushime y col., 1991), mientras que una rápida degradación de las ciclinas A y B, mediada por procesos de ubicuitinación, es esencial para permitir la salida de las células de la fase de mitosis (Irniger y col., 1995; King y col., 1995; Murray, 1995)

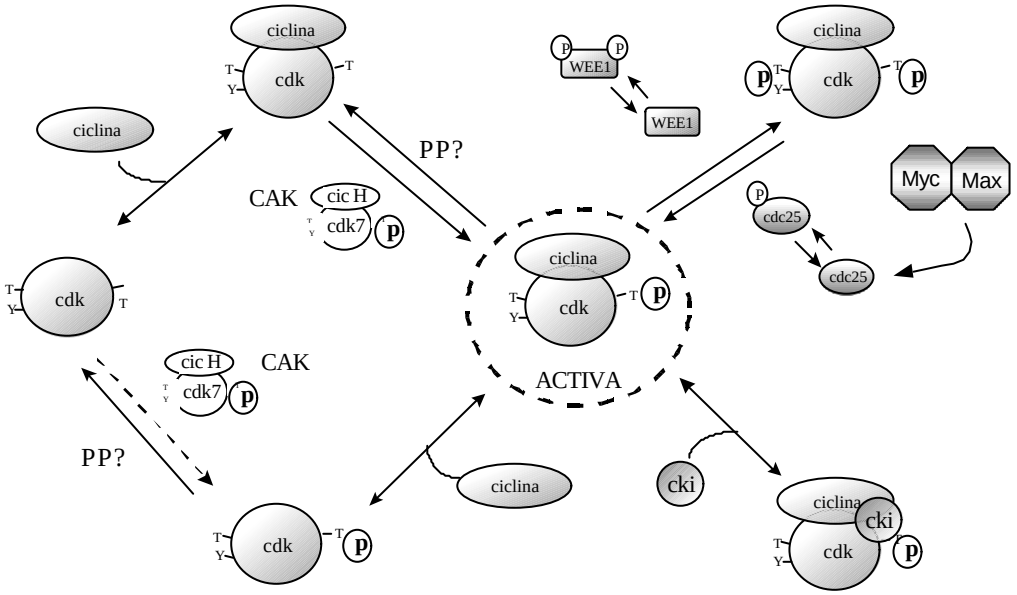


Figura 8. Puntos de la regulación de los complejos ciclina-cdk. La existencia de innumerables puntos de regulación de estos complejos es necesaria para asegurar la elevada sensibilidad del ciclo celular para detectar los estímulos externos, así como para asegurar la correcta duplicación celular. Cdk, quinasa dependiente del ciclo celular; CAK, quinasa activada por el ciclo celular; PP, proteína fosfatasa; P, punto de fosforilación; cki, inhibidor de las cdk; T, residuo de treonina; Y, residuo de tirosina.

Los complejos ciclina-cdk son regulados posteriormente por fenómenos de fosforilación y defosforilación en residuos conservados de las subunidades cdk. Se ha propuesto que la fosforilación del residuo treonina T(160) mediada por la quinasa CAK (*Cell cycle-activated kinase*) induce un posterior cambio conformacional y permite la correcta exposición del centro catalítico, favoreciendo así la unión del substrato al mismo (Jeffrey y col., 1995). Esta fosforilación permite también la estabilización de los complejos ciclina-cdk. La quinasa CAK es un complejo enzimático multimérico compuesto por la ciclina H y la cdk-7 (Fisher y Morgan, 1994; Makela y col., 1994; Solomon y col., 1993) (Fig. 8).

Otro punto de regulación de las cdks es su fosforilación en residuos tirosina conservados, T(15) en la cdk-1. El residuo T(15) está localizado dentro del centro catalítico del enzima (Dunphy, 1994). En organismos eucariotas superiores la T(14) adyacente constituye también una diana de fosforilación. A diferencia de lo que ocurre con la T(160), la fosforilación en los residuos T(15) y T(14) inhibe la actividad de los complejos ciclina-cdk. Estos residuos son fosforilados por quinasas emparentadas con Wee1. Las fosfatasas de la familia cdc25 son las responsables de la defosforilación de dichos residuos y, por tanto, de la activación de las cdks (Dunphy, 1994; Hoffman y Karsenti, 1994) (Fig. 8).

La función última de la actividad de los complejos ciclina-cdk es regular el estado de fosforilación de la proteína del retinoblastoma (pRb). La pRb es fosforilada por los complejos ciclina-cdk, particularmente por los complejos ciclina D-cdk4/6 y ciclina E-cdk2. La pRb regula la progresión a través de la fase G_1 del ciclo celular (Weinberg, 1995) permitiendo, o no, la expresión de genes esenciales para la replicación del DNA y, por tanto, para la proliferación celular. La forma hipofosforilada de pRb es la forma activa e inhibitoria de la proliferación celular, mientras que la forma hiperfosforilada es inactiva. Cuando la célula alcanza el punto R (punto de restricción) podrá continuar a través del ciclo celular, sólo si pRb se encuentra hiperfosforilada, gracias a la actividad de los complejos ciclina-cdk. De este simple modelo, se deduce fácilmente que aquellos factores que modulen la fosforilación de pRb son cruciales para la regulación de la progresión del ciclo celular.

La pRb se une, y presumiblemente, controla la actividad de diversas proteínas. La más importante de éstas, en el contexto de la regulación del ciclo celular, es la familia de proteínas E2F. Estas proteínas son factores de transcripción que regulan la expresión de diversos genes requeridos para la progresión a través del ciclo celular, entre los cuales se incluyen ciclinas y la timidina quinasa, entre otros (DeGregori y col., 1995; Muller, 1995; Nevins, 1992).

2.2.3. Inhibición de la actividad de los complejos ciclina-cdk

Se ha descrito un elevado número de proteínas capaces de inhibir la actividad de los complejos ciclina-cdk, ejerciendo así un punto adicional de regulación y aumentando la complejidad en el control del ciclo celular (Sherr y Roberts, 1995). Hasta la actualidad, siete moléculas inhibidoras han sido aisladas en las células de mamífero. Esas proteínas inhibidoras pueden ser clasificadas en dos grandes familias en función de su homología y modo de acción (Tabla II)

Tabla II. Cki (Inhibidores de los complejos ciclina-cdk).

	Diana	Fase del ciclo	Función
p15/Ink 4b	cdk-4/6	G_1	Respuesta al TGFβ
p16/Ink 4a	cdk-4/6	G_1	Transición G_1/S
p18/Ink 4c	cdk-4/6	G_1	?
p19/Ink 4d	cdk-4/6	G_1	Oncogénesis
p21/Waf-1/Cip-1	Todos los complejos ciclina-cdk	G_1, G_1/S, G_2, M	Senescencia, Inducido por p53, Diferenciación y desarrollo
p27/Kip-1	cic D-cdk-4/6, cic E-cdk-2	G_1, G_1/S	Respuesta al TGFβ y al AMPc
p57/Kip-2	cic D-cdk-4/6, cic E-cdk-2, cic A-cdk-2	G_1, G_1/S, S	Desarrollo

2.2.3.1. Familia INK4

Esta familia de inhibidores incluye a $p16^{ink4a}$ y otros tres polipéptidos emparentados: $p15^{ink4b}$, $p18^{ink4c}$ y $p19^{ink4d}$ (Chan y col., 1995; Guan y col., 1994; Hannon y Beach, 1994; Hirai y col., 1995; Kamb y col., 1994; Serrano y col., 1993). Estas proteínas están formadas por cuatro dominios del tipo anquirina (*ankirin-like*), los cuales son importantes para la interacción con las cdks. Una característica de esta familia de inhibidores es su especificidad de unión a cdk-4 y cdk-6. La administración de p16 a los complejos ciclina D-cdk4 supone la disociación del complejo. Además, la unión de p16 a la cdk-4 monomérica impide su posterior asociación con la ciclina D. Por lo tanto, los inhibidores de la familia INK actúan principalmente impidiendo la asociación de las quinasas cdk-4 y cdk-6 con la ciclina D (Parry y col., 1995)

2.2.3.2. Familia CIP/KIP

Esta familia de inhibidores comprende los productos de tres genes distintos: $p21^{Waf1}$, $p27^{Kip1}$ y $p57^{Kip2}$. Los miembros de la familia CIP/KIP muestran un amplio abanico de especificidades, siendo capaces de inhibir a todos los complejos ciclina-cdk que operan en la fase G_1 y, en menor grado, a los complejos ciclina B-cdk-1 (Harper y col., 1995; Polyak y col., 1994a; Matsuoka y col., 1995). A diferencia de la familia INK4, esta familia tiene una mayor afinidad para unirse a los complejos ciclina-cdk ya formados en comparación con las quinasas monoméricas. Estos inhibidores también pueden prevenir la activación de las cdks por la CAK (Sherr y Roberts, 1995)

2.2.3.2.1. $p21^{Waf1}$

El cDNA de p21 fue clonado por diferentes grupos de investigación utilizando estrategias independientes. Mediante la búsqueda por proteínas híbridas en levaduras (*two-hybrid screen*) se identificó a p21 como una proteína que se unía a cdk, denominándola CIP-1 (*CDK-interacting protein 1*) (Harper y col., 1993). También fue identificada como un producto de un gen activado por p53, y se lo denomino WAF1 (*wild-type p53-activated factor*) (El-Deiry y col., 1993). Más tarde, p21 fue clonado

utilizando una genoteca de expresión (*expression screen*) para identificar inhibidores de la síntesis de DNA procedentes de fibroblastos senescentes y fue llamado, SDI1 (*senescent cell-derived inhibitor*) (Noda y col., 1994). Por último, mediante la técnica de hibridación substractiva, p21 fue purificado en células de melanoma humanas inducidas a diferenciarse, por lo que se le denomino MDA-6 (*melanoma differentiation-associated protein*) (Jiang y col., 1994).

La proteína $p21^{Waf1}$ contiene dos regiones funcionales independientes: un dominio inhibidor de las cdks en el extremo N-terminal (motivo común a $p27^{Kip1}$ y $p57^{Kip2}$), y un dominio único en su extremo C-terminal capaz de inhibir la replicación del DNA. Esta inhibición se produce mediante la formación de un complejo estable entre $p21^{Waf1}$ y PCNA (*DNA polymerase d processivity factor*) (Chen y col., 1995; Li y col., 1994; Luo y col., 1995; Waga y col., 1994). El aumento de $p21^{Waf1}$ actúa inhibiendo eficazmente los complejos cic/cdk formados por cdk-2, cdk-3, cdk-4 y cdk-6, los cuales ejercen una función en las fases G_1/S del ciclo celular. Aunque puede unirse a todas estas cdks, su efecto se asocia principalmente al complejo cicD1/cdk4.

Numerosos estudios han aportado funciones adicionales de esta proteína en relación al ciclo celular. Recientemente se ha descrito que $p21^{Waf1}$ podría interaccionar directamente con el factor de transcripción E2F (Delavaine y col., 1999). La posibilidad que $p21^{Waf1}$ funcione como un modulador directo de la transcripción consiste en el reciente descubrimiento del papel regulador negativo de esta molécula en la diferenciación (Di Cunto y col., 1998). También, se ha propuesto que $p21^{Waf1}$ interaccionaría con otros factores de transcripción modulando su función. En realidad, se ha descrito que $p21^{Waf1}$ se une a la región N-terminal de Myc, e interfiere en la asociación Myc-Max, suprimiendo la transcripción de aquellos genes que dependen de Myc. En contraposición, la asociación de $p21^{Waf1}$ con Myc podría contrarrestar directamente la inhibición de la síntesis de DNA dependiente de $p21^{Waf1}$, ya que Myc interacciona por la misma región C-terminal de $p21^{Waf1}$ compitiendo con PCNA (Kitaura y col., 2000). También se ha descrito su competición con otras proteínas como MCMT (*DNA-(cystosine-5) methyltransferase*) controlando los niveles de metilación del DNA durante la replicación, así como la reparación del DNA (revisado en Dotto, 2000).

Además, se ha propuesto que los niveles de $p21^{Waf1}$ controlan a los coactivadores transcripcionales, CBP o p300, potenciando su función (Perkins y col., 1997) y actúa en sinergismo con la proteína GADD45, una proteína nuclear implicada en la inhibición de la proliferación, apoptosis y estabilidad del DNA (Vairapandi y col., 1996).

A pesar de todas las funciones adicionales descritas, la mayoría del $p21^{Waf1}$ presente en las células proliferantes normales se encuentra formando parte de los complejos ciclina-cdk activos. Esto sugiere que, en condiciones normales, $p21^{Waf1}$ no es un inhibidor de dichos complejos, sino que debe jugar un papel en la progresión del ciclo celular (Zhang y col., 1994). La inhibición total de las cdks por $p21^{Waf1}$ requiere la unión de más de una molécula de $p21^{Waf1}$ a los complejos ciclina-cdk. Cuando incrementa la expresión de $p21^{Waf1}$, por ejemplo, durante la respuesta a una lesión en el DNA o debido a citocinas como el IFN-γ, la estequiometría p21:ciclina:cdk permitiría la inhibición de la actividad quinasa e induciría una parada del ciclo celular al nivel de la fase G_1 (Brugarolas y col., 1995). Recientemente, se ha demostrado que $p21^{Waf1}$ puede, en determinados modelos celulares, inhibir el ciclo celular a nivel de la fase G_2 (Waldman y col., 1996).

La expresión de $p21^{Waf1}$ puede ser inducida por diversos estímulos, incluyendo la activación transcripcional directa por p53 en respuesta al daño en el DNA (El-Diery y col., 1993) o la inducción por MyoD durante la diferenciación muscular (Parker y col., 1995) desempeñando un papel importante en la diferenciación terminal de estas células. Además en el promotor de este gen se ha descrito la presencia de innumerables cajas de respuesta a factores transcripcionales tales como STAT1, CREB, y otros, implicando la existencia de un elevado número de vías de señalización con capacidad para modular la expresión del RNA mensajero (Gartel y Tyner, 1999).

En los últimos años se ha implicado a $p21^{Waf1}$ con un papel en la regulación de la apoptosis a varios niveles y mediante mecanismos diversos. En muchas situaciones, especialmente en aquellas células con tasas de proliferación bajas, la activación de las cdks y el tránsito a través del ciclo celular representa un paso hacia

la apoptosis, ya sea antes o después de la activación de las caspasas. Este efecto se ha observado mediante la expresión de dominantes negativos de cdks, los cuales son capaces de inhibir la muerte celular. Por tanto, la inhibición de las cdk por $p21^{Waf1}$ podría explicar su efecto protector (Harvey y col., 2000). Otro mecanismo por el cual $p21^{Waf1}$ podría proteger de la apoptosis es a través de su interacción con moléculas implicadas en procesos apoptóticos como las propias caspasas 8 y 10 (Xu y El-Deiry, 2000), la caspasa 3 (Suzuki y col., 1998), o enzimas como las SAPKs (Shim y col., 1996) o MEKKs (ASK1) (Asada y col., 1999). La asociación de $p21^{Waf1}$ con estas moléculas podría indicar que $p21^{Waf1}$ es un substrato de la vía de las caspasas, y su localización citoplasmática dependería de la degradación del extremo C-terminal, que contiene las secuencias de localización nuclear (NLS) (Levkau y col.,1998; Zhang y col., 1999). Por último, $p21^{Waf1}$ podría inducir a la expresión de genes con actividad antiapoptótica (Chang y col., 2000). Trabajos de nuestro grupo, han demostrado que $p21^{Waf1}$ es el responsable de los efectos antiapoptóticos frente a estímulos como el LPS o la falta de factores de crecimiento mediados tanto por el IFN-γ como la adhesión a la decorina en macrófagos derivados de médula ósea (Xaus y col, 1999; 2001b), aunque en estos trabajos no se llegó a establecer el mecanismo por el cual $p21^{Waf1}$ ejercía su efecto inhibidor de la apoptosis.

2.2.3.2.2. $p27^{kip1}$

Descubierto en 1993, $p27^{Kip1}$ fue identificado como un inhibidor del complejo ciclina E-cdk2 (Polyak y col., 1994a). $p27^{Kip1}$ muestra una elevada homología con $p21^{Waf1}$ en su extremo aminoterminal pero no en el C-terminal, por lo que $p27^{Kip1}$ no interacciona con PCNA y, por tanto, no inhibe la replicación del DNA (Kato y col., 1994).

$p27^{Kip1}$,a diferencia de $p21^{Waf1}$, juega un papel principal en la regulación de respuestas debidas a señales externas. En realidad, mientras que la expresión de $p21^{Waf1}$ es normalmente reducida o inexistente en células quiescentes, $p27^{Kip1}$ es inducido en fibroblastos en ausencia de suero o factores de crecimiento y su expresión es requerida para la parada en G_1 del ciclo celular (Coats y col., 1996). En células normales, el aumento de la expresión de $p27^{Kip1}$ media la parada en la fase G_1

inducida por el TGF-β (Polyak y col., 1994b), la inhibición por contacto (Polyak y col., 1994a) o por la pérdida de adhesión a la matriz extracelular (Fang y col., 1996). Además, agonistas o agentes que aumentan los niveles de cAMP aumentan los niveles de $p27^{Kip1}$ inhibiendo el ciclo celular en macrófagos (Kato y col., 1994; Xaus y col., 1999c). $p27^{Kip1}$ es también inducido por otros factores inhibidores de la proliferación como el tratamiento con rapamicina en linfocitos T (Nourse y col., 1994) o por IFN-γ en células epiteliales de mamífero (Harvat y col., 1997). Por otro lado, a diferencia de $p21^{Waf1}$, la expresión de $p27^{Kip1}$ es inhibida cuando los fibroblastos quiescentes (Coats y col., 1996), células epiteliales (Sgambato y col., 1996) o linfocitos T (Nourse y col., 1994) son estimulados con factores de crecimiento. Por tanto $p27^{Kip1}$ juega un claro papel como regulador negativo del ciclo celular en una variedad de tejidos y de condiciones (Sherr y Roberts, 1995).

La regulación de $p27^{Kip1}$ se da principalmente a nivel postranscripcional, mientras que en el caso de $p21^{Waf1}$ ésta se produce principalmente por mecanismos de regulación trancripcional. En realidad, los niveles de mRNA de $p27^{Kip1}$ son constantes a lo largo del ciclo celular, mientras que la proteína es rápidamente degradada por la vía de ubicuitina-proteosoma cuando las células están proliferando mientras que la proteolisis de $p27^{Kip1}$ se encuentra reducida en células quiescentes (Pagano y col., 1995). $P27^{Kip1}$ actúa mayoritariamente inhibiendo la actividad de los complejos ciclina E/cdk2. Sin embargo, en contraposición, $p27^{Kip1}$ también puede actuar como substrato de estos complejos cicE/cdk2, resultando fosforilado en la T(187). Esta fosforilación es la responsable de la regulación de la degradación de la proteína. Por lo tanto, una vez activados los complejos cicE/cdk2 pueden mediar la degradación de $p27^{Kip1}$, haciendo así irreversible la entrada a la fase S del ciclo celular (Nguyen y col., 1999; Sheaff y col., 1997). En realidad, se ha visto que la expresión del dominante negativo de cdk-2 bloquea la fosforilación y degradación de $p27^{Kip1}$. Además la activación de la proteína Ras puede inducir la degradación de $p27^{kip1}$ en varios tipos celulares (Aktas y Cai., 1997; Takuwa y Takuwa, 1997) a través de la vía mediada por Rho, la cual es capaz de activar a los complejos cicE/cdk2 (Hu y col., 1999); o la vía de las MAPK (Kawada y col., 1997). La importancia de $p27^{Kip1}$ como regulador negativo del ciclo celular *in vivo* se demostró en estudios realizados con

ratones deficientes en este gen (*knock-out*). Estos animales son más grandes comparados con los ratones control, y presentan un alargamiento de los órganos internos, especialmente del timo, bazo y glándula pituitaria (Fero y col., 1996; Kiyokawa y col., 1996; Nakayama y col., 1996).

Otro tipo de evidencias sugieren el papel de $p27^{Kip1}$ en procesos de diferenciación en numerosos tipos celulares, como es el caso de la diferenciación de osteoblastos (Drissi y col., 1999) u oligodendrocitos (Casaccia-Bonnefil y col., 1997). En este sentido, la expresión ectópica de $p27^{Kip1}$ induce, en la línea celular mielomonocítica U937, la inhibición del ciclo celular y la inducción de marcadores de superficie específicos de macrófagos (Liu y col., 1996c).

2.3. Inhibidores de la proliferación inducida por el M-CSF

Existen diversos mecanismos de inhibición de la proliferación de los macrófagos inducida por el M-CSF. En general, los agentes que promueven la elevación de los niveles intracelulares de adenosina monofosfato cíclico (AMPc) son potentes inhibidores de la síntesis de DNA inducida por el M-CSF (Vairo y col., 1990). Un ejemplo natural de este tipo de inhibición lo constituyen la prostaglandina E_2 (PGE_2) y la adenosina, los cuales inducen una drástica elevación de los niveles intracelulares de AMPc en monocitos y macrófagos (Cheung y Hamilton, 1992; Xaus y col., 1999a; 1999c). El aumento de los niveles de AMPc activa a la proteína quinasa dependiente de AMPc (PKA), su principal diana. El aumento de la actividad de PKA disminuye la expresión de c-Myc, la ciclina D_1 y la quinasa cdk-4 en macrófagos estimulados con M-CSF (Vadiveloo y col., 1996; Vairo y col., 1996), así como incrementa los niveles de $p27^{kip1}$ (Xaus y col., 1999c).

Por otro lado, y como ya hemos comentado con anterioridad, la activación de los macrófagos supone una disminución de su capacidad proliferativa. Así, la activación de los macrófagos por interferones (IFN) de tipo I (IFN-α y -β) y II (IFN-γ), el factor de necrosis tumoral (TNF-α) α o el lipopolisacárido (LPS) también son potentes

inhibidores de la proliferación de los macrófagos, produciendo la detención de las células en la fase G_1 del ciclo celular (Vairo y col., 1996; Xaus y col., 1999b). En el caso del TNF-α y del LPS, la inhibición parece estar mediada por la inducción de la síntesis endógena de IFN-α y β, mientras que el IFN-γ bloquea la proliferación por un mecanismo diferente (Hamilton y col., 1996a). Aunque en ambos casos se observa una disminución de la actividad de los complejos ciclina-cdk y en los niveles de *c-myc*, se desconocen por el momento los mecanismos moleculares que median estos efectos.

3. ACTIVACIÓN DE LOS MACRÓFAGOS

La activación de los macrófagos es un proceso complejo y estrictamente controlado que consiste en una serie de modificaciones morfológicas, bioquímicas y funcionales que culminan en el aumento del potencial de la célula para ejercer funciones complejas, tales como la presentación de antígenos, la lisis de células tumorales y la actividad bactericida. Los agentes más potentes en la activación de los macrófagos son la citocina IFN-γ y el lipopolisacárido (LPS), aunque también otros agentes como el GM-CSF, M-CSF, IL-1, IL-2, IL-4 y TNF-α pueden inducir algunos aspectos de la activación (Celada, 1994; Celada y Nathan, 1994; Hamilton y Adams, 1987)

3.1 El Lipopolisacárido (LPS)

3.1.1. Características bioquímicas y funcionales del LPS.

El LPS, también conocido como endotoxina, es uno de los componentes estructurales de la membrana exterior de las bacterias Gram negativas. Desde el punto de vista estructural, el LPS está formado por tres regiones distintas: una región

central compuesta por polisacáridos repetitivos, una estructura de tipo antígeno-O, ambas hidrofílicas, y un dominio hidrofóbico formado por seis cadenas de ácidos grasos, también denominado lípido A, que representa el componente biológicamente activo del LPS. Debido a su estructura, el LPS actúa como una molécula anfipática y muy poco soluble en soluciones acuosas (Ulevitch y Tobias, 1995). El LPS funciona como un antígeno timo-independiente, es decir, es capaz de estimular a los linfocitos B en ausencia de linfocitos T cooperadores. Además, el LPS activa a los linfocitos B sin la necesidad de interaccionar con las inmunoglobulinas presentes en la superficie de este tipo celular (Celada, 1994).

En los macrófagos, el LPS induce la síntesis de citocinas, tales como TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, IFN-α/β, TGF-β, metabolitos del ácido araquidónico (prostaglandinas y leucotrienos) y otros lípidos bioactivos (factor activador de plaquetas, etc), péptidos quimiotácticos como MIP-1α (*macrophage inflammatory protein*), y especies reactivas del oxígeno y del nitrógeno. La mayor parte de estos productos inducidos por el LPS actúan de forma autocrina sobre los propios macrófagos y algunos de ellos, como por ejemplo, los metabolitos del ácido araquidónico o el IFN-α/β, controlan la expresión de genes tardíos en el contexto de la respuesta al LPS. Sin embargo, no todos estos productos ejercen un efecto positivo sobre la respuesta de los macrófagos. Por ejemplo, la IL-10 forma parte de un mecanismo autoinhibidor que controla la producción de otras citocinas como el TNF-α (Sweet y Hume, 1996). También se ha descrito un efecto negativo del LPS sobre algunas de las actividades inducidas por el IFN-γ, tales como la expresión de las moléculas del complejo principal de histocompatibilidad (MHC) de clase II o la expresión de los receptores *scavenger* (Celada y col., 1989; Koerner y col., 1987; Roselaar y Daugherty, 1997; Steeg y col., 1982; Xaus y col., 2000a)

Mientras que una respuesta inflamatoria local generada por el LPS es beneficiosa para la lucha contra las infecciones de bacterias Gram negativas, la respuesta inmunológica generalizada y exagerada debido a la presencia de LPS en la circulación puede conducir a un shock endotóxico, capaz de causar la muerte del individuo (Wright y Kolesnick., 1995)

3.1.2. El receptor del LPS

El receptor de alta afinidad del LPS pertenece a una familia denominada *Toll-like receptor* (TLR). Los TLRs son proteínas transmembranarias de tipo I que estan altamente conservadas en los mamíferos y en los insectos. Estos receptores presentan una alta homología en la región citoplasmática con los receptores de la interleucina IL-1 (IL-1R) porque ambos presentan unos dominios conservados denominados TIR (*Toll/IL-1R homology*). En cambio, en la región extracelular, los TLRs contienen dominios ricos en leucina (LRR), importantes en la interacción proteína-proteína, mientras que los IL-1Rs contienen tres dominios emparentados con las inmunoglobulinas. Hasta el momento se han descrito diez miembros diferentes de TLRs que se expresan diferencialmente a lo largo de los diversos tipos celulares del sistema inmunológico y responden a diferentes estímulos. Los TLRs más estudiados corresponden al TLR4, como receptor específico del LPS presente en las bacterias Gram negativas; y el TLR2, como el receptor de péptido-glicanos y lipopéptidos de las bacterias Gram positivas. La especificidad de los TLRs por los diferentes ligandos, permite a la célula recibir información acerca de la naturaleza del antígeno y transmitirlo dentro de la célula. Con esta información, el sistema inmunológico innato puede responder directamente contra una amenaza de infección. Por ejemplo, la expresión de diferentes TLRs en diferentes poblaciones de células dendríticas puede condicionar el desarrollo de una respuesta linfocitaria del tipo T_H1 o T_H2. (Akira y col., 2001; Pulendran y col., 2001; Underhill y col., 1999; Underhill y Kocinsky, 2002) (Fig. 9).

Para que se produzca el reconocimiento del LPS, y posterior señalización intracelular por parte del receptor TLR4, es necesario que el LPS se encuentre unido a una proteína sérica denominada LBP (*LPS-binding protein*) (Viriyakosol y Kirkland, 1995). LBP funciona como una opsonina de partículas que contienen LPS, incluyendo a bacterias Gram negativas enteras, facilitando la unión de estas partículas con el CD14 presente en las células mieloides (Ulevitch y Tobias,1995). El CD14 es una glicoproteína que puede encontrarse anclada a una molécula de glicofosfatidilinositol (GPI) en la membrana de monocitos, macrófagos y leucocitos polimorfonucleares, o bien, soluble en la circulación (Ulevitch y Tobias, 1995). El CD14 se asocia

físicamente con un complejo proteico que incluye al TLR4 y una proteína accesoria, MD-2 (Fig. 9). A pesar de que MD-2 haya perdido el dominio transmembranario, se mantiene asociada con la célula a través de la interacción con el dominio extracelular del TLR4. Parece ser que el complejo TLR4/MD-2 es el responsable del reconocimiento directo de diferentes estructuras del LPS, que varían en función de la especie, proporcionando una alta especificidad al receptor (Akashi y col., 2000; Shimazu y col., 1999).

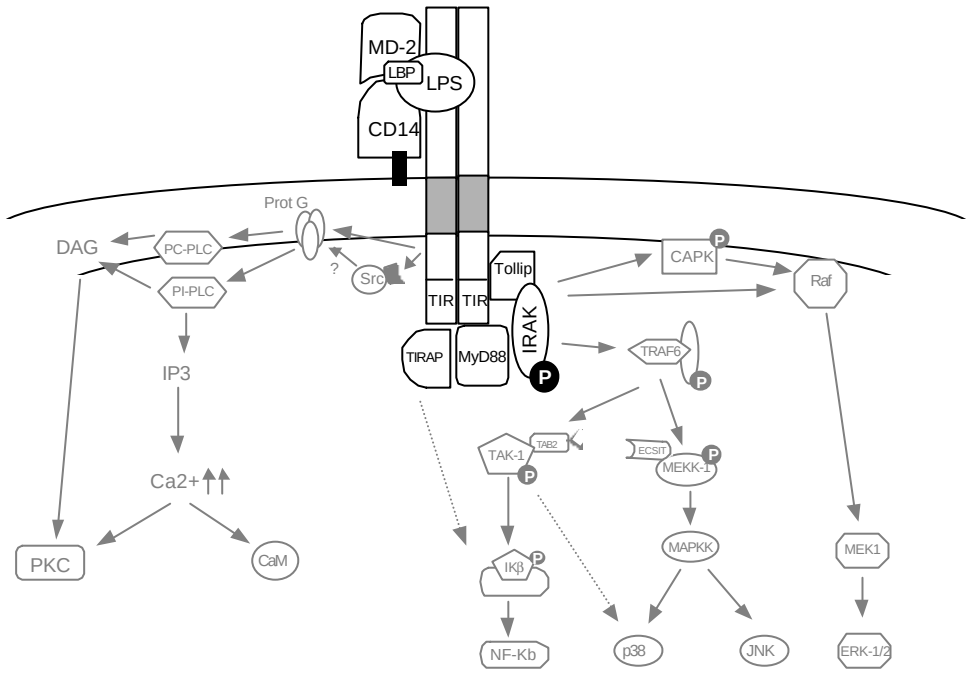


Figura 9. Vías de señalización por el receptor del LPS. CAPK, ceramida-activated protein kinase; ERK, *extracellular-regulated kinase*; IP3, inositol trifosfato; JNK, *c-jun* N-terminal quinasa; MEK, *MAPK/ERK kinase*; P, punto de fosforilación; PC-PLC, fosfolipasa C específica de fosfatidilcolina; PI-PLC, fosfolipasa específica de fosfatidilinositol; PKC, proteína quinasa C; Src, proteína quinasa de la familia Src; TIR, *Toll/IL-R homology element*; TAK, *TNF-activated kinase*; TRAF, *TNF-receptor-activated factor*.

Aunque el LPS es el ligando específico del TLR4, se ha descrito que este receptor puede reconocer y mediar la respuesta inflamatoria frente a otras moléculas, como son el componente anti-tumoral taxol, la proteína HSP60 (*Heat-shock protein* 60) liberada en condiciones de necrosis celular, el EDA (*extra domain A*) producido

por la degradación de la fibronectina en lesiones tisulares, y la proteína F del *respiratory syncitial virus* (RSV) (Underhill y Ozinsky, 2002). Así como no se descarta la existencia de otras moléculas no vinculadas al receptor capaces de reconocer, con baja afinidad, al LPS e, incluso, transmitir algún tipo de señal a altas concentraciones de LPS (Kielian y Blecha, 1995). Por ejemplo, se ha descrito que el complejo CD11b-CD18 puede reconocer a dos ligandos del TLR4, el LPS y el Taxol (Bhat y col., 1999; Wright y Jong, 1986)

3.1.3. Transducción de la señal del LPS

Una vez el LPS interacciona con todo el complejo proteico del receptor TLR4, se produce la dimerización del receptor y el consiguiente reclutamiento de una proteína adaptadora, MyD88. MyD88 contiene un dominio TIR que le permite unirse al receptor y un "dominio de muerte" (DD, *Death domain*) (ver apartado 4.2.2), que se unirá con otro dominio DD de la serina/treonina quinasa de la familia IRAK, produciéndose así la fosforilación y activación de esta quinasa (Aderem y Ulevitch, 2000; Akira y col., 2001). Recientemente, se ha descrito otra molécula, Tollip, que se encuentra constitutivamente asociada a IRAK y que puede interaccionar con los dominios TIR del receptor de TLR4 (Bulut y col., 2001; Burns y col., 2000). Una vez fosforilada, IRAK puede disociarse del receptor y de Tollip y pasa a asociarse con el Factor activado por el receptor del TNF 6 (*TNF-receptor-activated factor 6, TRAF6*). Esta asociación permite que TRAF6 interaccione con una MAPKK Kinasa, TAK-1 a través de una proteína adaptadora denominada TAB2. TAK-1 está implicada en la activación del factor de transcripción NF-κB mediante la fosforilación del inhibidor IκB (Muzio y col., 1998b; Ninomiya-Tsuji y col, 1999). La activación de TRAF6 puede también transmitir la señal iniciada por el LPS a través de la proteína adaptadora ECSIT (*evolutionarily conserved signaling intermediate*) que actuaría de puente entre TRAF6 y la activación de MEKK1 (Kopp y col., 1999). El resultado final de este proceso conduce a la activación de dos vías; por un lado la activación de las MAP quinasas JNK y p38 y por otro lado hacia la familia Rel de factores de transcripción, entre ellos NF-κB (Fig. 9).

Recientemente, se ha demostrado la existencia de otra vía de señalización mediada por el LPS independiente de la proteína adaptadora, MyD88. Esta vía fue identificada al detectarse tan solo una inhibición parcial de la activación de NF-κB en células deficientes en MyD88, mientras que en células deficientes en el receptor TLR-4, la inhibición era total (Kawai y col., 1999). La proteína implicada en la activación de esta vía corresponde a TIRAP o MAL, con un dominio TIR en el extremo carboxi-terminal parecido al presente en MyD88. Se conoce muy poco sobre la función específica y la vía de señalización de TIRAP/MAL. Se ha descrito que también puede activar a NF-κB pero independientemente de MyD88, y que puede interaccionar con otros miembros de la familia IRAK (Fitzgerald y col., 2001; Horng y col., 2001). Por otro lado, la vía de TIRAP/MAL se ha implicado con la activación de otros substratos como la proteína serina/treonina quinasa activada por RNA de doble hebra (dsRNA) (PKR) (Horng y col., 2001). PKR ha estado implicada en la activación de p38 MAP quinasa y en la regulación de la expresión de la óxido nítrico sintetasa inducible (iNOS) (Goh y col., 2000; Uetani y col., 2000) (Fig. 9).

Aunque el mecanismo exacto de transducción de la señal por el receptor del LPS no está bien definido en algunos casos, se han descrito numerosas vías de señalización intracelular activadas tras la estimulación de los macrófagos con LPS (Sweet y Hume, 1996). Así, la unión del LPS con su receptor (MD-2/CD14/TLR4) induce la activación transitoria de tirosina-quinasas de la familia Src, como $p53/56^{lyn}$, $p58/64^{hck}$ y $p59^{c\text{-}fgr}$ (English y col., 1993; Stefanova y col., 1993; Weinstein y col.,1992), proteínas G heterotriméricas con un amplio espectro de funciones, miembros de la familia PKC de serina/treonina quinasas, e induce también la formación de substratos importantes para la activación de PKC y de la proteína quinasa activada por ceramida (CAPK) (Yao y col., 1995).

3.1.3.1. Activación de proteínas G, PKC y PKA

La activación de proteínas G heterotriméricas, formadas por una subunidad catalítica, α, y dos subunidades reguladoras β y γ, implica la unión de esta proteína a una molécula de GTP y la consiguiente disociación de la subunidad α de las subunidades reguladoras. A continuación, la subunidad α es capaz de activar a otros efectores, como la fosfolipasa C (PLC). El LPS activa sobre todo a la PLC específica de fosfatidilcolina (PC-PLC), pero también se ha detectado activación de la PLC específica de fosfatidilinositol (PI-PLC) (Fig. 9). En ambos casos, parece ser necesaria la activación previa de tirosina-quinasas. Tanto la activación de PC-PLC como la de PI-PLC culminan en la generación de DAG. Dicho DAG permite la activación de las PKC convencionales y nuevas. La actividad de PI-PLC también genera el segundo mensajero fosfatidilinositol 1,4,5-trifosfato (IP_3), capaz de estimular la movilización de iones calcio, contribuyendo a la activación de las isoformas de PKC dependientes de calcio (convencionales) y a la Calmodulina (apartado 3.1.3.3). Además, el propio LPS puede, por si solo, constituir un segundo mensajero. De hecho, el lípido A del LPS puede unirse a la fosfatidilserina de las PKC convencionales, ejerciendo una acción directa sobre estos enzimas (Ellis y col., 1987). La activación de las PKC por parte del LPS permite la producción de TNF-α e IL-1β en monocitos humanos (Shapira y col., 1994). En los macrófagos murinos estimulados con LPS, la PKC induce la síntesis de óxido nítrico y la adquisición del estado tumoricida (Celada y Schreiber, 1986; Fujihara y col., 1994; Novotney y col., 1991, Paul y col., 1995) y colabora en la reorganización del citoesqueleto (Li y Aderem, 1992; Seykora y col., 1991).

Los macrófagos expresan en la membrana plasmática niveles elevados de un subgrupo de proteínas G heterotriméricas denominadas $G_i\alpha 2$. Tras la estimulación con LPS, la $G_i\alpha 2$ es internalizada y asociada a las vesículas y las vacuolas (Sweet y Hume, 1996). La inhibición de $Gi\alpha$ bloquea la producción de IL-1, de derivados del ácido araquidónico y del óxido nítrico inducida por el LPS (Coffee y col., 1990; Daniel-Issakani y col., 1989; Jakway y DeFranco, 1986), mientras que potencia la producción de TNF-α en respuesta al LPS (Zhang y Morrison, 1993). Por otro lado, la simple

inhibición de Giα es capaz de imitar la citotoxicidad y la inhibición de la proliferación inducida por LPS (Hume y Denkins, 1989). Estos datos demuestran que una misma proteína G puede estar involucrada en la regulación positiva de algunas respuestas al LPS y en el control negativo de otras.

Además, el LPS también es capaz de inducir la activación de la proteína Gs, cuyo efector es la adenilato ciclasa, enzima responsable de la generación de AMPc (Hepler y Gilman, 1992). De hecho, la producción de AMPc por este enzima y la consiguiente activación de PKA, ha sido descrita en la línea de macrófagos J774 estimulados con LPS y parece funcionar como un mecanismo coestimulador de la activación del factor de transcripción NF-κB, así como la expresión de cierto grupo de genes, entre los que se encuentra la IL-6 (Fujihara y col., 1993; Muroi y Suzuki, 1993). Sin embargo, la estimulación de esta vía debe estar estrictamente controlada en el tiempo, ya que una activación prolongada de la PKA inhibe la síntesis de citocinas como TNF-α y otros aspectos de la activación del macrófago (Zhong y col., 1995).

3.1.3.2. Activación de MAP quinasas

La activación del receptor del LPS induce la fosforilación en tirosina de un gran número de proteínas citoplasmáticas y la consiguiente activación de múltiples MAP quinasas. JNK y p38 son las principales MAP quinasas activadas en respuesta al LPS y a otros estímulos de estrés celular. En contraposición, también se ha descrito que el LPS es capaz de activar a las quinasas ERK-1/2, a pesar de que estas proteínas han sido ampliamente relacionadas con procesos de proliferación (ver apartado 2.1.3.2 de la Introducción)

3.1.3.2.1 Activación de JNK (*c-jun NH2-terminal kinase*)

La quinasa JNK, también conocida como SAPK, representa el segundo grupo de MAPKs que han sido identificadas en mamíferos. Las proteínas JNK están

codificadas por tres genes. Los genes Jnk1 y Jnk2 se expresan de forma ubicua en todos los tejidos. En contraposición, el patrón de expresión de Jnk3 se encuentra restringido a cerebro, corazón y testículos. Estos genes, mediante procesamiento (*splicing*) alternativo, generan unas diez isoformas diferentes de JNK, de unos 46 a 55 KDa. La especificidad de estas isoformas determina el substrato sobre el cual actuarán (Gupta y col., 1996). Por ejemplo, se ha descrito que el factor de transcripción c-Jun interacciona y es fosforilado principalmente por JNK1, mientras que ATF2 es preferencialmente activado por JNK2 (Kallunki y col., 1994; Sluss y col., 1994).

La cascada de señalización de la vía JNK empieza con la activación del primer módulo MAPKKK (Fig. 10). Mediante ensayos de transfección y actividad quinasa *in vitro*, se han descrito las proteínas implicadas en este primer módulo. Así, en este primer módulo encontramos a los miembros de la familia MEKK (MEKK1-4), los de la familia MLK (*mixed-lineage protein kinase*) (MLK1-3, DLK y LZK), de la familia ASK (*Apoptosis signal-regulating Kinase*) (ASK1-2), TAK (*TGF-b-activated kinase-1*) y Tpl-2. En muchos de estos casos, la activación de estas proteínas dependerá del tipo de estimulo que reciba la célula (Ichijo y col., 1997; Tanaka y Hanafusa, 1998; Tibbles y col., 1996; Salmeron y col., 1996; Shirakabe y col., 1997).

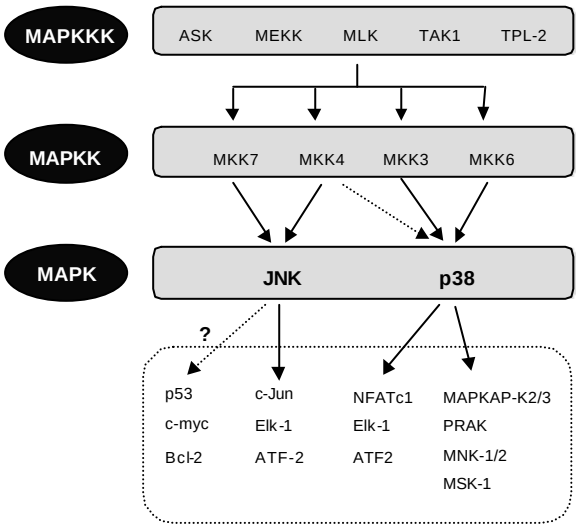


Figura 10. Módulos de activación de JNK y p38 MAP quinasas. JNK**,** ASK, apoptosis signal-regulating kinase; *c-jun N-terminal kinase*; MAPK, *mitogen-activated protein kinase*; MAPKK, *MAPK Kinase*; MAPKKK, *MAPKK Kinase;* MEK, *MAPK/ERK kinase*; MLK, *mixed-lineage protein kinase*; TAK, *TGF-b-activated kinase.*

Numerosos estudios han implicado a las proteínas GTPasas de la familia Rho, como iniciadoras de la activación de este primer módulo MAPKKK. Estas proteínas, relacionadas con la regulación del citoesqueleto, pueden ser activadas por tirosina quinasas de receptores extracelulares. Los substratos potenciales de esta familia de GTPasas incluyen a los miembros de la familia MLK y de MKK (Fanger y col., 2000; Schleissinger, 2000). Por otro lado, la activación de JNK por citocinas inflamatorias parece mediada por la proteína adaptadora TRAF (Liu y col.,1996b). La activación del receptor del TNF-α, mediante el reclutamiento de TRAF2, podría inducir la activación de MEKK1 y ASK1 (MAPKKK), con la consiguiente activación de JNK (Baud y col., 1999; Nishitoh y col., 1998). En el caso de la citocina IL-1, la proteína TRAF6 sería la responsable de la activación de este primer módulo a través de dos posibles mediadores, ECSIT (Baud y col., 1999; Lomaga., 1999) o TAB2 (Takaesu y col., 2000).

Finalmente, las proteínas JNK podrán ser activadas por fosforilación en residuos treonina y tirosina por un segundo módulo MAPKK. A este grupo pertenecen las isoformas de MKK4 (también conocida como SEK1) y MKK7 (Fig. 10). Estas isoformas son diferencialmente activadas por las diversas MAPKKK (Tournier y col., 1999). La proteína quinasa MKK7 es preferencialmente activada por citocinas (TNF-α e IL-1β), mientras que la activación de MKK4 desempeña un papel importante en situaciones de estrés. Las comparaciones bioquímicas de MKK4 y MKK7 demuestran que aunque ambas proteínas pueden fosforilar a JNK, estas quinasas presentan especificidades diferentes por otros substratos. Por ejemplo, MKK4, pero no MKK7, puede, además de fosforilar a JNK, activar a p38. Esta diferencia de especificidad sugiere que en algunas circunstancias, MKK4 y MKK7 pueden cooperar para activar a JNK (Lawler y col., 1998).

Entre los substratos de JNK, encontramos al factor de transcripción c-Jun. JNK también puede activar a otros factores, como JunB, JunD y Elk-1, participando de forma directa e indirecta en la activación del complejo transcripcional AP-1 (*Activator-protein-1*) implicado en diversos procesos celulares como la proliferación, la diferenciación y la adhesión (Ip y Davis, 1998; Yang y col., 1997). Además JNK

participa en la activación de ATF2, el cual heterodimeriza con c-Jun y estimula la expresión del propio gen *c-jun* en un bucle de retroalimentación (*feedback*) positivo (Van Dam y col., 1995) (Fig. 10).

Por otro lado, JNK juega un papel en la señalización de los procesos apoptóticos de la célula, aunque éste no está todavía muy bien definido. Dependiendo del contexto celular, JNK ha estado implicada tanto en la regulación de la apoptosis como de la supervivencia. Entre los posibles candidatos a la regulación de los procesos de muerte celular por JNK incluye a la proteína supresora de tumores p53. En este sentido, JNK contribuiría en la regulación de la estabilidad de esta proteína (Fuchs y col., 1998). Por último, existen observaciones acerca de que JNK es requerida en los procesos apoptóticos, como por ejemplo para la liberación del citocromo c de las mitocondrias y posterior activación de la vía de las caspasas (Chen y Tan., 2000; Hatai y col., 2000; Tournier y col., 2000). También se ha relacionado a JNK con la fosforilación y consiguiente inactivación de miembros de la familia Bcl-2 (Yamamoto y col., 1999). Finalmente, y en el lado opuesto, se ha descrito un posible papel de JNK regulando señales de supervivencia a través de integrinas y adhesión celular (Almeida y col., 2000).

3.1.3.2.2. Activación de p38

Este último subgrupo de las MAP quinasas comprende a cuatro miembros de serina/treonina quinasas de 38 KDa, de ahí su nombre. Estas isoformas son conocidas como p38α (también denominada *CSAIDs binding protein* ,CSBP o SAPK2a), p38β (SAPK2b o p38-2), p38γ (SAPK3 o ERK6) y p38δ (SAPK4). Al igual que la subfamilia de JNK, las p38s son fuertemente activadas *in vivo* por condiciones de estrés en el entorno celular y/o citocinas inflamatorias, aunque también se ha descrito su activación por la insulina y algunos factores de crecimiento (revisado en Kyriakis y Avruch, 2001). En casi todos los casos, los mismos estímulos que activan a JNK también pueden activar a p38, aunque hay algunas excepciones. En condiciones de isquemia, por ejemplo, se produce la activación de p38 pero no es detectable la activación de JNK (Kyriakis y Avruch., 1996)

En este caso, el módulo MAPKKK lo comprenden miembros de las familias MEKK, ASK y TAK. Al igual que en la vía de JNK, los miembros de la familia Rho de las GTPasas, entre otros efectores, activan este primer módulo (Zhang y col., 1995). La familia MEKK ejerce un papel promiscuo en la activación de la vía de las MAP quinasas. MEKK-1 puede fosforilar tanto a los miembros MKK7 y MKK4, del segundo módulo de activación de las JNK, mientras que MEKK-2 y MEKK-3 pueden activar a las tres subfamilias (ERK, JNK y p38) (Johnson y col., 1996) (Fig. 10).

Las proteínas MKK3 (también MEK3 o SKK2) y MKK6 (MEK6 o SKK3) del segundo módulo MAPKK son mayoritariamente las responsables de la fosforilación de p38; aunque en algunos casos, MKK4 (SEK1) puede también activar a p38 (Derijard y col., 1995). Al igual que en JNK, estas quinasas presentan diferentes especificidades por el substrato. Por ejemplo MKK3 es activada preferentemente en condiciones de estrés físico/químico y ejerce un efecto directo sobre p38α y p38β. En cambio, todos los agentes capaces de activar a p38 también pueden activar a MKK6, la cual es capaz de activar a todas las isoformas conocidas de esta quinasa (Cuenda y col., 1997).

Por último, los principales substratos de las p38 son las serina/treonina quinasas de la familia MAPKAR-K2 ,-3 (*MAPK-Activated Protein Kinase*) y PRAK (*p38-regulated/activated kinase*). La acción de estas quinasas sobre la proteína de shoc térmico HSP27 contribuye a la reorganización del citoesqueleto afectando a la motilidad celular (Huot y col., 1997). Por otro lado, p38 modula directamente a las quinasas MNK-1/2 y MSK-1 (*mitogen- and stress-activaded protein kinases*). Estas quinasas pueden ser activadas tanto por ERK-1/2 (en respuesta a estímulos mitogénicos) como por p38 (en respuesta a estrés celular). Las MNK-1/2 intervienen en la regulación traduccional mediante la activación del factor eIF-4E (Waskiewicz y col., 1999). Por otro lado, MSK-1 es un potente activador *in vitro* de la quinasa CREB, y por tanto, controla la regulación transcripcional de los genes inducidos por este factor (Deak y col., 1998). Otros factores de transcripción activados por p38 incluyen a NFATc1 (NFAT2) que es crucial en la diferenciación de los linfocitos T_H0 a un fenotipo efector T_H2 (Chow y col., 2000) y a los factores ATF2 y AP-1 mediante la fosforilación

directa del factor ATF2 o a la estimulación de la expresión de los genes que forman AP-1 (Gupta y col., 1995; Karin y col., 1997) (Fig. 10).

3.1.3.3 Otras vías de transducción de la señal (segundos mensajeros**)**

El LPS induce la activación de la esfingomielinasa, un enzima encargado de degradar la esfingomielina y generar ceramida. La ceramida actúa como un segundo mensajero para la activación de la proteína quinasa CAPK y de la proteína-fosfatasa activada por ceramida, CAPP. Uno de los substratos directos de CAPK puede ser el propio Raf-1, contribuyendo de este modo a la activación de la cascada Raf/MEK/ERK (Yao y col., 1995). El lípido A y la ceramida presentan un elevado grado de homología estructural, por este motivo se ha descrito que el LPS es capaz de unirse y activar directamente a CAPK (Joseph y col., 1994; Wright y Kolesnick, 1995). Además, el tratamiento de los macrófagos con análogos permeables de la ceramida, así como con esfingomielinasa, es capaz de estimular algunos de los efectos del LPS. Sin embargo, en los macrófagos de la cepa C3H/HeJ, que no pueden responder al LPS por una mutación a nivel del CD14, los análogos de ceramida no inducen tales efectos (Barber y col., 1995). Esto sugiere la necesidad de un CD14 funcional para los efectos de la ceramida aunque éstos se produzcan a nivel intracelular.

Finalmente, algunas respuestas al LPS requieren la movilización del calcio intracelular. La elevación de los niveles citoplasmáticos de calcio constituye un mecanismo de activación de algunas isoformas de PKC y de la calmodulina. En los macrófagos no estimulados, encontramos elevados niveles de calmodulina libre en el citoplasma. Tras un incremento de los niveles de calcio intracitoplasmáticos, la calmodulina experimenta un cambio conformacional asociado a la unión de los iones de calcio. En este estado activo, la calmodulina se une a diversas proteínas, incluyendo quinasas y fosfatasas dependientes de calmodulina, como la Ca-calmodulin quinasa II (CaMKII) o la calcineurina, y regula la actividad enzimática de las mismas (Hook y Means, 2001).

La Calcineurina, también conocida como PP2B, es un heterodímero compuesto por una subunidad catalítica A (60 KDa) y una subunidad reguladora B (17 KDa). La subunidad A es la que se une a la calmodulina, mientras que la subunidad B se une a iones Ca^{2+}. La subunidad A presenta baja actividad fosfatasa basal y ésta es estimulada mediante la asociación con la calmodulina. A su vez, la subunidad B asociada a Ca^{2+} potencia la activación de la subunidad A. La calcineurina también puede ser activada por otros cationes divalentes, tales como Ni^{2+} y Mn^{2+}. Entre los substratos defosforilados por la calcineurina encontramos a la subunidad reguladora de PKA, a MAP-2 y a Tau (Shenolikar y Nairn, 1991). En linfocitos T, la calcineurina está involucrada en la activación del factor de transcripción NF-AT, el cual está presente en el citoplasma de células no estimuladas. La defosforilación de NF-AT mediada por esta fosfatasa induce la translocación del mismo al núcleo, donde puede asociarse al dímero AP-1 y controlar así la transcripción de genes importantes, tales como IL-2 y TNF-α (Goldfeld y col., 1994; Rao y col., 1997). Además, la calcineurina también induce la translocación al núcleo de otro factor de transcripción importante en los linfocitos T. Se trata del factor NF-P, necesario para la transcripción de IL-4 (Kubo y col., 1994). La actividad de esta fosfatasa puede ser inhibida por los agentes inmunosupresores Ciclosporina A (CsA) y FK506. Estos agentes se unen a receptores intracelulares conocidos como inmunofilinas, y los complejos resultantes se asocian e inhiben a la calcineurina (Schreiber y Crabtree, 1992).

En los macrófagos el papel del calcio y de las proteínas implicadas en su señalización es confuso, aunque se ha descrito que el bloqueo de la movilización de calcio inhibe la producción de citocinas como TNF-α y de substancias procoagulantes. Sin embargo, los agentes que inducen una elevación de calcio intracelular en los macrófagos, similar a la observada tras el tratamiento con LPS, son insuficientes por si solos para generar las respuestas asociadas a la activación de este tipo celular (Lo y col., 1996; West y col., 1996).

3.2. El Interferón-gamma (IFN-g)

3.2.1. Características bioquímicas y funcionales del IFN-g

El IFN-γ es una glicoproteína homodimérica formada por dos subunidades de 21 a 24 KDa, ambas codificadas por el mismo gen y asociadas de forma no covalente (Celada, 1994). El IFN-γ es producido por linfocitos T $CD4^+$ del subtipo T_H1 y por linfocitos T $CD8^+$ de fenotipo T_C1, todos ellos activados a través de la interacción entre el receptor de los linfocitos T (TCR) y el complejo antígeno-MHC (de clase I o II, según el tipo celular) presente en la superficie de una célula presentadora de antígeno. También los linfocitos *Natural Killer* (NK) producen IFN-γ en respuesta a la IL-12 y al TNF-α (Boehm y col., 1997).

Inicialmente se atribuyó al IFN-γ un efecto principalmente antivírico. Sin embargo, actualmente se sabe que esta citocina juega un papel crítico como modulador de la respuesta inmunitaria (Goes y col., 1995). Todas las células del organismo poseen receptores específicos para el IFN-γ, lo que condiciona que esta citocina tenga un cierto efecto pleiotrópico sobre distintos tipos celulares. En linfocitos B, promueve la diferenciación y el cambio de isotipo de las inmunoglobulinas (Snapper y Paul, 1987), así como la expresión de las moléculas de clase II del complejo mayor de histocompatibilidad (MHC) (Wong y col., 1983). En los linfocitos T $CD4^+$, estimula la proliferación de las células con subtipo T_H1 y bloquea la de las células T_H2, favoreciendo aquellos procesos asociados a la respuesta inmunológica específica mediada por células. En los neutrófilos y en los linfocitos NK, desencadena el proceso de activación, promoviendo la respuesta inmunológica inespecífica. A nivel de células endoteliales, el IFN-γ induce un aumento de la expresión de selectinas en la superficie celular con el fin de facilitar la extravasación de linfocitos T desde el torrente sanguíneo a los tejidos (Bach y col., 1997).

Pese a este efecto pleiotrópico, la diana por excelencia del IFN-γ son los monocitos/macrófagos, sobre los que actúa como el principal inductor del proceso de activación (Farrar y Schreiber, 1993). En estas células, induce la expresión de los

genes del MHC de clase II, promoviendo la función presentadora de antígeno de los macrófagos. Además, induce la expresión de la sintetasa del óxido nítrico inducible (iNOS), la generación de intermediarios reactivos del oxígeno, aumenta la expresión de moléculas de adhesión y de receptores FcγRI de alta afinidad, así como induce la síntesis y secreción de citocinas, tales como TNF-α, IL-1β, IL-6 e IL-12, y de componentes del sistema del complemento, por ejemplo C3 (Bohem y col., 1997; Celada y col., 1989). En particular, la inducción de la síntesis de IL-12 representa un mecanismo de retroalimentación (*feedback*) positivo en el contexto de la inmunidad específica mediada por células, ya que esta interleucina actúa como un potente inductor de la generación y proliferación de linfocitos T $CD4^+$ del subtipo T_H1 (Bellardelli, 1995).

3.2.2. El receptor del IFN-g /Transducción de la señal del IFN-g

El receptor del IFN-γ es un heterodímero formado por dos cadenas polipeptídicas, α y β, que se encuentran disociadas en el estado inactivo. Tras la unión con el ligando, las cadenas del receptor oligomerizan y dan lugar al tetrámero $\alpha_2\beta_2$ (Fig. 11). La cadena α del receptor es la responsable de la interacción con la molécula de IFN-γ, mientras que la cadena β está únicamente involucrada en la transmisión de la señal hacia el interior de la célula. En el estado inactivo la cadena α se encuentra asociada a la quinasa *Janus* (JAK)-1, y la cadena β a la quinasa JAK-2. Tras la unión con el ligando y la consiguiente oligomerización, estas quinasas interaccionan entre sí y se activan por fosforilación recíproca. A continuación, fosforilan determinados residuos tirosina que se encuentran en la región intracitoplasmática de la cadena α del receptor. Dichos residuos tirosina fosforilados son reconocidos por el dominio SH2 de la proteína STAT1α, entre otras proteínas con dominios SH2 (Fig. 11).

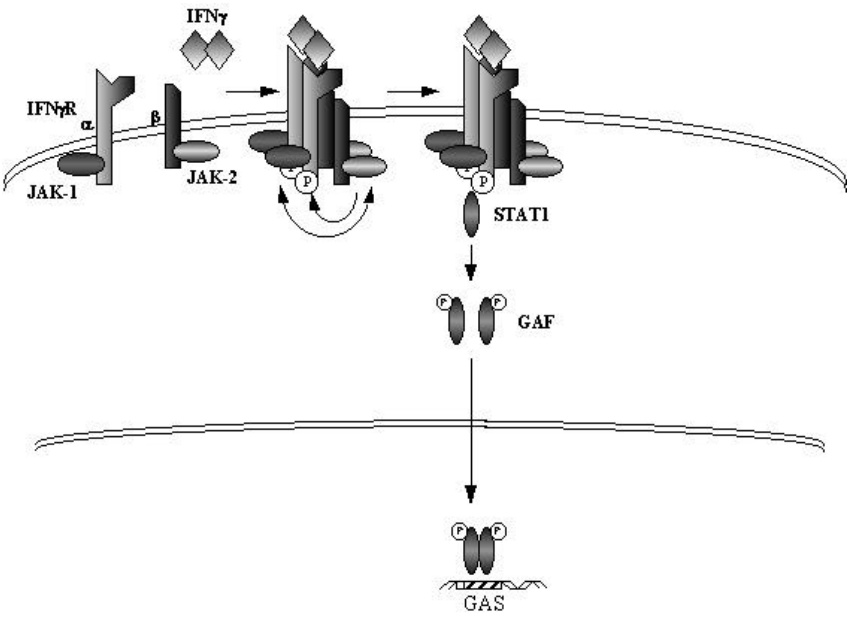


Figura 11. Transducción de la señal del IFN-g. GAF, *gamma-interferon activated factor*; GAS, *gamma-interferon activated sequence*; IFN-γ, interferon gamma; IFN-γR; receptor del IFN-γ; JAK, *Janus kinase*; P, punto de fosforilación; STAT, *signal transducer and activator of transcription* (Valledor, 1998).

Una vez que STAT1α es reclutada por el complejo receptor-JAKs activado, experimenta una fosforilación mediada por las JAKs. La fosforilación induce la dimerización de STAT1α, dando lugar al complejo transcripcional GAF (*Gamma-interferon Activated Factor*), que es translocado al núcleo donde induce la transcripción de numerosos genes mediante la unión a una secuencia de DNA específica denominada GAS (*Gamma-interferon Activated Sequence*) (Bach y col., 1997; Ihle y Kerr, 1995; Schindler y Darnell, 1995).

Entre los genes controlados por GAF, encontramos aquellos que codifican para el receptor de alta afinidad de la fracción constante de las inmunoglobulinas G (FcγRI); el transactivador de los genes del MHC de clase II CIITA (*Class II Transactivator*), necesario para la inducción de la expresión de las moléculas del MHC

de clase II; factores de transcripción de la familia IRF1, necesarios para la regulación de la expresión de genes con secuencias ISRE (*IFN-stimulated response elements*) en sus promotores, como, por ejemplo, IFNα/β, MHC clase I o la iNOS. La activación de STAT1α es transitoria y está controlada negativamente por la vía del proteosoma-ubiquitina. De esta manera, la activación de STAT1α alcanza un nivel máximo a los 15-30 min tras la estimulación con IFN-γ y disminuye hasta niveles basales en 1-2 h, permitiendo la atenuación de la respuesta al IFN-γ (Boehm y col., 1997).

Por otro lado, tras la unión del IFN-γ a su receptor, el complejo formado por la subunidad α y el ligando se internaliza, formando parte de un compartimento acídico intracelular. Allí el complejo se disocia y el IFN-γ libre es finalmente degradado en los lisosomas. El receptor disociado entra a formar parte de un reservorio intracelular de subunidades α y puede ser reciclado a la superficie (Celada y Schreiber, 1987)

Pese a que la vía de activación de JAK/STATs es el único mecanismo de señalización bien descrito para el IFN-γ, recientemente se han descrito diversas actividades mediadas por IFN-γ a través de vías alternativas a STAT1, afectando tanto a procesos de proliferación como de activación celular (Gil y col., 2001; Ramana y col., 2000; 2001).

3.3. Inhibidores de la activación

Existen diversos mecanismos de inhibición de la activación de los macrófagos inducida por el LPS o el IFN-γ, debido al carácter potencialmente dañino de los mediadores liberados tras la activación de estas células. En general, los agentes que promueven la elevación de los niveles intracelulares de adenosina monofosfato cíclico (AMPc) son potentes inhibidores de la activación de los macrófagos. Un ejemplo natural de este tipo de inhibición lo constituyen la prostaglandina E_2 (PGE_2) y la adenosina, los cuales podrían actuar como mecanismos atenuadores de la activación

de los monocitos y macrófagos en los procesos inflamatorios (Cheung y Hamilton, 1992; Xaus y col., 1999a; 1999c). Por ejemplo, la adenosina, a parte de inhibir la expresión de las moléculas de clase II inducidas por el IFN-γ, inhibe también otros aspectos de la activación de los macrófagos como son la expresión de la enzima iNOS y de citocinas pro-inflamatorias como la IL-1β y el TNF-α (Xaus y col., 1999a).

Otros grupo de inhibidores de la activación lo constituyen las citocinas como la IL-10 que es inducida por los macrófagos posteriormente a la expresión de TNF-α inducida en respuesta al LPS o por el IFN-γ, y por linfocitos T activados, pudiendo actuar como un mecanismo atenuador de la actividad de estas células (O'Farrell y col., 1998). El TGF-β es otra citocina inhibidora de la actividad de los macrófagos, y de otras células del sistema inmunológico, la cual es producida de forma autocrina por los macrófagos. Se han identificado cinco genes distintos en vertebrados y tres de estos (TGF-β1, -β2 y -β3) son expresados en mamíferos. En la bibliografía han sido descritos numerosos efectos producidos por el TGF-β. Así el TGF-β inhibe la proliferación de los linfocitos T y B, antagoniza la producción de citocinas, y previene la inducción de los receptores de IL-1 e IL-2. También suprime la producción de las inmunoglobulinas por los linfocitos B, inhibe la adhesión de los linfocitos T y los neutrófilos a las células endoteliales y suprime la actividad de las células NK (Kehrl y col., 1986; Rook y col., 1986; Wahl y col., 1988).

En los macrófagos, se ha descrito que el TGF-β inhibe la activación de estas células mediada tanto por IFN-γ como por LPS. En este sentido, el TGF-β inhibe la inducción por el IFN-γ de la producción de H_2O_2, de óxido nítrico y la expresión de iNOS, la liberación de TNF-α o la capacidad de matar a bacterias intracelulares (Bogdan y col., 1992; Vodovotz y col., 1993). Además, tras la estimulación de los macrófagos con IFN-γ, el TGF-β inhibe la expresión de las moléculas del MHC de clase II y, por tanto, la presentación antigénica. Este efecto sobre el MHC de clase II se encuentra mediado a través de unas secuencias conservadas en el promotor proximal de los genes de clase II (Reimold y col., 1993). Esta inhibición general de la vía de señalización del IFN-γ por parte del TGF-β se cree que es debida a la interacción y competición recíproca entre los factores de transcripción de la familia

STAT y SMAD activados por ambas citocinas (Pitts y col., 2001; Ulloa y col., 1999). Junto a los efectos sobre la vía de activación del IFN-γ, el TGF-β es capaz de inhibir la expresión de iNOS inducida por el LPS o la expresión de citocinas pro-inflamatorias, aunque en estos casos los mecanismos de acción son menos conocidos (Imai y col., 2000; Werner y col., 2000).

Finalmente, y aunque tanto el LPS como el IFN-γ son potentes estimuladores de la activación de los macrófagos, se ha descrito que la estimulación previa de los macrófagos con LPS puede disminuir o inhibir algunos aspectos de la activación posterior con IFN-γ. Este es el caso, por ejemplo, de la expresión de iNOS o de IL-1β inducida por el IFN-γ, la cual es reducida tras el tratamiento previo con LPS (Chujor y col., 1996). Un efecto similar ha sido descrito para los genes del MHC de clase II (Xaus y col., 2000a). En este caso se ha asociado el efecto inhibidor del LPS al bloqueo del ciclo celular de los macrófagos, previo a la estimulación con IFN-γ, (Xaus y col., 2000a).

4. MUERTE CELULAR PROGRAMADA O APOPTOSIS

El término apoptosis deriva de la palabra griega que describe el proceso de caída de una hoja de un árbol o del pétalo de una flor y fue introducido por Kerr y col., (1972) para definir cómo mueren las células en determinadas condiciones fisiológicas y patológicas.

4.1. Características morfológicas de la apoptosis

La apoptosis es un tipo particular de muerte celular, definida por una secuencia de cambios morfológicos, bioquímicos y funcionales específicos. Tal como se muestra en la Figura 12, el proceso de apoptosis comienza con una condensación

del citoplasma y una reducción del volumen celular. Este fenómeno va acompañado de la condensación progresiva de la cromatina en la cara interna de la membrana nuclear, de manera que el núcleo puede variar su aspecto morfológico. A continuación, en muchos casos, se produce una fragmentación nuclear como consecuencia de que las masas densas de la cromatina emergen hacia el citoplasma en forma de pequeñas masas rodeadas de membrana nuclear. La membrana plasmática engloba a cada uno de estos fragmentos nucleares junto con regiones del citoplasma que contienen orgánulos intactos. Seguidamente se produce la separación de los fragmentos nucleares rodeados de membrana plasmática dando lugar a los cuerpos apoptóticos. Los cuerpos apoptóticos expresan marcadores de superficie que les permite ser reconocidos y fagocitados por las células vecinas y por los macrófagos. Por ello, el resultado final del proceso de apoptosis es la desaparición de la célula sin que se libere su contenido (gránulos cargados de toxinas catiónicas, enzimas degradativos y otros agentes altamente reactivos) al espacio extracelular (Gregory, 1995; Martin, 1997). La eliminación de células por apoptosis garantiza la integridad de la célula muerta y su eficaz eliminación sin afectar a las células vecinas y sin desencadenar procesos inflamatorios.

Durante la apoptosis, las alteraciones morfológicas ocurren de forma paralela a una serie de cambios bioquímicos esenciales. Uno de los rasgos característicos del proceso apoptótico es la participación activa de la célula afectada, ya que ésta moviliza una cascada de sucesos que culminan en su propia desintegración física. El concepto de muerte activa está apoyado por la existencia de moléculas pro- y anti-apoptóticas que participan en la ejecución del proceso o en su regulación (Gregory, 1995; Martin, 1997).

En contraposición a la apoptosis, las células pueden experimentar una muerte pasiva, denominada generalmente oncosis o necrosis. Este tipo de muerte celular se observa en situaciones extremas que producen lesiones graves en la célula de manera que ésta no tiene capacidad para responder activamente. Durante la necrosis (Fig. 12), la célula y los orgánulos se hinchan y revientan como consecuencia de un incremento de la permeabilidad de la membrana plasmática tras el fallo de sus bombas iónicas (Majno y Joris, 1995). No obstante, existen muchas situaciones donde

la muerte celular es atípica y no comparte estrictamente los criterios morfológicos de la apoptosis ni los de la necrosis.

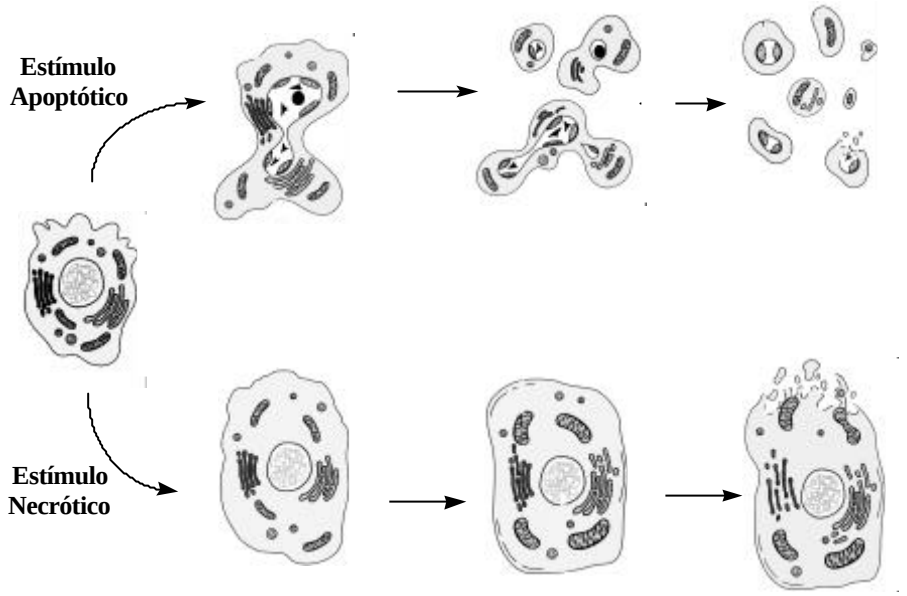


Figura 12. Características morfológicas de la muerte celular, apoptosis *versus* necrosis

4.2. Características bioquímicas de la apoptosis

Gran parte de los conocimientos que se disponen actualmente sobre las moléculas que intervienen en la apoptosis de los vertebrados deriva de los estudios genéticos realizados en el nemátodo *Caenorhabditis elegans*. Tres productos génicos de *C.elegans* son esenciales para la regulación de la apoptosis: CED-3 y CED-4, los cuales promueven la apoptosis y CED-9, un inhibidor de la misma (Hengartner y Horvitz, 1994). Los vertebrados han desarrollado familias enteras de proteínas que simulan a los genes apoptóticos de *C. elegans*. Las caspasas de los mamíferos son similares a CED-3 (Thornberry y Lazebnik, 1998). Apaf-1 es el único homólogo de CED-4 conocido hasta el momento en mamíferos (Zou y col., 1997). Los productos de la familia de genes Bcl-2 están relacionados con CED-9, pero incluyen dos subgrupos

de proteínas, las cuales tanto inhiben como promueven la apoptosis (Adams y Cory, 1998; Yang y Korsmeyer, 1996).

4.2.1. Caspasas

Las caspasas son una familia de proteasas que cortan específicamente tras un residuo de aspártico. Reconocen un motivo de al menos cuatro aminoácidos situado antes del punto de corte. El motivo tetrapeptídico preferido para cada caspasa difiere entre ellas y esto explica la diversidad de sus funciones biológicas (Thornberry y col., 1997).

Las caspasas mantienen similitudes entre ellas a nivel de su secuencia aminoacídica, estructura y especificidad para el substrato (Nicholson and Thornberry, 1997). Todas ellas están constitutivamente presentes en muchas células, residiendo en el citosol como proenzimas de cadena simple (de 30 a 50 kDa) que contienen tres dominios: un dominio N-terminal (pro-dominio), un dominio que dará lugar a una subunidad grande (20 kDa) y un tercer dominio correspondiente a una subunidad pequeña (10 kDa). Su activación se debe al procesamiento proteolítico de estos dominios, seguido por la asociación de las subunidades grande y pequeña para formar un heterodímero (Fig. 13). A continuación, dos de estos heterodímeros se asocian para formar un tetrámero, con dos sitios catalíticos que funcionan de forma independiente (Rotonda y col., 1996; Walker y col., 1994; Wilson y col., 1994). Dentro de cada dominio catalítico, la subunidad grande y la pequeña se encuentran íntimamente asociadas, contribuyendo ambas a la unión del sustrato y de su catálisis posterior.

La cascada de activación de la apoptosis empieza por las caspasas iniciadoras. Son caspasas con grandes pro-dominios y con regiones de interacción interproteica que se activan a sí mismas por autoproteolisis. Por ejemplo, la agregación de varias moléculas de pro-caspasa 8 es suficiente para permitir la autoproteolisis y activación de esta caspasa (Muzio y col., 1998a; Yang y col., 1998). Los efectos de la activación de las caspasas iniciadoras se ven amplificados por la

proteolisis y activación de otras caspasas con pro-dominios cortos y sin motivos de interacción interproteicos (caspasas 3, 6, 7) situadas por debajo de la vía de señalización de la apoptosis y que reciben el nombre de caspasas efectoras. Existen dos rutas distintas de activación de las caspasas. La vía dependiente de receptores de la membrana celular (*death receptors*) y aquella derivada de la liberación de citocromo C por parte de la mitocondria en la que intervienen la familia de proteínas Bcl-2 (apartado 4.2.3).

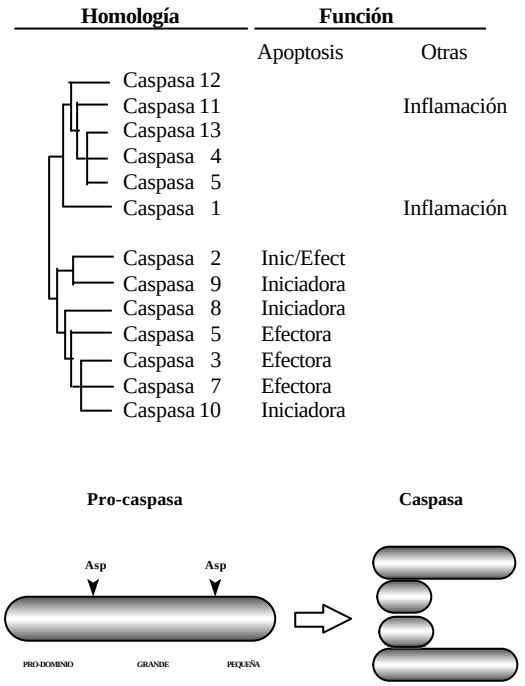


Figura 13. Función y estructura de la familia de las caspasas. Hasta la fecha se han identificado 13 caspasas distintas en mamíferos (las caspasas 11 y 12 pertenecen a ratón y todavía se desconoce su homólogo humano). Las funciones de las caspasas se han deducido a partir de experimentos con ratones *knock-out* (Thornberry y Lazebnik, 1998).

La activación de las caspasas y la proteolisis de sus dianas específicas son los responsables últimos de todas las modificaciones morfológicas y bioquímicas que se observan en la célula que sufre apoptosis. Entro los substratos de las caspasas encontramos a I^{CAD}/DFF45, un inhibidor de la nucleasa responsable de la fragmentación del DNA, (CAD, *caspase-activated deoxyribonuclease*) (Liu y col., 1997); componentes estructurales como la lámina nuclear (Orth y col., 1996; Takahashi y col., 1996), o proteínas implicadas en la regulación del citoesqueleto, incluyendo a la gelsolina (Kothakota y col., 1997), la FAK (*focal adhesion kinase*)

(Wen y col., 1997) o la PAK-2 (*p21-activated kinase* 2) (Rudel y Bokoch, 1997); o de la membrana plasmática la cual pierde la asimetría en la distribución de los fosfolípidos debido a un fallo en la enzima dependiente de ATP que, en condiciones normales, transporta los fosfolípidos cargados negativamente hacia el interior celular (Fadok y col., 1992). Además, las caspasas inactivan algunas proteínas implicadas en la reparación del DNA, como la proteína DNA-quinasa (DNA-PK), proteínas implicadas en el procesamiento de los mRNA (como la U1-70K) o en la replicación del DNA (como el factor de replicación C) (Cryns y Yuan, 1998).

4.2.2. *Death Receptors*

En los mamíferos, una de las vías de actuación de las caspasas es inducida tras la activación de algunos receptores de membrana de la familia del receptor del TNF-α (TNFR). Las proteínas implicadas en esta vía presentan dos tipos de dominios de interacción entre proteínas: el "*death domain*" (DD) y el "*death efector domain*" (DED). El DD se ha encontrado en la región intracelular de algunos receptores de la familia del TNFR, concretamente en CD95 (también denominado Fas o Apo-1) y en CD10a (TNFR1, el receptor p55 de TNF-α). También se ha descrito en algunas proteínas adaptadoras citoplasmáticas, como FADD, TRADD o RIP, las cuales, a través de este dominio, interaccionan tanto con los receptores activados como entre ellas mismas. Por su parte, el motivo DED se encuentra también en algunas de dichas proteínas adaptadoras y en las caspasas 8 y 10. La unión de estas caspasas a las proteínas adaptadoras a través de las regiones DED tiene lugar tras la activación de los receptores de la familia del TNFR, y supone el autoprocesamiento de las caspasas, su activación y, por lo tanto, la inducción de apoptosis (Fig. 14).

Por tanto, la vía de inducción de apoptosis a través de los receptores de la familia del TNFR (*death receptors*) es una vía muy rápida y de escasa sofisticación basada en el reclutamiento de moléculas adaptadoras mediante sus dominios DD o DED, cuya única función es aproximar y activar a una caspasa iniciadora, como la caspasa 8. Sin embargo, estos receptores a su vez son capaces de transmitir otras

vías de señalización más complejas, las cuales pueden ser tanto activadoras como inhibidoras de la apoptosis dependiendo del tipo celular, y que incluyen a vías o proteínas de la familia de JNK o IKK, y donde intervienen proteínas adaptadoras de la familia TRAF (Beg y Baltimore, 1996; Chinnaiyan y col., 1995; Hsu y col., 1996) (Fig. 14).

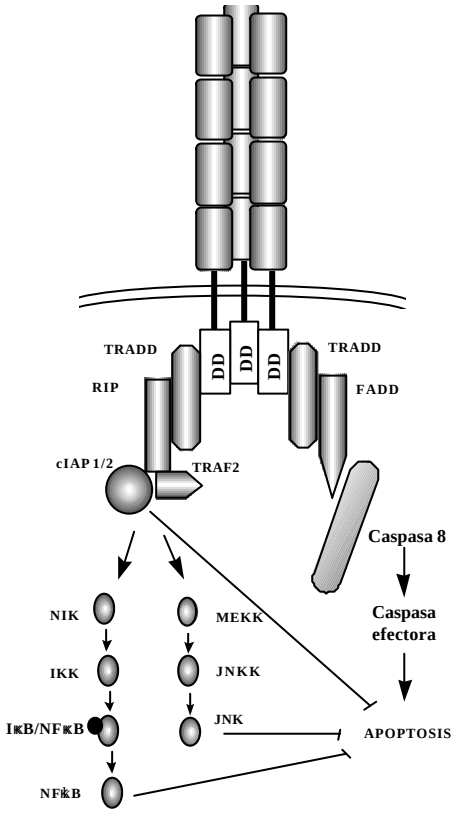


Figura 14. Señalización inducida por el receptor del TNF-α. TRADD, *TNF receptor-associated death domain*; DD, *death domain*; FADD, *Fas-associated death domain*; RIP, *receptor-interacting protein*; NIK, *NF-κB inducing kinase*; IKK, *IκB kinase complex*; IκB, *inhibitor of κB*; NFκB, *nuclear factor κB*; JNKK, *JNK kinase*; JNK, *c-Jun kinase*.

4.2.3. Familia de proteínas Bcl-2

La secuenciación del gen *ced-9* en *C. elegans* reveló que se trataba de un homólogo estructural y funcional del proto-oncogén humano *bcl-2* (Hengartner y Horvitz, 1994). En mamíferos existe toda una familia de proteínas homólogas al proto-oncogén Bcl-2 (Chao y Korsmeyer, 1998; Cory, 1995). Todos los miembros de esta familia poseen entre uno y cuatro motivos conservados, denominados dominios

homólogos a Bcl-2 (BH1-BH4). Estas proteínas se pueden clasificar en dos categorías funcionales: unas con capacidad para inhibir la apoptosis (Bcl-2, Bcl-X_L, Bcl-w, A1) y otras con capacidad para promoverla (Bax, Bak, Bik1, Bad y Bcl-X_S) (revisado en Yang y Korsmeyer, 1996).

Bcl-2 y sus homólogos anti-apoptóticos ejercen funciones clave durante el proceso apoptótico en vertebrados. Por una parte, pueden formar canales que permiten el transporte de iones y proteínas a través de las membranas intracelulares, preferentemente en el retículo endoplasmático, en la membrana nuclear (Minn y col., 1997; Reed, 1997; Schendel y col., 1997) y en la membrana mitocondrial externa, donde, por ejemplo, controlan la liberación del citocromo C (Kluck y col., 1997; Yang y col., 1997a). Por otra parte, actúan como proteínas adaptadoras, siendo capaces de interaccionar con al menos once proteínas diferentes, entre ellas Raf-1 (Wang y col., 1994; 1996), Apaf-1, una proteína que colabora en la activación de las caspasa de manera análoga a lo que ocurre en *C.elegans* entre CED-9 y CED-4 (Hengartner, 1997; Vaux, 1997) y componentes de su propia familia. En este sentido, los miembros de la familia Bcl-2 pueden formar homodímeros y heterodímeros, existiendo una relación antagónica entre las dos categorías de componentes de la familia. En muchas situaciones el destino final de la célula está determinado por la relación entre los niveles de los componentes promotores de apoptosis y los componentes inhibidores de la misma (Antonsson y col., 1997; Boise y col., 1993; Gajewski y Thompson, 1996; Oltvai y col., 1993; Reed, 1997; Sato y col., 1994; Sedlack y col., 1995; Yang y col., 1995).

4.3 Modelo de integración de la apoptosis

La muerte apoptótica puede ser desencadenada en respuesta a diferentes estímulos, tanto intracelulares como extracelulares. Así, se observa apoptosis en distintas situaciones fisiológicas en las que se produce la eliminación de células que ya no son necesarias o que representan un peligro para el organismo. Esto sucede, por ejemplo, durante el desarrollo embrionario (Jacobson y col., 1997), durante el recambio, atrofia y regresión tisular en el adulto (Carson y Ribeiro, 1993; Rotello y

col., 1989; Walker y col., 1989), como mecanismo de eliminación de timocitos que reconocen antígenos propios del individuo o bien de células que contienen lesiones genéticas (Kaufman y Paules, 1996; Shi y col., 1989) y como consecuencia de las acciones de los linfocitos T citotóxicos y de las células NK sobre sus células diana (Don y col., 1977; Kausalya y col., 1994).

Por otro lado, existen diferentes situaciones patológicas que pueden inducir la muerte apoptótica de determinadas poblaciones celulares, como es el caso de algunas infecciones víricas (Meyaard y col., 1992; Taga y Kishimoto, 1992), determinadas enfermedades degenerativas del sistema nervioso central (Raff y col., 1993) y la acción de agentes anticancerígenos y otros agentes tóxicos sobre las células en las que actúan. En cada una de estas situaciones, las vías de señalización celular que median la apoptosis pueden ser distintas (McConkey y Orrenius, 1994). En la actualidad se han descrito dos vías básicas de iniciación de la apoptosis. Una primera vía inducida por estímulos externos a la célula y en la que participa la activación de receptores específicos y otra interna, producida en respuesta a la liberación del citocromo C procedente de la mitocondria.

En el primer caso, una vez que los receptores Fas o TNFR interaccionan con su ligando específico, reclutan y oligomerizan proteínas adaptadoras y pro-caspasas. La proteína FADD, la cual contiene un dominio DED, es reclutada directamente por la molécula Fas e indirectamente por TNFR1. A continuación se produce el reclutamiento y autoactivación de la caspasa 8. De forma similar, la proteína adaptadora RAIDD (con un dominio CARD) se asocia a TNFR1 y así promueve la agregación y activación de la caspasa 2 (Fig. 15).

Por otro lado, ciertos estímulos apoptóticos inducen un colapso de la membrana interna mitocondrial y/o a una caída del potencial de membrana debido a la apertura transitoria de poros permeables en la membrana mitocondrial (Green y Reed, 1998) produciendo la liberación de citocromo C, que en consecuencia induce la asociación de Apaf-1 con la pro-caspasa 9 mediante la interacción de dominios CARD. Una segunda región en Apaf-1 permite la asociación de varias moléculas de Apaf-1,

provocando la agregación indirecta y activación de la caspasa 9 (Srinivasula y col., 1998).

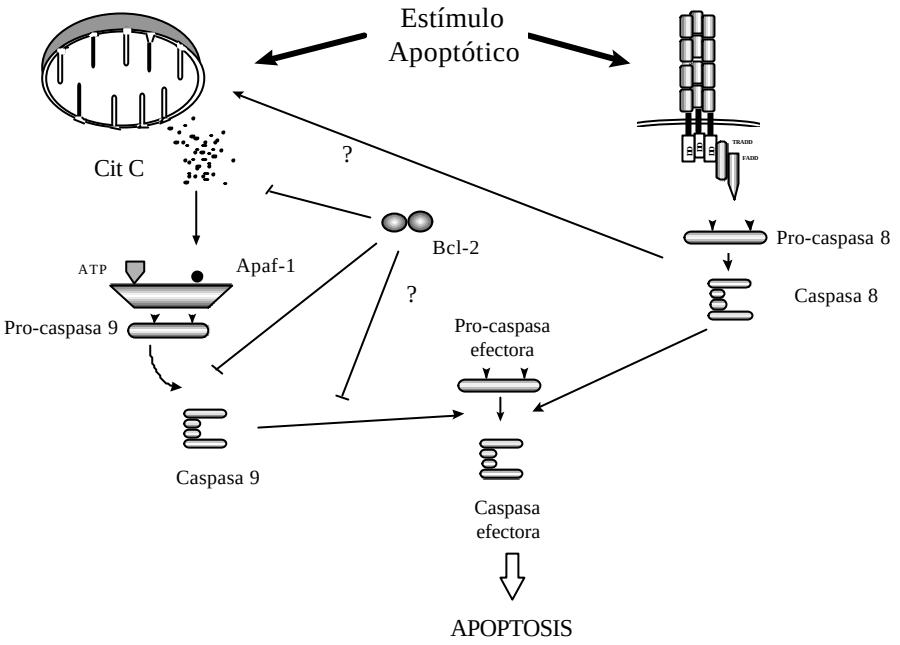


Figura 15. Modelo de integración de la apoptosis. Existen dos vías independientes capaces de iniciar la apoptosis que convergen en la activación final de las caspasas efectoras (caspasas 3, 6 y 7), la degradación de sus substratos y, por tanto, la muerte celular (Green, 1998).

Estas dos vías básicas de iniciación de la apoptosis permiten hacer predicciones de cómo ésta es regulada en determinadas circunstancias. Las proteínas de la familia Bcl-2 anti-apoptóticas (Bcl-2, Bcl-X_L) se unen a la mitocondria e inhiben la liberación del citocromo C (Kluck y col., 1997; Yang y col., 1997a). En contraposición, la proteína pro-apoptótica Bax induce de forma directa que la mitocondria libere citocromo C (Jurgensmeler y col., 1998). Sin embargo, en algunas situaciones la activación de Fas y TNFR1 puede inducir también la liberación de citocromo C, de manera que existe la posibilidad de un *cross-talk* entre las dos vías. La activación de Bid, un miembro pro-apoptótico de la familia Bcl-2, por la caspasa 8, vía receptores de

membrana, podría conducir a la activación de la caspasa 9 por la vía mitocondrial y amplificando la señal (Li y col., 1998; Luo y col., 1998). Probablemente, sólo en aquellas células en las que este bucle de amplificación mitocondrial sea importante, los miembros de la familia Bcl-2 anti-apoptóticos pueden inhibir la apoptosis mediada por Fas/TNFR1.

La inhibición de las caspasas protege totalmente de la muerte celular inducida por la vía de los receptores de membrana, pero no de la inducida a través de la vía mitocondrial. En este último caso, la célula muere posteriormente por un proceso que no coincide propiamente con la apoptosis y que es debida a la disfuncionalidad de las mitocondrias y de la cadena respiratoria (Cecconi y col., 1998). Por este motivo, las células tumorales son seleccionadas para expresar niveles elevados de las proteínas anti-apoptóticas de la familia Bcl-2, las cuales aseguran el correcto funcionamiento de la mitocondria a la vez que inhiben varios de los procesos apoptóticos, manteniendo así la viabilidad de las células

4.4. Papel de los macrófagos en la apoptosis

4.4.1. Los macrófagos como fagocitadores e inductores de apoptosis

Los macrófagos son los fagocitos profesionales encargados de la eliminación de las células apoptóticas intactas o de los cuerpos apoptóticos presentes en los tejidos. Por este motivo, los macrófagos juegan un papel trascendental en aquellos tejidos donde la carga de células apoptóticas es importante como, por ejemplo, el timo, los tejidos linfoides periféricos y los puntos de inflamación. No todas las células del linaje macrofágico son igualmente eficientes en la fagocitosis de células apoptóticas. Por ejemplo, los macrófagos activados con citocinas como el GM-CSF, IFN-γ, IL-1 y TNF-α u otras citocinas expresadas en los puntos de inflamación son capaces de fagocitar gran número de células y neutrófilos apoptóticos (Arai y col., 1990; Newman y col., 1982). Sin embargo, los monocitos recién aislados no presentan esta capacidad, aunque sí pueden fagocitar otras partículas.

El reconocimiento de las células que deben ser eliminadas por parte de los fagocitos se produce gracias a la presencia de determinadas moléculas en la superficie de las células apoptóticas. Estas moléculas interaccionan con receptores específicos en los macrófagos e inducen los procesos fagocíticos Por ejemplo, encontramos la interacción entre lectinas fagocitarias (Duvall y col., 1985), la asociación de la tromboposdina con el receptor de la vitronectina en los fagocitos (Savill y col., 1990), el reconocimiento mediado por CD36 (Hughes y Savill, 1993; Savill y col., 1992;) y la interacción de fosfatidilserina con su receptor específico en la membrana de los macrófagos (Fadock y col., 1992). Además, la naturaleza de la población macrofágica determina el mecanismo predominante por el cual será reconocida la célula apoptótica (Fadock y col., 1992). Los macrófagos peritoneales utilizan principalmente el sistema de reconocimiento de la fosfatidilserina, mientras que los macrófagos derivados de la médula ósea llevan a cabo el reconocimiento gracias al receptor de la vitronectina. Por ultimo, es posible que las células apoptóticas liberen factores quimiotácticos capaces de atraer a los macrófagos, aunque la naturaleza no inflamatoria de la apoptosis indica que estos factores no pueden actuar como mediadores inflamatorios convencionales.

Por otro lado, los macrófagos han sido implicados como potentes inductores de apoptosis de las células tumorales (Chambers y Weiser, 1972), de las células endoteliales vasculares (Peri y col., 1990), de los linfocitos T periféricos en desarrollo (Munn y col., 1996; Zheng y col., 1995) y de los linfocitos B en la médula ósea (Osmond y col., 1994) y han sido implicados en la regresión de varios tejidos, aunque generalmente se había asumido que su papel era meramente como limpiadores pasivos de los restos celulares (Ellis y col., 1991). Actualmente, no está del todo claro qué moléculas producidas por los macrófagos son responsables de la inducción de apoptosis por parte de estas células. La variedad de mediadores intercelulares producidos por los macrófagos sugiere la posible existencia de múltiples mecanismos (ver apartado 1.2 de la introducción).

Una de las muchas funciones del TNF-α es su implicación en la citotoxicidad mediada por los macrófagos (Beutler y Cerami, 1992). La muerte inducida por el TNF-α es debida a procesos de apoptosis y ha sido descrita tanto en células normales

como tumorales (Leist y col., 1995; Robaye y col., 1991). Muchos de los efectos biológicos del TNF-α soluble, incluyendo la inducción de apoptosis, estan mediados a través de su receptor p55 o TNFR1 (Tartaglia y col., 1993a; 1993b). Sin embargo, Grell y col. (1995) han mostrado recientemente que el precursor del TNF-α asociado a la membrana puede activar al receptor TNFR2 (p75) e inducir apoptosis incluso en aquellas células resistentes a los efectos citotóxicos del TNF-α soluble. Así, la presencia de TNF-α asociado a la membrana de los macrófagos puede ser crítico en la inducción de apoptosis, de forma consistente con la observación de que el contacto célula-célula es esencial para la apoptosis mediada por este tipo celular (Bernasconi y col., 1991; Le y Vilcek, 1987; Peri y col., 1990; Webb y col., 1991).

Las formas reactivas del oxígeno y del nitrógeno son también responsables de numerosos procesos apoptóticos inducidos por los macrófagos (Lewis y McGee, 1992; Martin y Edwards, 1993). Por ejemplo, los intermediarios reactivos del oxígeno, generados a través de la cadena respiratoria de los macrófagos, pueden inducir apoptosis en las células NK (Hansson y col., 1996). La producción de óxido nítrico (NO) por los macrófagos estimulados con diversas citocinas promueve la apoptosis de células tumorales (Cui y col., 1994; Keller y col., 1990) principalmente mediante la inducción de lesiones en el DNA (Messmer y col., 1994; Nguyen y col., 1992). Los macrófagos también secretan la citocina IL-1β la cual es capaz de inducir apoptosis en líneas celulares de melanoma (Onozaki y col., 1985) y en células de timoma de ratón (Fratelli y col., 1995).

4.4.2. Apoptosis en los macrófagos

A pesar de que los macrófagos juegan un papel clave en la fagocitosis de los cuerpos apoptóticos o en la inducción de apoptosis sobre otros tipos celulares, poco se conoce acerca de los estímulos que modulan la apoptosis de los propios macrófagos.

Al igual que el resto de células del organismo, incluyendo a las células hematopoiéticas normales, los macrófagos también requieren factores de crecimiento específicos que mantengan su viabilidad (Raff, 1992; Sachs y Lotem, 1993). Los macrófagos requieren de M-CSF tanto para proliferar como para sobrevivir (ver apartado 2.1 de la Introducción). La ausencia de este factor de crecimiento induce la muerte por apoptosis de los macrófagos y células del linaje macrofágico (Fuller y col., 1993; Gehrmann, 1993; Tomozawa y col., 1996). El mantenimiento de la viabilidad celular inducida por el M-CSF parece estar mediada por la señalización a través de la enzima PI-3K (Carpenter y Cantley, 1996; Minshall y col., 1996). Aunque el M-CSF es el principal factor de crecimiento y diferenciación de los macrófagos, otros factores de crecimiento como el GM-CSF o la IL-3 pueden mantener la viabilidad de los macrófagos o de los monocitos recién aislados en ausencia de M-CSF (Bach y Brashler, 1995; Kinoshita y col., 1995).

La infección por bacterias y otros microorganismos ejerce potentes efectos moduladores de la apoptosis en los macrófagos. En este sentido, la infección por *Shigella flexneri, Mycobacterium tuberculosis, Salmonella typhimurium, Actinobacillus actinomycetem-cominants o Bordetella pertusis* induce apoptosis tanto en los macrófagos de ratón como en los monocitos humanos (Hisaeda y col., 1997; Keane y col., 1997; Khelef y col., 1993; Monack y col., 1996; Rojas y col., 1997; Zychlinsky y col., 1994); mientras que la infección por *Candida albicans, Mycobaterium bovis o Leishmania donovani* inhibe la apoptosis de los macrófagos inducida por la ausencia de factores de crecimiento o por el tratamiento con TNF-α (Heidenreich y col., 1996; Kremer y col., 1997; Moore y Matlashewski, 1994). Los mecanismos implicados en la apoptosis inducida por la infección de los macrófagos son muy diversos, e incluyen la activación de la adenilato ciclasa (Khelef y col., 1993), la producción de NO (Rojas y col., 1997) o la interacción con componentes propios de los microorganismos (Chen y col., 1996; Rojas y col., 1997; Zychilinsky y col., 1994).

Por otro lado, existe un gran número de substancias capaces de inducir la muerte de los macrófagos por apoptosis, entre las que se incluyen substancias minerales como la sílice o el asbestos (Hamilton y col., 1996b); drogas como la bleomicina (Hamilton y col., 1995), gadolinium (Mizgerd y col., 1996) o los

bifosfonatos (Rogers y col., 1996) y la morfina (Singhal y col., 1998); u otros compuestos como la ricina (Khan y Waring, 1993).

Al igual que ocurre con otras células del sistema inmunológico, existe una relación entre los procesos de activación de los macrófagos y la modulación de los procesos apoptóticos. Así, la activación de la iNOS ha sido correlacionada con la consecuente inducción de apoptosis en los macrófagos (Albina y col., 1993; Messmer y Brüne, 1996; Sarih y col., 1993). Sin embargo, existen evidencias de que dosis moderadas de NO pueden tener un papel más bien protector que inductor de la apoptosis en este tipo celular (Mangan y col., 1991; Brune y col., 1996). El tratamiento con citocinas como el TNF-α, GM-CSF, IL-3, IL-1β o la estimulación de CD-40 tiene un efecto protector de la apoptosis en monocitos humanos y otras células del linaje macrofágico por (Kinoshita y col., 1995; Mangan y col., 1991; Mangan y Whal, 1991; Poe y col., 1997). Finalmente, una serie de trabajos recientes de nuestro grupo han demostrado que la activación de los macrófagos con IFN-γ protege a estas células de la muerte por apoptosis inducida por diversos estímulos como la ausencia de factores de crecimiento o la estimulación con LPS (Xaus y col., 1999b).

5. MATRIZ EXTRACELULAR

La regulación de la proliferación y del crecimiento celular no sólo requiere la interacción de los factores de crecimiento con sus respectivos receptores, sino también de los cambios producidos en las macromoléculas del entorno celular, que afectan la adhesión, la migración, la diferenciación, la proliferación, la activación y la apoptosis de las células. Por lo tanto, la matriz extracelular circundante a las células es de vital importancia para las funciones celulares.

La matriz extracelular es un complejo macromolecular formado por una mezcla de largas glicoproteínas como el colágeno, fibronectina, lamininas y proteoglicanos,

así como elastina e hialurantes que se ensamblan, predominantemente por interacciones no covalentes, en fibrilas u otras estructuras complejas. La matriz extracelular no sólo es una barrera física por la cual, las células se extienden formando los tejidos, sino que presenta una funcionalidad activa. Primero, es considerada como un reservorio de factores de crecimiento y citocinas modulando su concentración y actividad. Por ejemplo, algunas moléculas de la matriz pueden secuestrar y modificar (ej. por proteolisis) factores de crecimiento, alterando su bioactividad para que posteriormente puedan ser presentados a sus receptores específicos. Segundo, intervienen en la integridad estructural de los tejidos y los cambios dinámicos en la adhesión celular. Estos cambios dependen de la interacción directa de las moléculas de la matriz extracelular con las células, ya sea vía interacciones de receptores específicos, como las integrinas, o a través de receptores de los propios factores de crecimiento. Cabe mencionar que, la integridad estructural de los tejidos u órganos también depende de la interacción célula-célula mediada por las cadherinas, promoviendo la compactación y reagrupamiento celular (Couchman y col., 2001; Gumbiner, 1996; Ornitz, 2000; Schonherr y Hausser, 2000; Yamaguchi, 2000).

La adhesión de las células a la matriz y la consiguiente activación del factor de crecimiento específico es necesaria para pasar de la fase G_1 a S del ciclo celular (Meredith y Schwartz, 1997; Ruoslahti y Reed, 1994). Las principales moléculas implicadas en esta adhesión son las integrinas, una familia de proteínas transmembranarias heterodiméricas que, en mamíferos, comprende por lo menos 16 subunidades α y 8 subunidades β .Las diferentes combinaciones de dímeros de las cadenas sencillas α y β forman, por lo menos, unos 22 receptores distintos expresados diferencialmente en los tejidos y con distintas especificidades por las proteínas de la matriz extracelular. Por ejemplo, la expresión de la subfamilia CD11/CD18 representada por las integrinas, LFA-1 (*lymphocite function-associated antigen-1*), Mac-1, p150,95 y $\alpha d\beta 2$, los cuales comparten la subunidad $\beta 2$, está restringida a leucocitos (Harris y col., 2000). Por otro lado, la fibronectina, una proteína de la matriz extracelular que puede ser secretada de forma autocrina por los macrófagos, puede interaccionar con estas células induciendo la migración celular a

través de integrinas específicas de cadena β1 (α5β1, α4β1) (Fougerolles y col, 2000; Hershkoviz y col, 1992; Wesley y col, 1998).

Es interesante destacar que muchas de las vías de señalización activadas por las integrinas, son también activadas por los factores de crecimiento, sugiriendo que entre estas dos vías existe una intercomunicación o sinergismo. En realidad, durante la angiogénesis inducida por los factores de crecimiento o por las células tumorales se ha detectado en las células endoteliales vasculares un aumento de la expresión de los receptores específicos de integrinas (αvβ3). La inhibición de estos receptores con anticuerpos bloqueantes previene la formación de vasos sanguíneos, sugiriendo que estas integrinas desempeñan un papel importante en los procesos tumorales (Eliceiri, 2001; Schwartz y Assoian, 2001).

5.1. Proteoglicanos

De todas las moléculas de la matriz extracelular, los proteoglicanos representan las proteínas más estudiadas y las más complejas. Los proteoglicanos son macromoléculas compuestas de un cuerpo proteico el cual está unido covalentemente a una o más cadenas de glicosaminoglicanos (GAG). Los GAGs son cadenas de carbohidratos formadas por repeticiones de disacáridos unidos y que existen en cuatro formas distintas: sulfato de heparán (HS) y heparina; sulfato de condroitín (CS, principalmente CSA Y CSC y sulfato de dermatán, CSB); sulfato de queratán; y ácido hialurónico (HA). Las primeras tres formas se encuentran predominantemente formando complejos con proteínas y contienen un grupo sulfato, mientras que el ácido hialurónico se encuentra en forma libre y ha perdido el grupo sulfato. Las formas sulfatadas proporcionan a los GAG una fuerte carga negativa. Esta carga permite a los GAGs unirse a diversos substratos, entre los cuales hay factores de crecimiento (revisado en Iozzo, 1998).

La alta complejidad de los proteoglicanos viene dada por las combinaciones de estos GAGs con diferentes tipos de proteínas, formando una gran variedad de proteoglicanos con localizaciones y funciones distintas (Ruoslahti, 1989). Hasta el

momento se han identificado unos 30 miembros de esta superfamilia localizados en la matriz extracelular, en la superficie de las células o en el interior de las mismas (Elenius y Jalkanen, 1994; Gressner, 1994; Kjellen y Lindahl, 1991; Iozzo y Murdoch, 1996). Las funciones de estas macromoléculas son muy diversas, pudiendo actuar como organizadoras del tejido, influenciando el crecimiento celular y la maduración de los tejidos especializados. Además, los proteoglicanos tienen un papel importante como filtros biológicos y modulan las actividades de los factores de crecimiento; regulan la fibrilogénesis del colágeno y la resistencia de la piel; afectan al crecimiento de las células tumorales y la invasión o metástasis; e influencian la transparencia de la córnea y el crecimiento de las neuritas. Los estudios con animales deficientes, indican que algunos proteoglicanos son esenciales para la vida mientras que otros son redundantes (revisado en Iozzo, 1998).

Los proteoglicanos han sido agrupados en diferentes familias y subfamilias de genes proporcionando una nomenclatura más simplificada en función de su estructura proteica. Sin embargo, la relación estructura-función en algunos casos es paradigmática y complica su clasificación. Los proteoglicanos de la matriz extracelular han sido agrupados en tres clases (Iozzo, 1998) (Tabla III). El primer grupo está formado por proteoglicanos asociados a las membranas basales de tejido epitelial y vascular. A este grupo corresponden, el perlecán, agrín y bemacán. Los dos primeros presentan cadenas de sulfato de heparán, mientras que el segundo cadenas de sulfato de condroitín. Las funciones descritas de estos tres proteoglicanos son muy diversas y dependen del contexto celular, haciendo muy difícil atribuir un papel funcional común a este grupo.

El segundo grupo lo forman los lecticanos, que son proteoglicanos multidiméricos formados por una estructura tridimensional común. La región aminoterminal contiene un dominio globular que interacciona con hialurán, un dominio central que lleva las cadenas de GAGs, como sulfato de condroitín o sulfato de queratán y una región C-terminal que interacciona con las lectinas. Los miembros de esta familia son el agrecán, versicán, neurocán y brevicán. Los dos últimos son característicos del tejido neuronal. Se ha descrito que una de las funciones de estos

lecticanos es proporcionar a los tejidos elasticidad y resistencia contra las fuerzas compresivas.

TABLA III: Clasificación de los proteoglicanos de la matriz extracelular
(Iozzo, 1998.)

FAMILIA PROTEOGLICANOS		NOMBRE	GLICOSAMINOGLICANO (número)
Asociados a membranas basales de tejido epitelial y vascular		Perlecano	Sulfato de heparano/Condroitino (3)
		Agrín	Sulfato de Heparano (3)
		Bamacano	Sulfato de Condroitino (3)
Lecticanos		Versicán	Sulfato de dermatano/Condroitin (10-30)
		Agrecano	Sulfato de condroitino (100)
		Neurocano	Sulfato de condroitín (3-7)
		Brevicano	Sulfato de condroitín (1-3)
SLRP	CLASSE I	Decorina	Sulfato de Condroitín/Dermatano (1)
		Biglicano	Sulfato de Condroitín/Dermatano (2)
	CLASSE II	Fibromodulina	Sulfato de queratano (2-3)
		Lumicano	Sulfato de queratano (3-4)
		Queratocano	Sulfato de queratano (3-5)
		PRELP	Sulfato de queratano (2-3)
		Osteodherin	Sulfato de queratano (2-3)
	CLASE III	Epiglicano	Sulfato de dermatano/Condroitina (2-3)
		Osteoglicina	Sulfato de queratano (2-3)

La tercera clase la forma una familia compuesta de pequeños proteoglicanos ricos en leucinas (SLRP; *small leucine-rich proteoglycans*). Esta familia tiene en

común, una estructura formada por un dominio aminoterminal que contiene cadenas de GAGs compuestas de sulfato de condroitín/dermatán o cadenas de sulfato de queratán. Esta región de la molécula, unida a cuatro residuos de cisteína conservados, está involucrada en la interacción con dominios catiónicos de proteínas de la superficie celular o de la matriz extracelular. El dominio central contiene entre 8 y 10 repeticiones en tándem de leucinas (LRR; *leucine-rich repeats*). Esta familia contiene por lo menos nueve miembros, que han sido separados en tres clases basados en su homología proteica y genómica. La primera clase incluye la decorina y el biglicán. La segunda clase esta compuesta por la fibromodulina, el lumicán y el queratocán. Estos proteoglicanos juegan un papel clave en la regulación del ensamblaje de fibrilas de colágeno de diferentes tejidos como la piel, hueso, tendones y córnea; y participan en el control de la proliferación celular (Iozzo, 1998; 1999)

A esta alta complejidad en la distribución de las familias se le suman otros proteoglicanos de la matriz extracelular como el fosfacán, o el neuroglicán entre otros, que aún no han sido agrupados dentro de estas familias. También existe un gran número de proteínas de la matriz extracelular que están, o no, unidas a GAGs dependiendo del estado de desarrollo de la proteína. Algunos ejemplos son el CD44, el M-CSF, el precursor de proteína amiloide y los colágenos tipo IX, XII, XIV, y XVIII (Kresse y Schonherr, 2001)

5.2. Decorina

Dentro de la familia de SLRPs, la decorina es el proteoglicano que más extensamente ha sido estudiado. Este proteoglicano se encuentra de forma predominante en una elevada variedad de tejidos como la piel (Choi y col., 1989), cartílago (Rosenberg y col., 1985) o hueso (Fisher y col., 1983). Está compuesto de un núcleo proteico de unos 40 kDa y una sola cadena de GAG anclada a un residuo de serina en la parte aminoterminal de la proteína. El núcleo proteico de la decorina es dominado por una región central compuesta de unas diez unidades repetidas de leucinas de unos 21-26 residuos cada unidad. Muchos de los efectos biológicos de la decorina son atribuidos, en parte, a este dominio rico en leucinas (LRR). Este módulo

estructural permite el reconocimiento molecular en una gran variedad de procesos como adhesión celular, señales de transducción, reparaciones de DNA y procesamiento de RNA (Iozzo, 1997; Weber y col., 1996).

Una de las principales funciones asignadas a la decorina es la regulación del ensamblaje de la matriz extracelular mediante su interacción con las fibrilas de colágeno, principalmente de tipo I y presenta la capacidad de retardar la velocidad de degradación y de la fibrilogénesis *in vit*ro. Los estudios genéticos utilizando ratones deficientes en decorina proporcionan una clara información del papel de este proteoglicano en la homeostasis del colágeno. A pesar de que estos animales crecen sin experimentar ninguna patología, un análisis extenso revela un fenotipo de fragilidad de piel, donde el colágeno dérmico exhibe una organización aberrante de fibrilas con un empaquetamiento anormal (Danielson y col., 1997). Además la decorina puede interaccionar con los colágenos de tipo II (Vogel y col., 1984), III (Thieszen y Rosenquist, 1994) y VI (Bidanset y col., 1992), fibronectina (Schmidt y col, 1987), trombospondina (Winnemoller y col, 1992) , C1q (Krumdieck y col, 1992) y el factor de transformación tumoral (TGF-β) (Yamaguchi y col, 1990), modulando a diferentes niveles la producción y ensamblaje de la matriz extracelular y, por lo tanto, el remodelaje del tejido conectivo.

La implicación de la decorina en el control del crecimiento celular ha estado ampliamente estudiada. En condiciones normales, en los fibroblastos quiescentes se detecta un aumento de la expresión de la decorina que esta potenciada con el tratamiento con IL-1β o de la dexametasona e inhibida por factores de crecimiento (como TGF-β, EGF), TNF-α y el ácido retinoico (Kahair y col., 1995; Laine y col., 2000; Mauviel y col., 1995, 1996). Sin embargo, la importancia de la decorina reside en su papel en la regulación del crecimiento tumoral. Las células epiteliales transformadas en una amplia variedad de carcinomas humanos incluyendo páncreas, próstata y mama raramente expresan decorina (Iozzo y Cohen, 1993). Por otro lado, la expresión ectópica de decorina recombinante induce la supresión de la proliferación de las células neoplásicas de varios orígenes histogénicos, y es atribuida en parte a la inducción de la expresión de $p21^{Waf1}$, el inhibidor de los complejos ciclina-cdk (Santra

y col.,1997). La decorina inhibe el fenotipo maligno de la línea carcinómica A321 (y otras líneas tumorales) mediante la activación del receptor de EGF (*Epidermal growth factor*). La dimerización y aumento de la fosforilación del receptor induce la activación de la vía de las MAP quinasas, una movilización de calcio intracelular y la inducción de la expresión de $p21^{Waf1}$ (Moscatello y col., 1998). Otro mecanismo de inhibición de la proliferación mediado por la decorina se ha descrito en células CHO (*Chinese hamster ovary*). Este efecto es provocado porque la decorina secuestra el TGF-β, un factor que induce la proliferación de este tipo celular (Yamaguchi y Ruoslahti, 1988; Yamaguchi y col., 1990).

La habilidad de la decorina para inhibir el crecimiento tumoral abre la posibilidad de utilizarla como terapia contra numeroso tipos de cánceres. De hecho se ha visto que para contrarrestar la invasión de las células tumorales, en los lugares periféricos al tumor se detecta una acumulación de la expresión de decorina. Por otro lado, el papel de la decorina como modulador de la formación de fibrilas de colágeno y su acción sobre el TGF-β, principal inductor de la secreción de proteínas de la matriz extracelular, también ha llevado a la utilización de la decorina en ensayos clínicos como posible terapia para patologías fibróticas (Santra y col, 2000; Stander y col, 1998).

Objetivos

Objetivo general:

El objetivo general al iniciar este trabajo fue el estudio de las relaciones existentes entre los procesos de proliferación, activación celular y apoptosis en los macrófagos, prestando una especial atención en las vías de señalización encargadas de regular ya sea de forma conjunta o independientemente cada uno de estos procesos.

Objetivos concretos:

1. El papel de las quinasas MEK/ERK en los procesos de activación y proliferación.
2. Las vías de señalización implicadas en la supervivencia de los macrófagos inducidas por los factores de crecimiento como el M-CSF.
3. El mecanismo de inhibición de la proliferación inducido por el IFN-γ.
4. El efecto de drogas inmunomoduladoras como la ciclosporina A y el FK506 sobre la proliferación.
5. El efecto de componentes de la matriz extracelular como la decorina sobre la proliferación y activación de los macrófagos.
6. La inducción de la apoptosis por el LPS, y las vías de señalización implicadas.

Resultados

La supervivencia, pero no la proliferación, de los macrófagos en respuesta al M-CSF depende de la inducción de $p21^{waf1}$ a través de la vía PI-3K/Akt

La homeostasis celular es un proceso crítico de los organismos multicelulares para mantener un balance entre la supervivencia, proliferación y muerte de las células. Los macrófagos derivados de la médula ósea necesitan el requerimiento constante de M-CSF para proliferar, sobrevivir y diferenciarse. Cuando el M-CSF es eliminado del medio, los macrófagos paran su ciclo celular a nivel de la fase G_1. Si las condiciones de ausencia de factores de crecimiento persisten en el tiempo, los macrófagos experimentan una muerte celular por procesos de apoptosis.

En este trabajo, nos hemos centrado en intentar discernir si las vías de señalización implicadas en la proliferación y supervivencia de los macrófagos inducidas por el M-CSF son iguales o por el contrario se trata de vías independientes. En este sentido, hemos visto que el M-CSF induce la activación de las quinasas ERK-1/2 y de PI-3K. Mediante la utilización de inhibidores específicos de dichas vías de señalización, hemos podido demostrar que la proliferación y la supervivencia de los macrófagos inducidas por el M-CSF están reguladas por vías de señalización distintas e independientes. Así, mientras que la activación de ERK-1/2 es necesaria para la proliferación de los macrófagos, la supervivencia depende de la activación de la vía de señalización iniciada por PI-3K. Además, hemos podido descartar la implicación en la supervivencia de otras proteínas activadas por el M-CSF como JNK, p38 y la $p70^{S6}$ quinasa.

Trabajos previos de nuestro grupo demostraron que la inducción del inhibidor del ciclo celular $p21^{waf1}$ por el IFN-γ es capaz de proteger a los macrófagos de la apoptosis inducida por el LPS o por la falta de factores de crecimiento. La expresión de $p21^{waf1}$ y dicho efecto protector de la apoptosis también se observa tras la adhesión de los macrófagos al proteoglicano decorina presente en la matriz extracelular de la mayoría de los tejidos de los mamíferos. Puesto que hemos observado que el M-CSF también induce la expresión de $p21^{waf1}$, decidimos analizar si esta expresión de $p21^{waf1}$ inducida por el M-CSF también podía jugar un papel en la señalización hacia la supervivencia inducida

por este factor de crecimiento. El M-CSF induce la expresión de $p21^{waf1}$ a través de la activación de la vía de PI-3K/Akt, sin estar afectada por la activación de ERK, sugiriendo que la expresión de $p21^{waf1}$ depende de las vías de señalización hacia supervivencia pero no de la vía hacia proliferación.

Finalmente, para determinar si la inducción de $p21^{waf1}$ es un mecanismo general utilizado para proteger a los macrófagos de la apoptosis inducida por la falta de factores de crecimiento, hemos analizado otros estímulos que inducen supervivencia en los macrófagos. Así, tanto la ciclosporina A como la decorina protegen de la apoptosis inducida por la falta de factores de crecimiento mediante la inducción de la expresión de $p21^{waf1}$ a través de la vía de señalización iniciada por PI-3K/Akt en respuesta a estos estímulos.

M-CSF-dependent survival of macrophages, but not proliferation, involves $p21^{Waf1}$ induction mediated by the PI-3 kinase/AKT pathway

Abstract

A variety of mammalian cells undergo apoptosis when deprived of growth factors. Macrophages derived from bone marrow are not an exception to this rule. For proliferation and survival M-CSF requires two independent pathways. M-CSF-dependent proliferation requires the Raf-1/MEK/ERK pathway while for M-CSF-dependent survival PI-3K is necessary. Moreover, we have discarded other signaling pathways that although activated by M-CSF did not directly participate in these responses, such as JNK, p38 or $p70^{S6}$ kinases. M-CSF also induces the expression of the cdk inhibitor $p21^{Waf\,1}$. M-CSF-induced $p21^{Waf\,1}$ expression is not involved in proliferation but in the apoptosis inhibition induced by M-CSF. Thus, M-CSF-induced $p21^{Waf\,1}$ expression is mediated by PI-3K/Akt but not ERK pathways. Finally, PI-3K inhibitors, such as wortmannin, inhibit both $p21^{Waf\,1}$ expression and M-CSF-induced survival without modifying cell cycle progression. In accordance with our results, we have demonstrated that the induction of $p21^{Waf\,1}$ is a general mechanism, also induced in response to other agents such as decorin and cyclosporin A, that protects the apoptosis induced by growth factor starvation mediating the induction of $p21^{Waf\,1}$ through the PI-3K/Akt pathway activation.

Introduction

The ability of multicellular organisms to maintain cellular homeostasis is critically dependent on a balance between cell survival, proliferation and cell death (apoptosis). The responsiveness of individual cells to death signals can vary greatly depending on the presence of continuos survival stimuli from extracellular environment. The perturbation of normal cell survival mechanisms, leading to an increase in cell death or in cell survival, plays an important role in the development of a number of disease states, including cancer (Jacobsen et al., 1997).

Endogenous apoptosis has been linked to a default pathway in that the apoptotic program is always ready to be executed unless it is continously inhibited by extracellular signals called “survival factors” (Evan et al., 1992; Raff, 1992). The viability of many, if not all the mammalian cells is dependent on these specific survival factors. Some examples include: cytokines, such as IL-2 in lymphoblasts (Duke and Cohen, 1986) or IFN-γ in macrophages (Xaus et al., 1999b); proteoglycans, such as decorin that protects apoptosis induced by growth factor starvation in macrophages and endothelial cells (Schonherr et al., 2001; Xaus et al., 2001b); or growth factors like platelet-derived growth factor (PDGF) or insulin-like growth factor 1 (IGF-1) which induce the survival of fibroblasts and smooth muscle cells, respectively (Lawlor and Rotwein, 2000; Romanshkova and Makarov, 1999). Macrophage-colony stimulating factor (M-CSF) also appears to be important in the survival of monocytes and macrophages (Becker et al., 1987; Stanley et al., 1997). However, the specific intracellular mediators induced by M-CSF to promote cell survival are not well determined.

M-CSF initiates a mitogenic response by binding to its receptor (M-CSFR), thereby activating the receptor’s intrinsic tyrosine kinase activity and initiating signaling via multiple effector-mediated pathways (Stanley et al., 1997). When macrophages are deprived of M-CSF, cell-cycle arrest occurs, while prolonged starvation leads to cell death through apoptosis (Xaus et al., 1999b). There is a continual requirement for M-CSF from initiation of immediate early gene expression through G_1, therefore it is believed that M-CSF acts as a progression factor as well as survival factor (Sherr, 1991). Recently, the ability of trophic factors to promote survival has been attributed, at least in part, to the phosphatidylinositide 3’-OH kinase (PI-3K)/Akt kinase cascade (Datta et al., 1999) and mitogen activated kinases (MAPK) (Borasio et al., 1989; 1993; Xue et al., 1999). A role in the regulation of apoptosis for other different members of the MAP kinase family, such as JNK and p38 has also been suggested (Kummer et al., 1997; Xia et al., 1995).

In previous studies, we have shown that the Raf1-MEK-ERK cascade is required for macrophage proliferation in response to M-CSF (Valledor et al., 2000b). The tight control of the time-course of the ERKs kinases is essential for the fate of several cellular processes, namely proliferation and activation (Treisman, 1996). A clear correlation exists between the time-course of ERK activation and the

decision of macrophages to either proliferate or become activated. An early peak of ERK activation is common to all the proliferative signals (M-CSF, GM-CSF, IL-3) and a more delayed peak is induced by activating agents, such as LPS (Valledor et al., 2000b). However, in macrophages, it is not clear if in response to the M-CSF the activation of the ERK kinases also controls the survival mechanisms.

The cdk inhibitor $p21^{Waf\,1}$ is one of the most potent regulators of the cell cycle and is known to inhibit in some cellular models proliferation (Sherr and Roberts, 1999). However, we have found using macrophages from $p21^{Waf\,1}$ KO mice that this cdk inhibitor is not involved in the IFN-γ inhibition of macrophage proliferation, but regulates the antiapoptotic effect related to IFN-γ (Xaus et al., 1999b).

In this work, we have determined that M-CSF used two different pathways for survival and for proliferation. M-CSF induces the $p21^{Waf\,1}$ transcription through the PI-3K/Akt-dependent and MAPK-independent pathways and that, despite $p21^{Waf\,1}$ is a cell cycle inhibitor, it is not involved in regulation of macrophage proliferation but play a critical role in cell survival. In counterpart, the Raf-1-MEK-ERK pathway plays a critical role in the macrophage proliferation and activation stimuli but its activation or inhibition does not modulate the survival of macrophages. Moreover, we also tested the role of other pathways such as JNK and p38 MAPKs and we have discarded the participation of them in the regulation of macrophage proliferation or survival induced by M-CSF.

Materials and Methods

Reagents

Recombinant M-CSF was a gift from DNAX (Palo Alto, CA). In some experiments we used L cell-conditioned medium as the source of this growth factor (Celada et al., 1984). Recombinant GM-CSF was obtained from Sigma (St. Louis, CO). Recombinant IL-3 was purchased from R&D systems Inc. (Minneapolis, MN). CsA was a kind gift from Novartis (Basel, Switzerland). Recombinant purified

decorin was a generous gift from Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA). The following selective inhibitors to block specific signal transduction pathways were used: PD98059, Rapamycin, SB203580 and Curcumin (Calbiochem, San Diego, CA) and wortmannin (Sigma). Deionized water further purified with a Millipore Milli-Q system (Bedford, MA) was used. All reagents were used following the manufacturer's recommendations.

Cell Culture

Bone marrow-derived macrophages were isolated from six-week old balb/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) as previously described (Celada et al., 1984). Cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 30% L-cell conditioned media as a source of M-CSF. Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. After 7 days of culture a homogeneus population of adherent macrophages was obtained (>99 % Mac-1^+). The purity of the culture was checked regularly by flow cytometry using anti-Mac-1 antibodies (BD Pharmingen, Heidelberg, Germany). To render cells quiescent, when macrophages were 80% confluent they were deprived of L cell-conditioned medium for 16 hours before carrying out the experiments and then were subjected to different treatments.

Antibodies and contructs

The phospho-p38 MAP kinase (Thr180/Tyr182) antibody and the phospho-Akt (Ser473) antibody were obtained from Cell Signalling (Beberly, MA). The p38 MAP kinase antibody (sc-535) and the anti-JNK1 antibody (sc-474) were purchased from Santa Cruz Biotechnology, INC. (Santa Cruz, CA).

The Pmh117 plasmid corresponds to the mouse $p21^{Waf\,1}$ full-length cDNA cloned in pEx-lox and was kindly provided by Dr. J. Massague (Sloan Kettering Institute, HHMI, New York, NY). The probe for the 18 S rRNA was obtained as described (Torczynski et al., 1983).

Proliferation assay

Cell proliferation was measured as described previously (Celada et al., 1984) with minor modifications. Quiescent cells (10^5) were incubated for 24 h in 24-well plates (Costar Corp., Cambridge, MA) in 1 ml of medium with the indicated concentrations of M-CSF. The medium was aspirated and replaced with 0,5 ml of medium containing [^{3}H]-thymidine (1 μCi/ml) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After 4-6 h of incubation at 37ºC, cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% trichloroacetic acid, cells were solubilized in 1% SDS and 0,3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter. Each point was performed in triplicate, and the results were expressed as the mean ± S.D.

Apoptosis assay

Low molecular apoptotic DNA caused by internucleosomal cleavage was measured as described (Xaus et al., 1999b), using an ELISA technique based on the detection of histone-assossiated DNA fragments (Cell Death Detection ELISA plus, Roche Diagnostics, Mannheim, Germany). Each point was performed in triplicate, and the results was expressed as the mean ± SD. In some cases, apoptotic DNA was also measured by gel electrophoresis or by flow cytometry with DAPI-stained cells as previously described (Xaus et al., 1999b) with similar results.

RNA Extraction and Northern Blot Analysis

Total cellular RNA (20 μg), extracted with the TRIZOL Reagent (Life Technologies, Grand Island, N.Y.) was separated in 1% agarose gel with 5 mM MOPS (3-[N-morpholino] propanesulfonic acid), pH 7,0/1 M Formaldehyde buffer. RNA was transferred overnight to a Hybond-XL nitrocellulose membrane (Amersham Pharmacia, Uppsala, Sweden) and fixed by UV irradiation (150 mJ). All the probes were labeled with ^{32}P-α-dCTP (ICN Pharmaceuticals) using the oligolabelling kit method (Amersham Pharmacia). After incubation of the membranes for 18 hours at 65ºC in hybridization solution (5 X standard sodium

citrate [SSC], 5 X Denhart, 1% SDS and 10^6 cpm/ml of ^{32}P-labeled probe), they were exposed to Kodak X-AR films (Kodak Company, Rochester, NY).

Western blot analysis

For phospho-p38 and phospho-Akt western blot equal amounts of protein (60 μg) extracted as previously described (Valledor et al., 1999) were boiled at 95ºC in Laemmli SDS loading buffer, and separated on a 10% SDS-PAGE. Then, the proteins were electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham Corp., Arlington Heigths, IL). The membranes were blocked for at least 1 hour at room temperature in Tris buffered saline-0.1% Tween-20 (TBS-T) containing 5% (w/v) non-fat dry milk and then incubated with TBS-T containing BSA 5% and the primary antibody (1/1000) overnight at 4ºC. After three washes of 5 minutes each with TBS-T, the membranes were incubated with peroxidase-conjugated anti-rabbit IgG (Cappel, Durham, NC) antibody for 1 hour. After three washes of 5 minutes with TBS-T, ECL detection was performed (Amersham) and the membranes were exposed to X-ray ECL films (Amersham). The bands of interest were quantified by densitometric analysis. Western blot of p38 MAP kinase was performed equally to phospho-p38 MAP kinase with a little modification: the incubation with primary antibody (1/2000) was performed with TBS-T without BSA 5%.

Determination of MAPK activities

The analysis of ERK activity was performed by in gel kinase assay as described, using 50 μg total protein and 0,1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel as a substrate (Valledor et al., 2000b).

Determination of JNK activity was performed as described (Caelles et al., 1997) with minor modifications. Briefly, cells were washed with PBS and lysed in cold lysis buffer (1% NP-40, 20 mM HEPES-Na pH 7,5, 10 mM EGTA, 40 mM B-glycerophosphate, 25 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM DTT, 0,5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide). 150 μg of total protein were mixed with 75 μl of 20% protein A-sepharose and 1 μl of anti-JNK1 antibody (sc-474, Santa Cruz) in a total volume of 500 μl. The samples were

rotated for 2 hours at 4ºC. The immunecomplexes were washed three times with cold PBS supplemented with 1% NP-40 and 2 mM sodium orthovanadate, once with cold JNK buffer (20 mM HEPES-Na pH 7,5, 20 mM B-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 2 mM DTT) and resuspended in JNK reaction buffer (JNK buffer supplemented with 1 µg GST-c-jun (1-169) (Calbiochem) as a substrate, 20 µM ATP, 1 µCi γ-^{32}P-APT). The reaction was allowed to proceed for 30 minutes at 30ºC and was then stopped by adding 12µl of 5xLaemli buffer. The samples were incubated for 3 minutes at 100ºC and separated by 10% SDS-PAGE. After the electroforesis, the gels were fixed in isopropanol:water:acetic acid (25:65:10), dried and exposed to Kodak X-AR films.

Results

All cell types require specific factors to remain viable, and the withdrawal of these factors results in apoptotic cell death (Nick et al., 1996). For these studies we used macrophages obtained from bone marrow cultures, because they represent a homogeneus population of primary cells that requires to proliferate and survive the presense of growth factors, such as macrophage colony-stimulating factor (M-CSF). Although M-CSF is the major and specific growth factor for macrophages (Stanley et al., 1997), these cells are also able to proliferate in dose-dependent manner to GM-CSF and IL-3 (Fig. 1A). We want to make clear that we used ^{3}H-Thymidine incorporation as an indicator of cell proliferation, since we have demonstrated previously that this is a valid method that correlates with an increase in the number of macrophages (Xaus et al., 1999b; 1999c; 2001b). However, in these experiments when we differentiate proliferation versus cell survival it is important to note that by proliferation we only can talk about cell cycle progression since it is difficult to obtain a real increase in cell population if simultaneously most of the cells are dying by apoptosis.

Elimination of the growth factors induces a loss in cell viability that is due to apoptosis as analyzed by quantification of the DNA fragmentation (Fig. 1B) or by other methods such as flow cytometry with DAPI-stained cells (data not shown). IL-3 and GM-CSF also protect macrophages from apoptosis induced by growth factor withdrawal in the same degree than M-CSF (Fig. 1B). Since the induction of

proliferation and inhibition of apoptosis is a general mechanism for the three growth factors (M-CSF, GM-CSF, IL-3) in the following experiments of this manuscript we mainly used M-CSF because is the major and specific macrophage growth factor (Stanley et al., 1997).

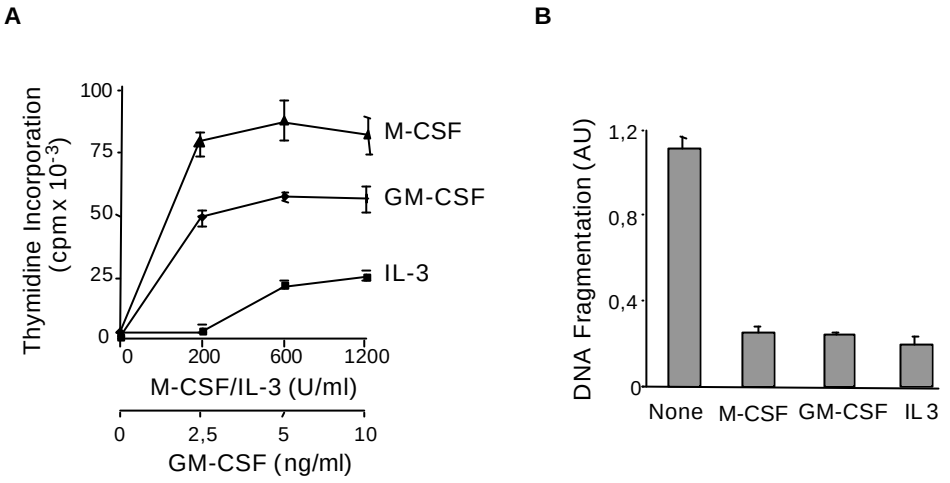


FIG 1. M-CSF, GM-CSF and IL-3 promote the proliferation and survival of macrophages. A) M-CSF- GM-CSF- and IL-3- dependent ^{3}H-Thymidine incorporation in macrophages. Cells were grown for 5 days before measuring thymidine incorporation as indicated in material and methods. Macrophages were incubated for 24 hours with the indicated concentrations of M-CSF, GM-CSF and IL-3. This figure represents one of four independent experiments with the mean ± SD of triplicates for each point. B) M-CSF, GM-CSF and IL-3 protect M-CSF starved macrophages from apoptosis. Macrophages were cultured without growth factors for 24 hours or in the presence of M-CSF (1200 U/ml), GM-CSF (10 ng/ml), or IL-3 (1200 U/ml). The induction of apoptosis measured as DNA fragmentation was analyzed using an ELISA kit. Each treatment was performed three times, and the results were represented as the mean value ± SD. These experiments were performed three times with similar results.

The first aim of this work was to explore if M-CSF regulates both proliferation and survival using the same pathway or, in contrast, this growth factor uses multiple pathways to modulate independently both processes. Several studies have suggested that Ras and its downstream mediators, the Raf-MEK-MAPK cascade, are critical for growth factor-induced survival in a number of different cell types (Borasio et al., 1993; Xue et al., 1999). Moreover, in previous works, we have been reported that the ERK-1/2 MAP kinases play a crucial role in macrophage proliferation and activation (Valledor et al., 1999; 2000a; 2000b). Therefore we explore whether ERK is also implicated in the regulation of macrophage survival in response to M-CSF. ERK phosphorylation was induced in macrophages by exposure to M-CSF (Fig 2A). The treatment of macrophages with the MEK-1

inhibitor, PD98059, blocked ERK-1/2 phosphorylation (Fig. 2A) as well as the M-CSF-induced proliferation (Fig. 2B). Interestingly, although PD98059 was able to block the proliferative effect of M-CSF, this inhibitor was unable to modify the apoptotic profile of macrophages in the presence or absence of M-CSF (Fig. 2C). Thus, ERK activity did not seems to be involved in the survival signaling of M-CSF, nor in the induction of apoptosis after groth factor withdrawal.

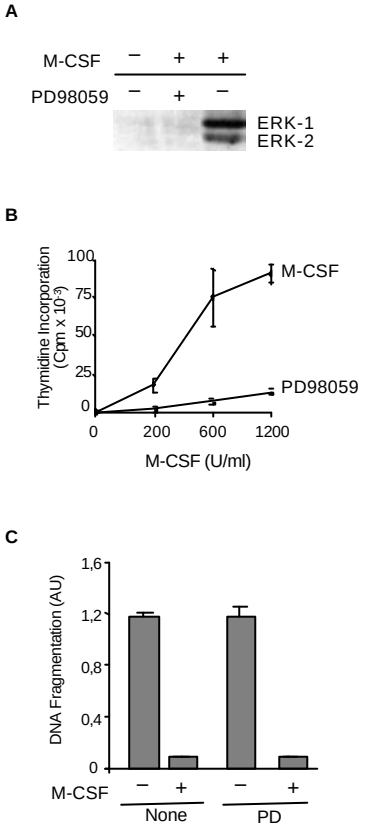


FIG. 2. Activation of the MEK/ERK pathway is necessary for M-CSF-induced macrophage proliferation but not survival. A) M-CSF activates ERK kinases. Quiescent macrophages were either left untreated or preincubated with PD98059 (50 μM) or vehicle (0,1% DMSO) for 1 h and then stimulated with M-CSF (1200 U/ml) for 5 min. ERK activity was analyzed by an in-gel kinase assay. Identical results were obtained with four independent experiments. B) PD98059 inhibits M-CSF-dependent proliferation. Quiescent cells were preincubated with PD98059 (50 μM) or with the vehicle for 1 h and then stimulated with the indicated concentrations of M-CSF for 24 hours. Thymidine incorporation was measured as described in material and methods. The figure shows the mean and SD of triplicate determinations. C) ERK inhibition does not induce apoptosis in macrophages. Cells were cultured in PD98059 (50 μM) or vehicle for 1 h and then stimulated with M-CSF (1200 U/ml) or in the absence of M-CSF for 24 h. Apoptosis was determined as in Fig. 1C. These experiments were performed three times with similar results.

The differences observed between the proliferation and survival responses suggest that an ERK-independent pathway is involved in M-CSF-dependent survival. In the next step we analyzed other MAP kinase cascades, such as JNK and p38 MAP kinases. In contrast with other cell types, where the activation of these kinases has only been observed after growth factor withdrawal (Eilers et al., 2001; Nick et al., 1996; Xia et al., 1995), M-CSF is able to estimulate the activation of both JNK and p38 MAPK in macrophages (Fig. 3A). Curcumin and SB203580 (SB) are specific inhibitors for either JNK or p38 MAPK. At a dose which were able to inhibit the kinase activity, any of these two inhibitors were able to block M-CSF-dependent macrophage proliferation (Fig. 3B). Also, these inhibitors did not

blocked the survival signaling of M-CSF (Fig. 3C). Thus, although the JNK and p38 MAP kinase pathways are activated in response to M-CSF, they are not involved in M-CSF survival or proliferation response.

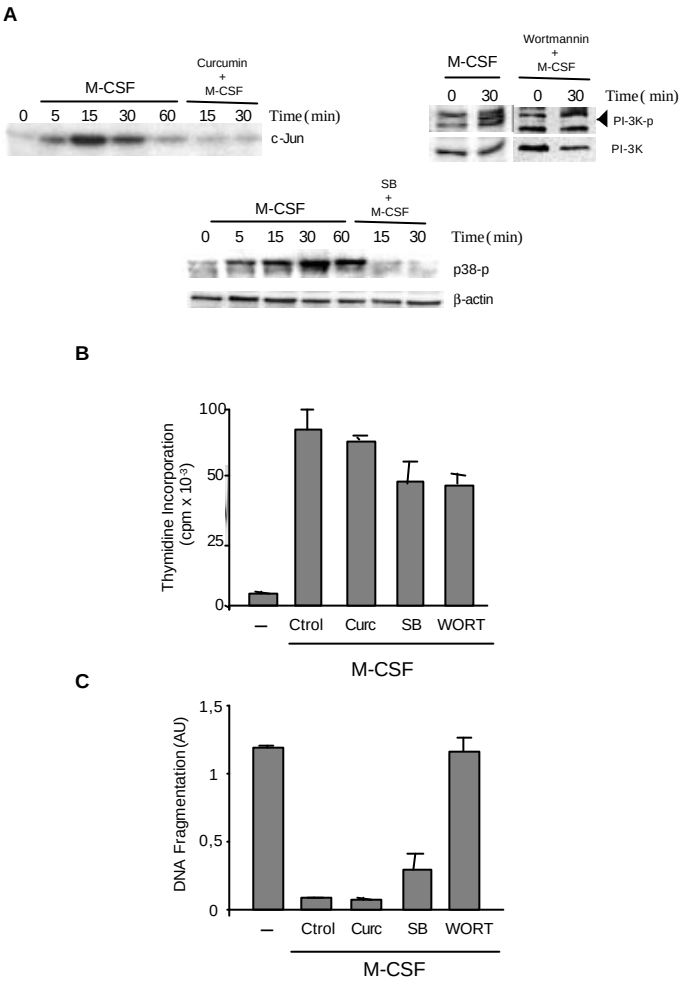


FIG. 3. PI-3 kinase is implicated in macrophage survival. A) M-CSF induces the activation of PI-3, JNK and p38 kinases. Quiescent bone marrow macrophages were either left untreated or preincubated with the following inhibitors: curcumin (5 μg/ml),SB203580 (5 μM) and wortmannin (100 nM) for 1 h and then stimulated with saturating amounts of M-CSF (1200 U/ml) for the indicated periods of time. JNK activation was measured as the capability of immunoprecipitated JNK to phosphorylate GST-c-jun *in vitro*. The p38 activation was analyzed as the expression of the p38 phosphorylated protein by Western blot. β-actin was used as control of protein transfer. PI-3K activity was mesuared by detection of tyrosine phosphorylation in immunoprecipitated PI-3K samples. B) PI-3K, JNK and p38 inhibition do not modify the proliferation induced by M-CSF. Quiescent cells were preincubated with curcumin (5 μg/ml),SB203580 (5 μM), and wortmannin (100 nM) for 1 h and then stimulated with M-CSF (1200 U/ml) for 24 h. Proliferation was measured as thymidine incorporation. C) PI-3K inhibition by wortmannin induces apoptosis in macrophages. Cells were cultured in the absence of M-CSF for 24 h or curcumin (5 μg/ml),SB203580 (5 μM), and wortmannin (100 nM) for 1 h and then stimulated with M-CSF (1200 U/ml). Apoptosis was determined as the amount of low molecular weight DNA using a comercial ELISA kit. Each experiment was performed at least in triplicates and represented as the mean ± SD. These experiments were performed three times with similar results.

Because it has been reported that PI-3 kinase is activated in response to growth factors (Brown and Shepherd, 2001) we decided to explore this pathway. Macrophages were not an exception and M-CSF also induced PI-3K activation in these cells (Fig. 3A). Again, the inhibition of PI-3 kinase with wortmannin, a well described PI-3K specific inhibitor (Arcaro and Wymann, 1993), did not significantly suppress the proliferation induced by M-CSF (Fig. 3B) but blocked completely the M-CSF-induced survival signals (Fig. 3C), suggesting that this pathway is involved in cell survival but not in proliferation.

We have reported in previous studies, that the cdk inhibitor $p21^{Waf\,1}$ is the mediator of IFN-γ- or decorin-induced protection against apoptosis (Xaus et al., 1999b; 2001b). Interestingly, although $p21^{Waf1}$ it has been involved in cell cycle inhibition, M-CSF also induced $p21^{Waf\,1}$ expression (Fig. 4A). Confirming this result, other macrophage growth factors, namely IL-3 and GM-CSF, also induced the expression of $p21^{Waf\,1}$ (Fig. 4B), suggesting that the expression of this cell cycle inhibitor could be necessary for growth factor responses regarding proliferation or cell survival in macrophages. To determine the pathway implicated in the expression of $p21^{Waf\,1}$ in response to M-CSF we used specific inhibitors. Only, wortmannin blocked the expression of $p21^{Waf\,1}$ induced by M-CSF treatment but not PD98059 or any other MAPK inhibitors (Fig. 4B). Therefore, all these results indicate that the signaling pathways that regulate $p21^{Waf\,1}$ expression in response to M-CSF are the same signaling the mechanism involved in the regulation of M-CSF-induced survival. This also suggests that $p21^{Waf\,1}$ could play an anti-apoptotic role in response to growth factors in macrophages.

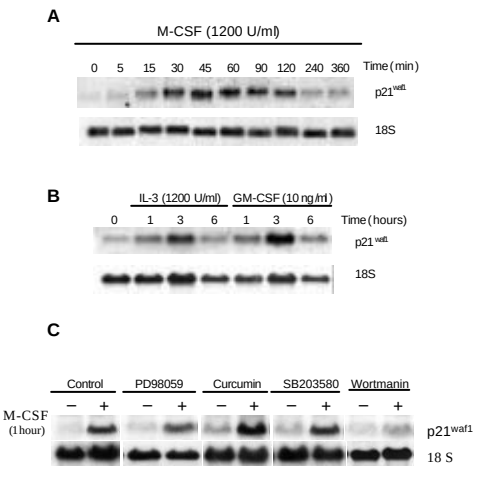


FIG. 4. M-CSF induces $p21^{Waf\,1}$ expression in macrophages. A) M-CSF induces $p21^{Waf1}$ expression. Quiescent bone marrow macrophages were incubated with M-CSF (1200 U/ml) for the indicated periods of time. $p21^{Waf1}$ mRNA expression was determined by Northen blot (20 μg total RNA per lane). Expression of 18 S was used as a control for RNA loading and transfer. B) GM-CSF and IL-3 induce $p21^{Waf1}$ expression. Quiescent cells were incubated with IL-3 (1200 U/ml) or GM-CSF (10 ng/ml) for the indicated periods of time. $p21^{Waf1}$ and 18 S mRNA expression was determined by Northen blot. C) PI-3K pathway mediate $p21^{Waf1}$ expression. Quiescent macrophages were preincubated with PD (50 μM), curcumin (5 μg/ml), SB203580 (5 μM), or wortmannin (100 nM) for 1h and then stimulated with M-CSF (1200 U/ml) for 1 h more. $p21^{Waf1}$ and 18S rRNA expression were measured by Northen blot.

PI-3K is able to regulate several targets (Rameh, et al., 1995) including Akt, which has been involved in the regulation of apoptotic processes (Datta et al., 1999). For this reason, we next investigated the effects of M-CSF treatment on the activation of the serine/threonin kinase, Akt. In macrophages after 5 minutes of treatment M-CSF led to an increase in Akt phosphorylation (Fig. 5), which has been linked to Akt activation. Akt phosphorylation was suppressed by preincubation of macrophages with the PI-3K inhibitor, wortmannin (Fig. 5), demonstrating that Akt phosphorylation in response to M-CSF was mediated through PI-3K.

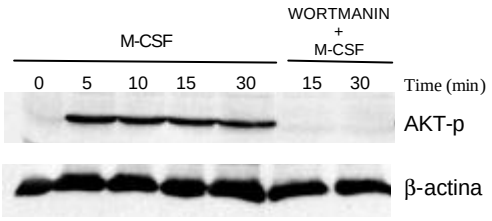


FIG. 5. M-CSF induces Akt activity. M-CSF induces Akt phosphorylation through PI-3K. Quiescent macrophages were pretreated or not with wortmannin (100 nM) for 1 h and then stimulated with M-CSF (1200 U/ml) for the indicated times. Phosphorylation of Akt was measured by Western blot using specific antibodies. β-actin expression was used as an indicator of protein loading and transfer. This experiment was performed three times with similar results

There are other targets of PI-3 kinase activity besides Akt such as the atipycal PKCξ and the $p70^{s6}$ kinase (Liu et al., 1998; Yao and Cooper, 1996). In our cellular model we discarded the PKCξ because we were unable to demonstrate the activity of this kinase in response to M-CSF (Valledor et al., 1999). The $p70^{S6}$ kinase is involved in the phosphorylation of the S6 protein in response to various stimuli, including growth factor and insulin, regulating cell cycle, transcription, and translation initiation (Proud, 1996). Therefore we tested the role of this kinase in the survival response induced by M-CSF. Rapamycin, a potent inhibitor of $p70^{S6}$ kinase activity, inhibited the proliferation of macrophages in a dose dependent way (Fig. 6A) but was unable to block the survival signaling in cells stimulated with M-CSF (Fig. 6B) or to inhibit the expression of $p21^{Waf\,1}$ (Fig. 6C). All together these results suggested that although $p70^{S6}$ kinase could play an important role on macrophage proliferation due to its effect on macromolecular synthesis, it is not involved in the survival of bone marrow macrophages in response to M-CSF. In consequence, the role of PI-3K in the inhibition of apoptosis mediated by the M-CSF-dependent

expression of p21$^{Waf\,1}$ seems to be only mediated by Akt. p70^{S6} kinase is not involved, and the other target of PI-3 Kinase, PKCζ it is not activated by M-CSF (Valledor et al., 1999).

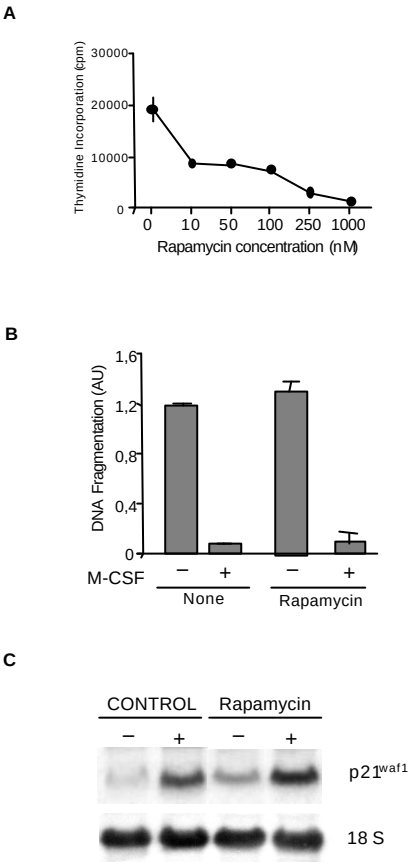


FIG. 6. p70^{S6K} is not involved in the survival of macrophages. A) Rapamycin inhibits M-CSF-dependent proliferation. Macrophages were grown for 5 days before measuring thymidine incorporation. Quiescent macrophages were pretreated with the indicated concentrations of rapamycin for 1 h and then incubated for 24 hours with 1200 U/ml of M-CSF. This figure represents one of three independent experiments with the mean ± SD of triplicates for each point. B) p70^{s6k} is not involved in the apoptosis induced by M-CSF starvation. Macrophages were cultured in the presence or the absence of rapamycin (500 nM) for 1 h and then stimulated or not with M-CSF (1200 U/ml) for 24 h. Apoptosis was determined using an ELISA and results represented as the mean ± SD of triplicates. C) Rapamycin does not block p21^{Waf1} expression. Quiescent macrophages were preincubated with rapamycin 500 nM) for 1h and then stimulated with M-CSF (1200 U/ml) for 1 more hour. p21^{Waf1} and 18S mRNA expression were measured by Northern blot. These experiments were performed three times with similar results.

To confirm that p21$^{Waf\,1}$ induced by the PI-3K/Akt pathway has a pivotal role as general inhibitor of apoptosis, we investigated other agents that play a protective effect against apoptosis induced by growth factor deprivation on macrophages. We observed in previous studies that decorin and cyclosporin A (CsA) inhibit the M-CSF starvation induced apoptosis (Xaus et al., 2001b; and *manuscript submitted*). CsA inhibited macrophage proliferation in a dose-dependent manner through the inactivation of ERK-1/2 kinases (Fig. 7A and B). CsA was also able to block the apoptosis induced by starvation (Fig. 7C). This effect may be related to the induction of p21$^{Waf\,1}$ in absence of M-CSF treatment (Fig. 7D). Moreover, inhibition of PI-3K with wortmannin abolished both the anti-apoptotic effect of CsA (Fig. 7C) as well as the induction of p21$^{Waf\,1}$ expression (Fig. 7E). Finally, CsA induced the activation of Akt in absence of M-CSF and this

activation was mediated through PI-3K since it could be inhibited by wortmannin (Fig. 7F).

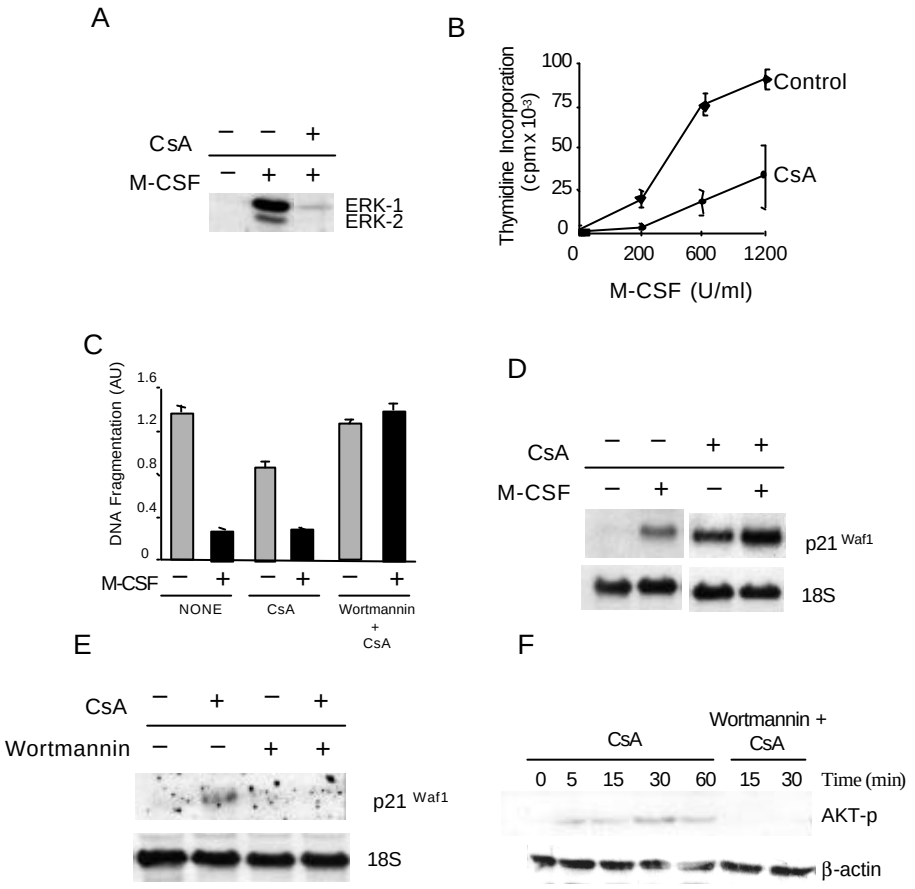


FIG. 7. Cyclosporin A protects apoptosis induced by M-CSF withdrawal through the PI-3K/Akt/p21^{Waf1} pathway. A) CsA inhibits the M-CSF proliferation in macrophages. Quiescent cells were preincubated with CsA (10 μg/ml) for 1 h and then stimulated with the indicated concentrations of M-CSF for 24 hours. Thymidine incorporation was measured as described in material and methods. The figure shows the mean and SD of triplicate determinations. B) CsA inhibits ERK-1/2 activity. Quiescent macrophages were preincubated with CsA (10 μg/ml) for 1 h and then stimulated with M-CSF (1200 U/ml) for 5 minutes. ERK-1/2 activity was measured by an in-gel kinase assay. C) CsA inhibits the apoptosis induced by M-CSF withdrawal. Macrophages were cultured in the presence or in the absence of wortmannin (100 nM) for 1 h and then stimulated with CsA (10 μg/ml) in the absence or presence of M-CSF for 24 h. Apoptosis was determined as indicated before. D) CsA induces p21^{Waf1}. Quiescent macrophages were preincubated with CsA (10 μg/ml) for 1h and then stimulated with M-CSF (1200 U/ml) for 1 h. p21^{Waf1} expression was analyzed by Northern blot (20 μg of total RNA per lane). E) CsA induces p21^{Waf1} expression through PI-3K. Cells were treated with CsA (10 μg/ml) for 1 hour in the presence or absence of the PI-3K inhibitor wortmannin (100 nM) and the expression of p21^{Waf1} mRNA was analyzed by northern blot. F) CsA activates the PI3K/Akt pathway. Quiescent macrophages were pretreated with wortmannin (100 nM) for 1 h and then stimulated or not with CsA (10 μg/ml) for the indicated periods of time. Phosphorylation of Akt was measured by Western blot. These experiments were performed four times with similar results.

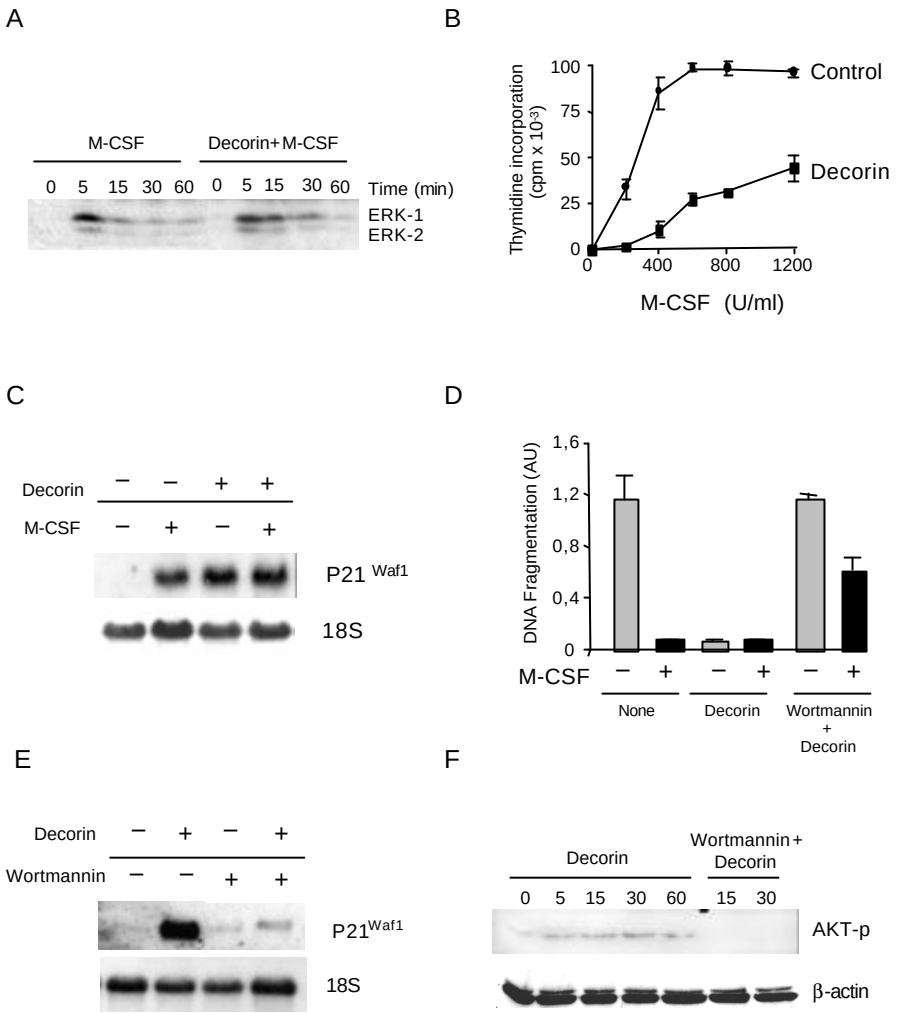


FIG. 8. Decorin protects apoptosis induced by M-CSF withdrawal through the PI-3K/Akt/p21^{Waf1} pathway. A) Decorin inhibits M-CSF-dependent proliferation in macrophages. Quiescent cells were preincubated in decorin precoated plates (10 μg/ml) for 1 h and then stimulated with the indicated concentrations of M-CSF for 24 hours. Thymidine incorporation was measured as described in material and methods. The figure shows the mean and SD of triplicate determinations. B) Decorin elongates ERK-1/2 activity. Quiescent macrophages were preincubated in decorin precoated plates (10 μg/ml) for 1 h and then stimulated with M-CSF (1200 U/ml) for 5 minutes. ERK-1/2 activity was measured by an in-gel kinase assay. C) Decorin inhibits the apoptosis induced by M-CSF withdrawal. Macrophages were cultured in the presence or in the absence of wortmannin (100 nM) for 1 h and then preincubated in decorin precoated plates (10 μg/ml) in the absence or presence of M-CSF for 24 h. Apoptosis was determined as indicated before. D) Decorin induces p21^{Waf1}. Quiescent macrophages were preincubated in decorin precoated plates (10 μg/ml) for 1h and then stimulated with M-CSF (1200 U/ml) for 1 h. p21^{Waf1} expression was analyzed by Northern blot (20 μg of total RNA per lane). E) Decorin induces p21^{Waf1} expression through PI-3K. Cells were preincubated in decorin precoated plates (10 μg/ml) for 1 hour in presence or absence of the PI-3K inhibitor wortmannin (100 nM) and the expression of p21^{Waf1} mRNA was analyzed by northern blot. F) Decorin activates the PI3K/Akt pathway. Quiescent macrophages were pretreated with wortmannin (100 nM) for 1 h and then preincubated or not in decorin precoated plates (10 μg/ml) for the indicated periods of time. Phosphorylation of Akt was mesuared by Western blotting.

Decorin a proteoglycan of the extracellular matrix in the presence of macrophages inhibits proliferation (Fig. 8A) that is related with the elongation of the ERK activity (19) (Fig. 8B). Decorin also protected macrophages from apoptosis induced by M-CSF withdrawal (Fig. 8D) and induced the expression of $p21^{Waf\,1}$ (Fig. 8C). These last two effects were blocked by wortmannin pretreatment (fig 8 D, E). Moreover, inhibition of PI-3K led to the inhibition of Akt activation induced by decorin treatment (Fig. 8F). Therefore, decorin protects macrophages from apoptosis mediating the induction of $p21^{Waf\,1}$ through the PI-3K/Akt pathway.

Discussion

One of the features of the immune system is the production of a large amount of any type of cells. Later, most unnecessary cells die through apoptosis. However, the small amount of cells required to develop a functional activity survive (Freitas and Rocha, 2000). The coordination and balance between cell growth, cell survival, and cell death requires a complex signaling network, including multiple checkpoints to determine cell fate. The decision between cell survival and cell death depends on the balance of constitutive and extracelullar signal-induced pro- and anti-apoptotic factors. Disturbances in the balance of pro- and anti-apoptotic factors can lead to either increased cell death or increased cell survival. In this sense, growth factors are needed for the continued proliferation and survival of certain populations of cells of the monocyte/macrophage lineage. Relatively little, however, is known about the signal transduction pathways activated by growth factors involved in maintaining cell survival or proliferation. Several lines of evidence have suggested that in the maintenance of survival and proliferation could be involved the activation of a single pathway (Bonni et al., 1999; Jung et al., 2000; Kinoshita et al., 1997) or, in contrast, they are independent processes mediated by different signaling pathways activated by the growth factors (Ballif and Blenis, 2001; Parrizas et al., 1997; Xue et al., 1999). To clarify this point, we focused this work on the signal pathways activated by M-CSF regarding macrophage proliferation and survival.

Although primary cultures are the best option in order to study the real mechanisms involved in macrophage proliferation and survival, their transfection is

very inefficient (Celada et al., 1996). For this reason, in our experiments we need to use chemical inhibitors to assay the involvement of specific molecules in macrophage biology.

In this study we found for the first time that M-CSF regulates proliferation and survival of macrophages through different pathways. Using inhibitors, we described that activation of the MEK/ERK pathway is necessary for macrophage proliferation in response to M-CSF (Valledor et al., 1999; 2000b). Although in some cell types the MEK/ERK pathway has also been implicated in the survival responses (Ballif and Blenis, 2001; Bonni et al., 1999; Xue et al., 1999) the blockage of ERK activation in macrophages did not induce apoptosis, suggesting that ERK activity is not necessary for the survival response of macrophages to growth factors.

Although JNK and p38 MAP kinases have been implicated in several apoptotic processes (Ip and Davis, 1998; Kyriakis and Avruch, 1996) and in contrast to other cell types (Xia et al., 1995), we did not detect JNK or p38 kinases activity when macrophages are deprived of M-CSF. Instead, their activity was induced after M-CSF treatment. Thus, although these MAPK play crucial roles in macrophage activation, we discard their involvement in the regulation of macrophage proliferation or survival.

Our results showed that the main pathway implicated in the survival responses is the recruitment of the p85 subunit of phosphatidylinositol 3-kinase (PI-3K) to the activated M-CSF receptor. Activated PI-3K generate phosphatidylinositol 3,4 bisphosphate (PI3,4P) and phosphatidylinositol 3,4,5 trisphosphate (PI3,4,5P). Once generated, these lipids function as signaling intermediates that regulates dowstream signal transduction cascades (Rameh and Cantley, 1999). They activate a number of cellular intermediates, including $p70^{s6k}$ (Proud, 1996; Yao and Cooper, 1996), atypical isoforms of PKC (Liu et al., 1998) and Akt (Datta et al., 1999; Rameh et al., 1995). In relation to the pro-survival signaling of M-CSF, we excluded the involvement of $p70^{S6K}$ and atypical PKCs, so Akt should be the main mediator of this effect. The activation of Akt inhibits the apoptosis induced by growth factor withdrawal or irradiation in neural cells, fibroblasts and lymphocytes (Coffer et al., 1998; Hemmings, 1997).

Several targets of the PI3K/Akt signaling pathway have been recently identified that may underlie the ability of this regulatory cascade to promote survival. These multiple substrates include two components of the intrinsic cell death machinery, BAD (Datta et al., 1997) and caspase 9 (Cardone et al., 1998); several transcription factors, including the forkhead factors (Brunet et al., 1999), E2F (Brennan et al., 1997); and a kinase, IKK, that regulates the NF-κB transcripiton factor (Madrid et al., 2000).

Although $p21^{Waf\,1}$ is a cell cycle inhibitor, there are a number of cell systems where his induction has been associated with cell cycle progression or proliferation (Harper et al., 1993; LaBaer et al., 1997; Li et al., 1994; Liu et al., 1996c; Zhang et al., 1994). In our experimental model $p21^{Waf\,1}$ expression is not involved in proliferation but in survival. This was supported by several observations: a) Macrophages obtained from mice deficient of $p21^{Waf\,1}$ proliferate in response to M-CSF; b) These macrophages were unable to protect from M-CSF dependent survival as well as in response to IFN-γ or decorin (Xaus et al., 1999b; 2001b); c) Inhibitors that block $p21^{Waf\,1}$ expression, such as wortmannin, did not inhibit M-CSF-dependent macrophage proliferation and d) An inhibitor of MEK, PD98059, that block the signaling pathway that regulates macrophage proliferation, does not interfere with the $p21^{Waf\,1}$ expression.

The expression of $p21^{Waf\,1}$ seems to be regulated by the same signaling pathways that regulate macrophage survival, namely PI-3K/Akt. This is supported because cyclosporin A and decorin, that induced survival also induced $p21^{Waf\,1}$ in a PI-3K-dependent manner. For all these, we suggest that $p21^{Waf\,1}$ is a general and critical molecule involved in macrophage survival induced by several stimuli, including growth factors, macrophage activators and components of the extracellular matrix. At the present we do not know how $p21^{Waf\,1}$ could protect macrophages from apoptosis. This cdk inhibitor could interact and inhibit apoptotic molecules, such as caspases 8 and 10 (Xu and El-Deiry, 2000), caspase 3 (Suzuki et al., 1999), SAPKs (Shim et al., 1996) or the MEKKs (ASK1) (Asada et al., 1999). Moreover, $p21^{Waf\,1}$ could be implicated in the regulation of anti-apoptotic gene expression (Chang et al., 2000).

In summary we have shown that in bone marrow-derived macrophages, separate pathways regulate proliferation and survival. Raf-1/MEK/ERK plays a crucial role regulating proliferation while PI-3K/Akt/$p21^{Waf\,1}$ regulates cell survival. Moreover, we have demonstrated that the PI-3K/Akt pathway and $p21^{Waf\,1}$ are a general survival mechanism on macrophages used for different agents such as growth factors, decorin or CsA. The regulation of cell survival and cell death involves different signaling pathways, and the decision between cell survival and cell death could require a crosstalk between these pathways and checkpoints where pro- and anti-apoptotic signals converge.

La activación breve o prolongada de ERK-1/2 se correlaciona con la proliferación o activación de los macrófagos

Los macrófagos, son células que actúan como reguladores de la homeostasis y como células efectoras en infecciones, heridas y crecimiento tumorales, jugando, por lo tanto, un papel crítico en el desarrollo de la respuesta inmunitaria. Los linfocitos en condiciones normales se encuentran en un estado de quiescencia o reposo y tras la activación experimentan un proceso de expansión clonal caracterizado por una elevada tasa de proliferación y posteriormente pasan a desarrollar sus funciones características. A diferencia de éstos, los macrófagos a nivel de los tejidos se encuentran normalmente proliferando, y tras su activación, dejan de proliferar y pasan a ejercer sus funciones específicas.

En este sentido, los macrófagos derivados de la médula ósea proliferan en respuesta a factores específicos, como el M-CSF, y otros factores inespecíficos capaces también de inducir proliferación en otros tipos celulares como la IL-3 o el GM-CSF. Cuando son estimulados con agentes activadores como el lipopolisacárido de las bacterias Gram negativas (LPS), los macrófagos paran de proliferar y pasan a desarrollar sus funciones de manera más eficiente como puede ser la producción de citocinas proinflamatorias como el TNF-α, IL-1β e IL-6.

Los estudios previos de nuestro grupo han demostrado que a pesar de inducir respuestas tan diferentes, tanto el M-CSF como el LPS, inducen la activación de las proteínas quinasas ERK-1/2; por lo que en este trabajo hemos querido estudiar el papel que juega esta vía de transmisión de señales en la regulación de estos procesos. Así, hemos establecido que el patrón de activación de estas quinasas es diferente frente ambos estímulos. Aunque en ambos casos la activación de las ERKs se produce de forma rápida y transitoria, la máxima activación de ERK-1/2 por el M-CSF se da a los cinco minutos mientras que por el LPS ocurre a los 15 minutos.

Además, hemos establecido una correlación entre el patrón de activación de ERK-1/2 y los fenómenos proliferativos y de activación. Otros factores de crecimiento no específicos, como el GM-CSF y la IL-3, así como los ésteres de

forbol (TPA) inducen también la proliferación de los macrófagos y un pico de activación de las ERK-1/2 a los cinco minutos, similar a lo observado con el M-CSF. Cuando se utiliza el PD98059, un inhibidor de la actividad ERK, los macrófagos en presencia de M-CSF, GM-CSF, IL-3 y TPA, dejan de proliferar, demostrando que la activación de estas quinasas en respuesta a factores de crecimiento es esencial para la proliferación de los macrófagos.

En el lado contrario, el tratamiento de los macrófagos con la fosfolipasa C específica de fosfatidilcolina (PC-PLC) inhibe la proliferación de los macrófagos, induce la activación de estas células analizada como la expresión de citocinas, e induce un pico de activación de las ERK a los quince minutos, equivalente al inducido tras el tratamiento con el LPS. Además, el tratamiento con PD98059 inhibe parcialmente la expresión de citocinas inducidas por el LPS o por PC-PLC, sugiriendo que la actividad de ERK también es requerida para la activación de los macrófagos con estos dos agentes.

Por último, hemos analizado la implicación de JNK, otro miembro de la superfamilia de las MAPK quinasas, también activada en los macrófagos. Tanto el M-CSF como el LPS y la PC-PLC inducen la activación de JNK con una cinética idéntica; en cambio, el tratamiento de los macrófagos con TPA no induce la activación de JNK. Por lo tanto, no hemos podido establecer ninguna correlación entre la activación de JNK y la decisión de los macrófagos sobre los procesos de proliferación y activación en los macrófagos.

Así, y en conclusión, la activación de ERK, o mejor dicho, el patrón de la cinética de activación de ERK juega un papel clave en la decisión de las actividades biológicas de los macrófagos en procesos tan importantes como la proliferación o la activación en respuesta a los estímulos externos, y su actividad es estrictamente necesaria para el desarrollo de ambos procesos.

The Differential Time-Course of Extracellular-Regulated Kinase Activity Correlates with the Macrophage Response Towards Proliferation or Activation

Abstarct

Bone marrow-derived macrophages proliferate in response to specific growth factors, including M-CSF. When stimulated with activating factors, such as LPS, macrophages stop proliferating and produce proinflamatory cytokines. Although triggering opposed responses, both M-CSF and LPS induce the activation of extracellular-regulated kinases (ERK)-1/2. However, the time-course of ERK activation is different; maximal activation by M-CSF and LPS occured after 5 and 15 minutes of stimulation, respectively. GM-CSF, IL-3 and TPA, all of which induced macrophage proliferation, also induced ERK activity, this being maximal 5 minutes post-stimulation. The use of PD98059, which specifically blocks ERK-1/2 activation, demonstrated that ERK activity was necessary for macrophage proliferation in response to these factors. The treatment with phosphatidylcholine-specific phospholipase C (PC-PLC) inhibited macrophage proliferation, induced the expression of cytokines and triggered a pattern of ERK-1/2 activation equivalent to that induced by LPS. Moreover, PD98059 inhibited the expression of cytokines induced by LPS or PC-PLC, thus suggesting that ERK activity is also required for macrophage activation by these two agents. Activation of the JNK pathway did not discriminate between proliferative and activating stimuli. In conclusion, our results allow to correlate the differences in the time-course of ERK activity with the macrophagic response towards proliferation or activation.

Introduction

Macrophages perform critical functions in the immune system. They act as regulators of homeostasis and as effector cells in infection, wounding and tumor growth (Celada and Nathan, 1994). Macrophages originate in the bone marrow and, through the blood stream, reach all the tissues in the organism. According to the specific needs, tissue macrophages either proliferate, further differentiate to more

specialized macrophagic populations or become activated. When there is no need of macrophages and macrophage-colony stimulating factor (M-CSF) is not locally produced, these cells undergo a process of apoptotic death (Xaus et al., 1999b).

M-CSF is the major and the only specific growth factor for this cell type (Stanley et al.,1997). The receptor for M-CSF is the product of the protooncogene *c-fms* (Vairo and Hamilton, 1991). The binding of M-CSF induces the autophosphorylation of the receptor and the subsequent recruitment of different signal transducing molecules (reviewed in Hamilton, 1997). One of the signalling cascades activated by M-CSF is the Raf/MEK/ERK pathway (Büscher et al., 1995; Jaworowski et al., 1996). Raf-1, a serine/threonine protein kinase, phosphorylates and activates the threonine/tyrosine protein kinase MEK-1 (Howe et al., 1992), which in turn phosphorylates and activates extracellular-regulated protein kinases (ERK)-1 and ERK-2 (Crews et al., 1992). These are proline-directed serine/threonine protein kinases, also known as p44- and p42-mitogen-activated protein kinases (-MAPK), respectively (Seger and Krebs, 1995). Active ERKs phosphorylate and regulate several cellular proteins, including additional protein kinases, cytoskeletal components, phospholipase A_2 and nuclear transcription factors, such as Elk1/TCF and c-Jun, which regulate the expression of immediate early genes (Seger and Krebs, 1995; Treisman, 1996).

Lipopolysaccharide (LPS) or endotoxin, a major component of the outer membrane of Gram-negative bacteria, activates macrophages and induces the secretion of arachidonic acid metabolites (e.g. prostaglandins, leukotrienes and platelet-activating factor), nitrogen intermediates and cytokines such as tumor necrosis factor (TNF)-α and interleukins IL-1 and IL-6 (Adams and Hamilton, 1984; Morrison and Ryan, 1987), which play important roles in the immune response. LPS triggers the activation of the Raf/MEK/ERK pathway in macrophages (Reiman et al., 1994; Weinstein et al., 1992).

Our aim is to determine the mechanism/s that induce macrophages to either proliferate or become activated. In this report we have found that both proliferating and activating processes in macrophages require the activation of the ERK cascade. However, the time-course of this activation is markedly different. Since

the time-course of ERK activity may determine the fate of many cellular responses (Marshall, 1995; Sarbassov et al., 1997; Tombes et al., 1998), we were interested in finding out a correlation between the pattern of ERK activation and the cellular response (proliferation *versus* activation) induced in macrophages. In this regard, the proliferating agents M-CSF, GM-CSF, IL-3 and TPA induced a peak of ERK activity at 5 minutes of stimulation. In contrast, the activating agents LPS or PC-PLC, both of which blocked macrophage proliferation, induced ERK activation more slowly, with maximal induction at 15 minutes post-stimulation. In contrast, we could not correlate the pattern of JNK activity with any particular macrophagic fate. In conclusion, in this report we show that differences in the time-course of ERK activity may be crucial to determine the macrophage response towards proliferation or activation, being the initial peak of ERK activity common to all the proliferative processes and the more delayed peak induced by activating agents.

Material and methods

Materials

Recombinant M-CSF was provided as a gift by DNAX (Palo Alto, CA). In some experiments, we used L-cell conditioned medium as the source of this growth factor. TPA and PC-PLC from *Bacillus cereus* were purchased from Calbiochem (San Diego, CA). Recombinant granulocyte/macrophage-colony stimulating factor (GM-CSF) and LPS were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant IL-3 was purchased from R&D systems Inc. (Minneapolis, MN). PD98059 was purchased from New England Biolabs Inc. (Beverly, MA). All reagents were used following the manufacturer's recommendations.

Cell Culture

Bone marrow-derived macrophages were obtained from six-to-ten-week-old Balb/c mice (Charles River Laboratories Inc., Wilmington, MA) as described (Celada et al., 1984). Macrophages were cultured in DMEM (Sigma), supplemented with 20% fetal bovine serum (FBS) (Sigma) and 30% L-cell conditioned medium as a source of M-CSF. Once macrophages were 80% confluent, normally after 6 days of culture,

they were deprived of L-cell conditioned medium for 16-18 hours and treated with either growth or activating factors in the presence or absence of selective inhibitors/activators. All treatments were not toxic for the cells as determined by trypan blue exclussion or by flow cytometry analysis.

Proliferation assay

Cell proliferation was measured as previously described (Celada and Maki, 1992; Celada et al., 1996) with minor modifications. Quiescent cells (10^5) were incubated for 24 hours in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA) in 1 ml of medium with the indicated concentrations of M-CSF. The media was aspirated and replaced with 0.5 ml of media containing ^{3}H-thymidine (1 μCi/ml) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After 4-6 hours of incubation at 37 ºC, the media was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% trichloroacetic acid (TCA), the cells were solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter. Each point was performed in triplicate and the results were expressed as the mean ± SD.

RNA extraction and Northern Blot analysis

The cells were washed twice in cold PBS and extraction of total RNA was performed as described (Chomcynski and Sacchi, 1987). Total RNA samples (15 μg) were separated on 1.2% agarose gels containing formaldehyde and transferred to nylon membranes (Genescreen, NEN Life Science Products, Boston, MA). For TNF-α mRNA detection, we used the EcoRI/HindIII fragment of pSP65/TNFα (kindly supplied by Dr. M. Nabholz, ISREC, Epalinges, Switzerland). To study the expression of IL-1β, we obtained a probe by digesting the construct pGEM1/IL-1β (kindly provided by Dr. R. Wilson, Glaxo Research and Dev. Limited, Greenford, UK) with EcoRI/PstI. The expression of IL-6 mRNA was analyzed by using as a probe the EcoRI/BglII fragment of pBS/IL-6 (kindly supplied by Dr. S. Rohatgi, Center for Blood Research, Boston, MA). To detect the L32 transcript, we used the EcoRI/HindIII fragment of pGEM1/L32 as a probe (Celada et al., 1989). All probes were labeled with α-^{32}P-dCTP (ICN Pharmaceuticals). After incubating in

hybridization solution (20% Formamide, 5X Denhart's, 5X SSC, 10 mM EDTA, 1% SDS, 25 mM Na_2HPO_4, 25 mM NaH_2PO_4 and 0.2 mg/ml salmon sperm DNA) at 65^oC, membranes were exposed to Kodak X-AR films (Kodak Company, Rochester, NY). Bands of interest were quantified with a Molecular Analyst System (Bio-Rad Labs., Richmond, CA).

Determination of ERK activity by in-gel-kinase assay

The cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide, 1mM PMSF). The analysis of ERK activity was performed as described (Chao et al., 1992). Briefly, 50 μg of total protein were separated by 12.5% SDS-PAGE containing 0.1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 hour at room temperature. The gel was then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol (buffer A) for 1 hour at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hour at room temperature and then renatured by incubating it with five changes of buffer A containing 0.04% Tween-20 for 16 hours at 4 ^oC. To perform the phosphorylation assay, the gel was first equilibrated in 40 mM Hepes-NaOH (pH 7.4) containing 2 mM DTT, 0.1 mM EGTA, 15 mM $MgCl_2$, 300 μM sodium orthovanadate for 30 minutes at 25 ^oC and then incubated in the same solution but also containing 50 μM ATP and 100 μCi γ-^{32}P-ATP (ICN). The reaction was terminated by washing the gel with 5% TCA containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to X-ray films (Kodak) and quantitated with a Bio-Rad Molecular Analyst.

Determination of JNK activity

This assay was performed as described (Caelles et al., 1997) with minor modifications. Briefly, the cells were washed with PBS and lysed in cold lysis buffer (1% NP-40, 20 mM Hepes-Na pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 25

mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide). 150 μg of total protein were mixed with 75 μl of 20% protein A-sepharose and 1 μl of anti-JNK1 antibody (sc-474, Santa Cruz Biotechnology, Santa Cruz, CA) in a total volume of 500 μl. The samples were rotated for 2 hours at 4 ^{o}C. The immunecomplexes were washed three times with cold PBS supplemented with 1% NP-40 and 2 mM sodium orthovanadate, once with cold JNK buffer (20 mM Hepes-Na pH 7.5, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 2 mM DTT), and resuspended in JNK reaction buffer (JNK buffer supplemented with 1 μg GST-c-Jun (1-169) (Calbiochem) as a substrate, 20 μM ATP, 1 μCi γ-^{32}P-ATP). The reaction was allowed to proceed for 30 minutes at 30 ^{o}C and was then stopped by adding 12 μl of 5xLaemli buffer. The samples were incubated for 3 minutes at 100^{o}C and separated by 10% SDS-PAGE. After the electroforesis, the gels were fixed in isopropanol:water:acetic acid (25:65:10), dried and exposed to Kodak X-AR films.

Results

In this work we have used bone marrow-derived macrophages because they represent an homogeneous population of primary macrophages that can either proliferate efficiently in response to M-CSF or become activated in response to LPS. Both agents induce the activity of ERK kinases. To determine the involvement of ERK activation in the response to M-CSF or LPS, we used the ERK kinase (MEK-1) inhibitor PD98059 (Dudley et al., 1995). ERK activation by M-CSF was abolished in the cells preincubated with PD98059 (Fig. 1A). M-CSF induced the proliferation of quiescent bone marrow macrophages in a dose-dependent manner as measured by ^{3}H-thymidine incorporation (Fig. 1B) and by cell counting (Fig. 1C). The addition of PD98059 inhibited almost completely the M-CSF-dependent proliferation of these cells.

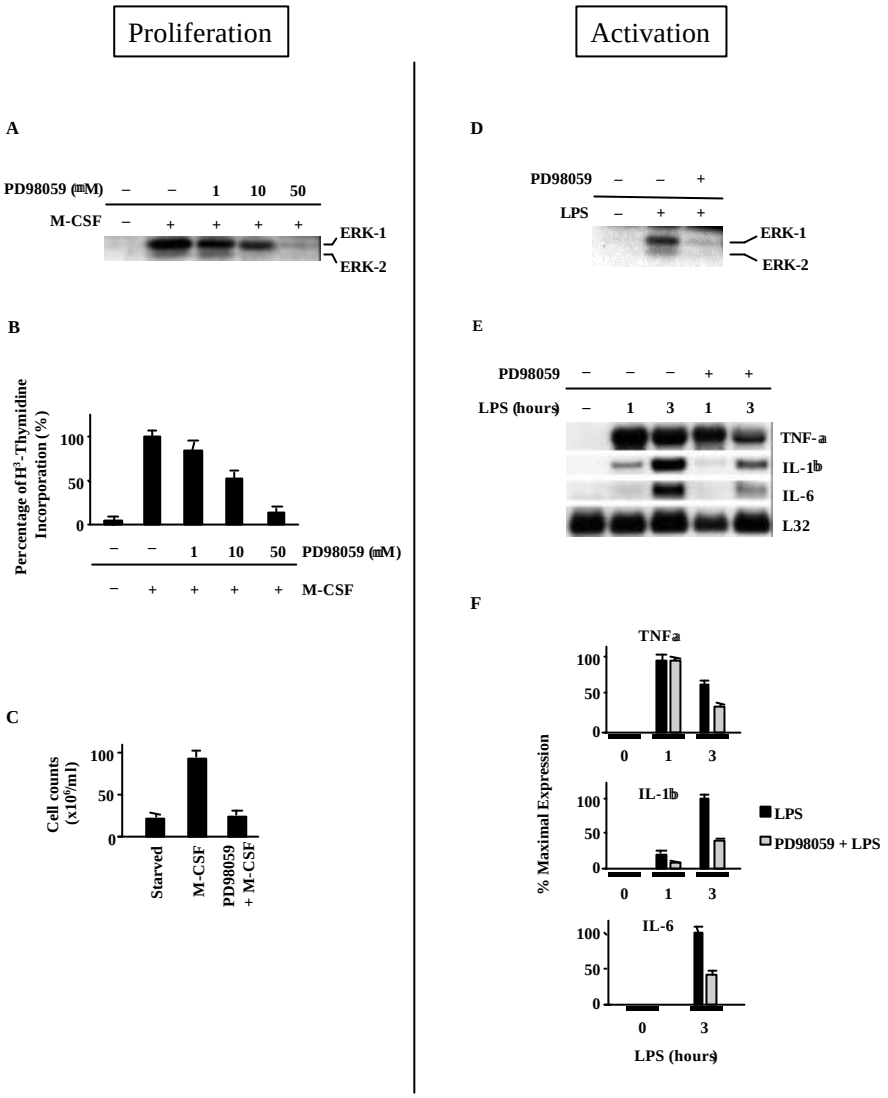


Figure 1. Activation of ERK-1/2 is required both for macrophage proliferation in response to M-CSF and for the correct induction of cytokines by LPS. A) Activation of ERK-1/2 by M-CSF was blocked by the inhibitor PD98059. The cells were untreated or preincubated with either PD98059 (50 μM) or vehicle (0.1% DMSO) for 1 hour and then stimulated with M-CSF (1200 U/ml) for 5 minutes. ERK-1/2 activity was analyzed by an in-gel-kinase assay. B) Quiescent macrophages were incubated for 24 hours with the indicated concentrations of M-CSF in the presence or absence of PD98059 (50 μM). The incorporation of ^{3}H-thymidine from triplicates was determined as described in Experimental Procedures and interpreted as a measure of macrophage proliferation. C) Quiescent cells were either not treated (starved) or incubated with M-CSF (1200 U/ml) for 24 hours in the presence of either vehicle or PD98059 (50 μM). Viable cells were counted after trypan blue staining and the mean ± SD of three independent experiments is represented. D) ERK activation by LPS was blocked by PD98059. The cells were preincubated with vehicle or PD98059 (50 μM) for 1 hour and then treated or not with LPS (100 ng/ml) for 15 minutes. ERK activity was measured by an in gel-kinase assay. E) PD98059 inhibits the induction of proinflammatory cytokines by LPS. The cells were not treated or preincubated with either PD98059 (50 μM) or vehicle (0.1% DMSO) for 1 hour and then stimulated with LPS (100 ng/ml) for 1 or 3 hours. The expression of TNF-α, IL-1β and IL-6 was determined by Northern blotting (15 μg of total RNA per lane). F) Normalyzed values of cytokine expression were represented. All images are representative of three independent experiments.

Once we had observed that ERK activation was required for the macrophage proliferation induced by M-CSF, we further extended our investigations to analyze the involvement of ERK-1/2 activity in the macrophage activation by LPS. Preincubation of macrophages with PD98059 also abolished the activation of ERK-1/2 by LPS (Fig. 1D). The treatment with this compound inhibited the LPS-induced expression of IL-1β and IL-6 (Fig. 1E and F). Besides, the blockage of ERK activation had also an inhibitory effect on the late induction of TNF-α by LPS. These results suggest that activation of ERK-1/2 is required for the correct induction of cytokines during the macrophagic response to LPS.

We next determined the time-course of ERK activation in response to M-CSF or LPS. Using an in-gel kinase assay, we detected ERK-1/2 activity as early as 2 minutes after the stimulation with M-CSF, it peaked at 5 minutes and decreased progressively thereafter (Fig. 2A). By contrast to the M-CSF signal transduction, we did not detect ERK activation within the first 5 minutes of stimulation with LPS (Fig. 2B). Instead, ERK-1/2 activity started to be detected at 10 minutes and peaked at 15 minutes of LPS treatment. Therefore, important differences were observed between the time-course of ERK-1/2 activity induced by M-CSF and LPS, being more rapidly induced by the proliferating factor than by the activating agent. These results were confirmed by mobility shift assays (data not shown).

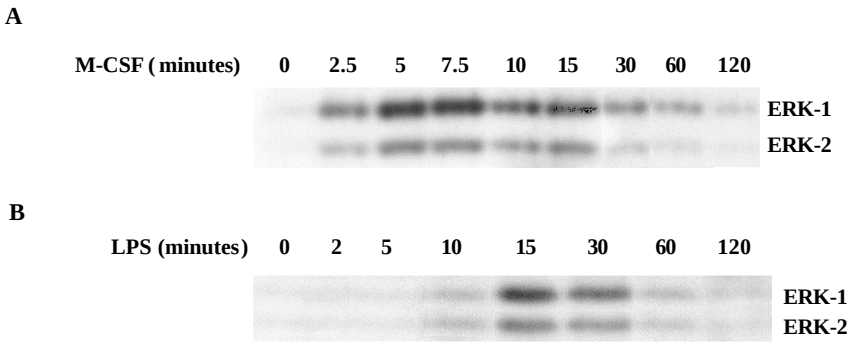


Figure 2. M-CSF and LPS induce distinct patterns of ERK-1/2 activity. Quiescent macrophages were treated with M-CSF (1200 U/ml) (A) or LPS (100 ng/ml) (B) for different periods of time. The activity of ERK-1/2 was analyzed by an in gel-kinase assay. The images are representative of three independent experiments.

In order to determine whether differences in the time-course of ERK activation had any specific relationship with the macrophagic response towards proliferation *versus* activation, we tested the effect of other growth factors. Although M-CSF is the major and specific growth factor for macrophages, these cells are also able to proliferate in response to GM-CSF and IL-3 (Fig. 3A and B). However, the signaling pathways induced after the binding of these growth factors to their specific receptors (deGroop et al., 1998; Miyahima et al., 1997) is different from that induced by the M-CSF receptor (Hamilton, 1997). As shown above for the M-CSF-induced response, the macrophage proliferation induced by GM-CSF or IL-3 was also blocked by the use of the specific MEK inhibitor PD98059 (Fig. 3A-D). This indicates that ERK activation is required for this process. The inhibition of macrophage proliferation is not due to a general toxic effect of PD98059, since in previous experiments this compound did not reduce macrophage viability or induce macrophage apoptosis (data not shown). In addition, a number of cellular responses, such as the induction of MAPK phosphatase-1 was not modified by this inhibitor (Valledor et al., 1999).

In order to determine any correlation between the earliest peak of ERK activity and macrophage proliferation, we studied the pattern of ERK activation in response to GM-CSF and IL-3. The treatment of macrophages with either one of these two growth factors induced the activation of ERK-1/2 within the first 5 minutes of stimulation (Fig. 3E and F). This activation was extended up for 10 more minutes and thereafter decayed to basal levels.

Moreover, we studied the effect of the phorbol ester TPA. This agent induced the proliferation of quiescent bone marrow macrophages (Fig. 4A). Interestingly, macrophage proliferation induced by TPA was inhibited by the pretreatment with PD98059 (Fig. 4A and B). We also compared the effect of TPA with that of LPS on macrophage activation and found that, in bone marrow macrophages, TPA was a much weaker inducer of the expression of the proinflammatory cytokines TNF-α and IL-1β (Fig. 4C).

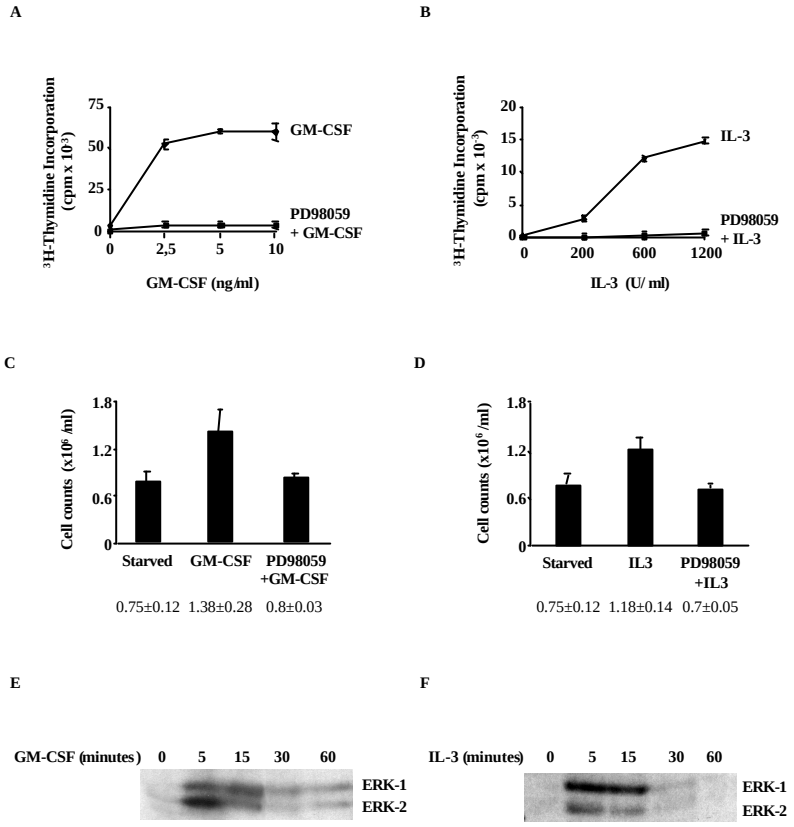


Figure 3. ERK activation is required for GM-CSF- or IL-3-induced macrophage proliferation. A and B) Quiescent macrophages were incubated with the indicated concentrations of GM-CSF (A) or IL-3 (B) in the presence or absence of PD98059 (50 μM). Macrophage proliferation was determined by measuring the incorporation of ^{3}H-thymidine. C and D) Quiescent cells were either not treated (starved) or incubated for 24 hours in medium containing GM-CSF (10 ng/ml) (C) or IL-3 (1200 U/ml) (D) in the presence of either vehicle or PD98059 (50 μM). Cell counting after trypan blue staining was performed and the mean of three independent experiments is represented. E and F) GM-CSF and IL-3 induce a time-course of ERK activity equivalent to that induced by M-CSF. Macrophages were incubated with GM-CSF (10 ng/ml) (E) or IL-3 (1200 U/ml) (F) for different periods of time. ERK-1/2 activity was measured by an in gel-kinase assay. The images are representative of two independent experiments.

Our results suggest that, in macrophages, TPA plays a more important role as an inducer of proliferation rather than as an activating factor. When the time-course of ERK activation was analyzed, a peak of ERK-1/2 activity was detected within the first 5 minutes of stimulation with TPA (Fig. 4D). Taken together, our results suggest that, in macrophages, the earliest peak of ERK activation is a common feature induced by mitogenic factors.

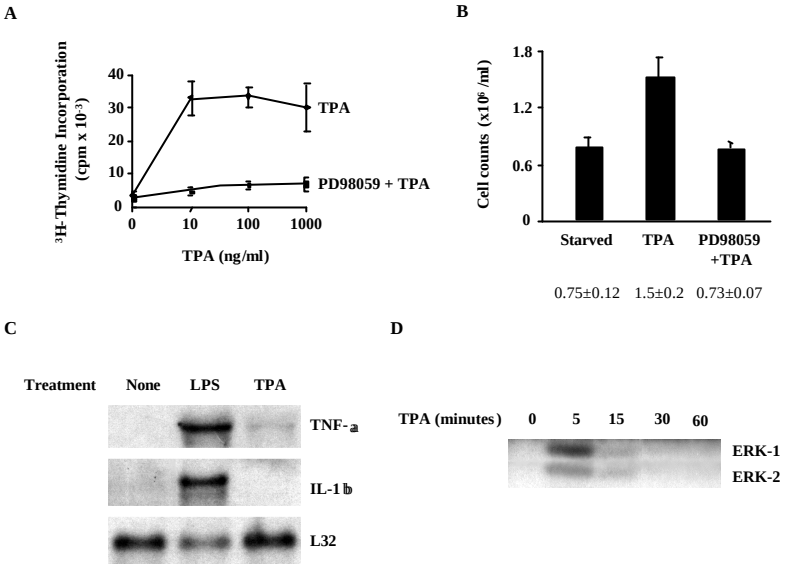


Figure 4. The treatment with TPA induces macrophage proliferation and an early peak of ERK activation. A) ERK activation is required for the proliferation of macrophages in response to TPA. Quiescent macrophages were treated with the indicated doses of TPA in the presence or absence of PD98059 (50 μM). Macrophage proliferation was determined from triplicates by the analysis of ^{3}H-thymidine incorporation. B) Quiescent cells were either not treated (starved) or incubated for 24 hours with TPA (100 ng/ml) in the presence of either vehicle or PD98059 (50 μM). Cell counting after trypan blue staining was performed and the mean of three independent experiments is represented. C) The TPA-induced expression of proinflammatory cytokines was compared to that induced by LPS in macrophages. The cells were either left untreated or incubated with LPS (100 ng/ml) or TPA (100 ng/ml) for 90 minutes. The expression of TNF-α and IL-1β was studied by Northern blotting (15 μg of total RNA per lane). The expression of the L32 transcript was analyzed to check for differences in RNA loading and transfer. D) TPA induces ERK activation within the first 5 minutes of stimulation. The cells were incubated with TPA (100 ng/ml) for the indicated periods of time. ERK activity was determined by an in gel-kinase assay.

Next, we were interested in assesing whether other agents that mimick the effect of LPS, induced also a similar pattern of ERK activation. Although IFN-γ is the major macrophage activating factor, it induces different functional activities than LPS. In fact, no ERK activity was detected in macrophages stimulated with IFN-γ (data not shown). Interestingly, the incubation of macrophages with exogenous PC-PLC from *Bacillus cereus* induced potently and very quickly the mRNA expression of the cytokines TNF-α and IL-1β (Fig. 5A). This induction was partially inhibited by the use of PD98059 (Fig. 5A) thus suggesting that ERK activation was involved in this event. The effect of PC-PLC on macrophage proliferation was also analyzed. The treatment with PC-PLC alone did not induce proliferation on quiescent macrophages (Fig. 5B). Since macrophage activation is linked to a loss of

proliferation, as demonstrated by the treatment of macrophages with M-CSF and LPS simultaneously (Fig. 5C), the effect of exogenous PC-PLC on the M-CSF-induced proliferation of macrophages was also studied. In fact, PC-PLC significantly inhibited macrophage proliferation in response to M-CSF (Fig. 5C).

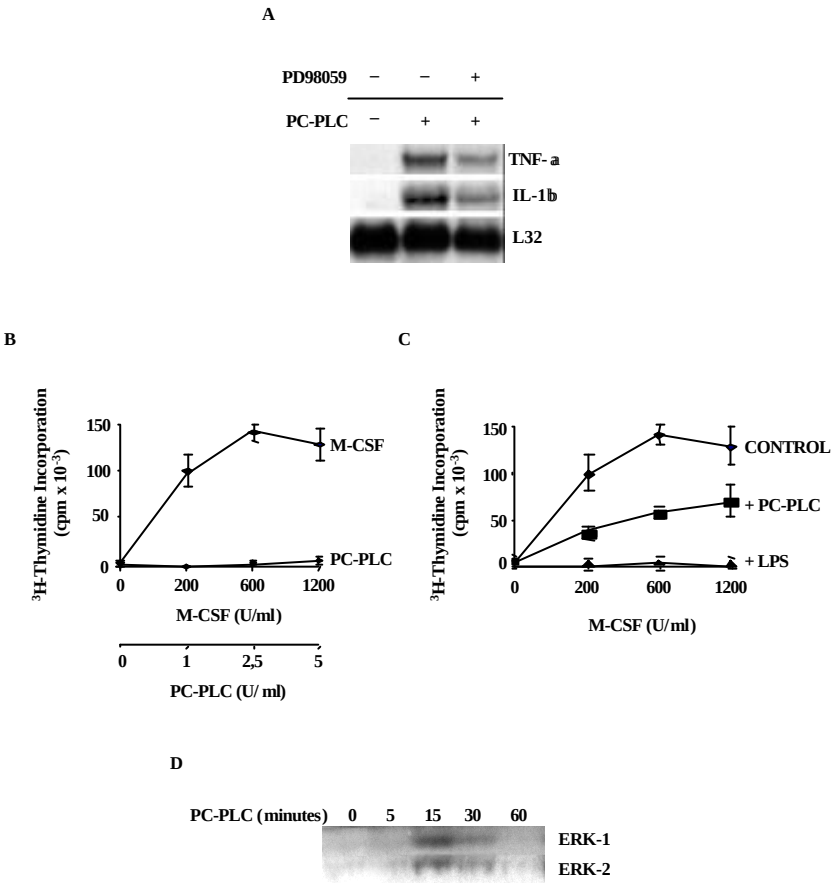


Figure 5. The treatment of macrophages with exogenous PC-PLC from *Bacillus cereus* induces the expression of proinflammatory cytokines. A) PD98059 inhibits the expression of TNFα and IL-1β induced by PC-PLC. Quiescent macrophages were either left untreated or incubated with PD98059 (50 μM) or vehicle for 1 hour and then treated with PC-PLC (5 U/ml) for 1 hour. The expression of TNF-α and IL-1β was assayed by Northern blotting (15 μg of total RNA per sample). The expression of the L32 gene was also analyzed to check for differences in RNA loading and transfer. B) PC-PLC does not induce macrophage proliferation. Quiescent cells were incubated with the indicated doses of M-CSF or PC-PLC for 24 hours. Macrophage proliferation was measured by studying the incorporation ^{3}H-thymidine. C) PC-PLC inhibits the macrophage proliferation induced by M-CSF. Quiescent cells were incubated with different concentrations of M-CSF in the presence or absence of PC-PLC (5 U/ml) or LPS (100 ng/ml). An assay of ^{3}H-thymidine incorporation was used to determine macrophage proliferation. D) PC-PLC mimicks the time-course of ERK-1/2 activation induced by LPS. The cells were exposed to exogenous PC-PLC for the indicated periods of time. ERK activity was analyzed by an in gel-kinase assay.

Taken together, these results indicate that exogenous PC-PLC acts as a macrophage activating agent. Therefore, we analyzed the time-course of ERK activation induced by this agent and compared it with that triggered by LPS. PC-PLC mimicked the time-course of ERK activity induced by LPS, with maximal activation after 15 minutes of stimulation (Fig. 5D). These results further supported our hypothesis that the pattern of ERK activation helped to define the macrophage response towards proliferation or activation.

We were also interested in analyzing whether the time-course of activation of the JNK pathway could also play a role in determining the macrophage dychotomy between proliferation and activation. Interestingly, both M-CSF and LPS induced JNK activity, as assessed by an *in vitro* kinase assay (Fig. 6). However, the time-course of this activation was virtually identical. JNK1 activity was not detected during the first 5 minutes of stimulation, peaked at 15 minutes, remained elevated at 30 minutes and thereafter decayed to basal levels. PC-PLC was also observed to induce JNK activity in a similar way. In contrast, TPA did not induce detectable JNK activity in macrophages. Therefore, we found no tight correlation between the capability of a certain agent to induce JNK activity and its effect on macrophage biology.

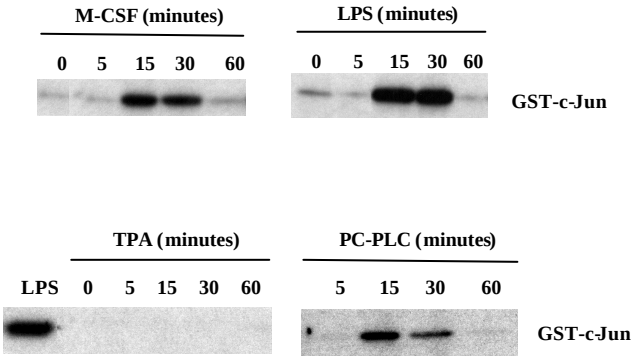


Figure 6. M-CSF, LPS and PC-PLC, but not TPA, induce JNK activation in bone marrow macrophages. Quiescent cells were incubated with M-CSF (1200 U/ml), LPS (100 ng/ml), TPA (100 ng/ml) or PC-PLC (5 U/ml) for the indicated periods of time. JNK activation was measured as the capability of immunoprecipitated JNK1 to phosphorylate GST-c-Jun *in vitro*.

Discussion

Lymphocytes undergo a clonal expansion when they are activated either after interaction with a peptide presented by the major histocompatibility complex (T cells) or after the direct recognition of an antigen (B cells) (Neuberger, 1997). In contrast, macrophages cannot simultaneously proliferate and become activated. In fact, macrophage activation is linked to a growth arrest and an enhancement of their ability to perform specialized functions in the immune system. Our goal is to determine the signaling mechanisms that induce macrophages to either proliferate or become activated.

In this report, we have used bone marrow-derived macrophages because they constitute an homogeneous population of primary macrophages. An advantage over macrophagic cell lines is that bone marrow macrophages can be rendered quiescent by removing M-CSF from the media and then induced to proliferate efficiently in response to growth factors. Besides, these cells become activated after exposure to activating agents, such as LPS. One of the main properties of the activation of macrophages by LPS is the production of proinflammatory cytokines that help resolve the immune response against microorganisms. However, although representing a suitable model for studying several aspects of the regulation of macrophage proliferation *versus* activation, at the present, transfection of these primary cultures is very unefficient (Celada et al., 1996). For this reason, we need to use chemical inhibitors to assay the involvement of specific molecules in macrophage biology.

In this report we have shown that, in macrophages, activation of ERK-1/2 is necessary for the proliferative processes induced by different growth factors including M-CSF, GM-CSF and IL-3 and by the tumor promoting agent TPA. The activation of these kinases is also necessary for the proliferation of other cell types in response to specific growth factors or serum (Brondello et al., 1995; Robinson and Cobb, 1997). The inhibition of this pathway led to a growth arrest of macrophages at the G_1 phase of the cell cycle, without inducing apoptosis (Valledor

et al., 1999). Besides, we have also demonstrated that activation of ERK-1/2 by LPS is required for the correct induction of the cytokines IL-1β, IL-6 and, to a lesser extent, TNFα. These results complement recent observations in human monocytes, in which blockage of the ERK pathway inhibited the LPS-induced secretion of IL-1β and TNF-α (Foey et al., 1998; Scherle et al., 1998). We have also found that the treatment with exogenous PC-PLC mimicks some of the aspects of the macrophage activation by LPS, including the growth arrest and the production of proinflammatory cytokines. Again, ERK activity was required for the normal expression of TNF-α and IL-1β induced by PC-PLC. These observations contrast with the mitogenic effect atributted to the treatment with exogenous PC-PLC in some other cellular system (Cai et al., 1993). Our results suggest that, in macrophages, PC-PLC plays a major role in the context of macrophage activation, which is in agreement with the findings that mouse septic shock during gram-negative bacterial infections can be downmodulated by the blockage of PC-PLC activation (Tschaikowsky et al., 1998).

The fact that both proliferating and activating agents induced ERK activity in macrophages does not reduce the importance of these kinases in the regulation of these two processes. In fact, we have shown that the time-course of ERK activity was markedly different in one case or the other. Our results confirm previous observations that LPS and M-CSF induced two distinc patterns of ERK activation in macrophages (Krause et al., 1998). For the first time, we have demonstrated that GM-CSF, IL-3 and TPA, which induce macrophage proliferation, trigger a similar pattern of ERK activation as that induced by M-CSF, with an early peak of ERK-1/2 activity within the first 5 minutes of stimulation. In contrast, LPS and PC-PLC, which inhibited macrophage proliferation and induced the expression of cytokines, triggered a more delayed peak of ERK activation, which was maximal after 15 minutes of stimulation. These observations agree with several reports that correlate the time-course of ERK activation with the fate of certain cellular responses (reviewed in Sarbassov et al., 1997).

The mechanisms by which the proliferating agents induce an earlier peak of ERK activation in comparison to that induced by the activating agents may be a consequence of the usage of different pathways to activate the ERK cascade. In

BAC1.2F5 macrophages, ERK activation by M-CSF was postulated to be mediated by both Ras-dependent and -independent mechanisms, whereas the activation by LPS partially involved the action of a PLC isoform specific for phosphatydilcholine (Büscher et al., 1995). These observations are in agreement with our finding that the treatment of macrophages with exogenous PC-PLC induces a similar time-course of ERK activation as that induced during the macrophage response to LPS. Our results also indicate that although M-CSF, GM-CSF and IL-3 have been shown to activate different signaling molecules, e.g. signal transducer and activator of transcription (STAT)-1 in response to M-CSF (Hamilton, 1997a; 1997b) *versus* STAT-5 in response to GM-CSF (Feldman et al., 1997) or IL-3 (Hara and Miyajima, 1996), convergence at the level of ERK activation exist between the different macrophage mitogenic networks.

We are currently investigating the mechanisms downstream or in parallel to ERK-1/2 that may help define a certain cellular response depending on the time-course of ERK activation. The fact that a particular pattern of ERK activity correlates with a specific macrophage response does not necessarily mean that ERK-1/2 are the unique regulators of that process. In fact, the blockage of ERK activation did not result in a complete inhibition of the expression of proinflammatory cytokines in any of the systems tested. Our results point to an involvement of ERK activity in the determination of the fate of the macrophage response rather than on the extent of the final response itself. We should also think on the existence of finely regulated interactions between active ERK-1/2 and other signaling molecules, most probably transiently switched on, in order to regulate gene transcription leading to a specific macrophage response. In this regard, other members of the MAPK group of kinases, such as JNK/SAPK, have been also implicated in the control of proliferation (Auer, et al., 1998; Bost et al., 1997) and the production of cytokines in different cell types (Hoffmeyer et al., 1999; Ishizuka et al., 1997). Unfortunately, we have not been able to demonstrate any tight correlation between the activation of the JNK pathway and the macrophage response towards proliferation or activation. However, we are currently assessing whether these kinases play a role in synergy to ERK-1/2 in the control of cytokine expression in response to LPS or PC-PLC.

Taken together, our results allow us to take two important conclusions. First, the ERK pathway is required both for macrophage proliferation and for the correct production of cytokines during macrophage activation. And second, a clear correlation exists between the time-course of ERK activation and the decision of macrophages to either proliferate or become activated, being the initial peak of ERK activation common to all the proliferative signals and the more delayed peak induced by activating agents. Although we cannot state that this pattern of activation is sufficient to induce a certain macrophage response, our results suggest a crutial role for the ERK pathway in the control of this dychotomy.

El IFN-g inhibe la proliferación de los macrófagos a través del alargamiento del patrón de activación de ERK-1/2, el cual regula la expresión de c-*myc*

El M-CSF es el factor de crecimiento específico de los macrófagos. La interacción del M-CSF con su receptor conduce a la activación de varias vías de señalización, entre ellas, la activación de las MAP quinasas, ERK-1/2. Como ya hemos mencionado anteriormente, el patrón de activación de ERK-1/2 es esencial en muchos procesos, regulando tanto los procesos de activación como los de proliferación de los macrófagos. La regulación negativa de la actividad de las ERK viene dada por la fosfatasa MKP-1, la cual es capaz de defosforilar e inactivar a ERK tanto *in vitro* como *in vivo*. La activación de las diversas vías de señalización en respuesta a factores de crecimiento, como el M-CSF, conduce finalmente a la activación del ciclo celular que regula y dirige todo el proceso de división celular. c-Myc es una proteína que juega un papel clave en la regulación de dicho ciclo celular.

En contraposición al M-CSF, el IFN-γ secretado por los linfocitos T activados o por las células NK es el principal activador y un potente inhibidor de la proliferación de los macrófagos. La mayoría de las respuestas inducidas por esta citocina se producen mediante la activación de una vía de señalización que conduce a la activación del factor de transcripción STAT1. Aunque se ha implicado a la expresión del inhibidor del ciclo celular $p21^{waf1}$ y/o a la inhibición de c-*myc* en la regulación negativa que el IFN-γ ejerce sobre la proliferación de los macrófagos, poco se conoce realmente sobre este proceso. Por tanto, el objetivo de este estudio era intentar determinar el mecanismo de acción del IFN-γ sobre la proliferación de los macrófagos.

Como muchas otras acciones mediadas por el IFN-γ, el efecto antiproliferativo de esta citocina depende de la activacion de STAT1, ya que en macrófagos deficientes (*knock-out*) de STAT1, el IFN-γ no es capaz de bloquear la proliferación inducida por el M-CSF. Además hemos visto que a pesar de que el IFN-γ induce de forma STAT1-dependiente la expresión del inhibidor del ciclo celular $p21^{waf1}$, éste no es el responsable de la inhibición de la proliferación inducida por esta citocina en los macrófagos. En contraposición, hemos observado

que el IFN-γ inhibe la expresión de la fosfatasa MKP-1 y que, por tanto, alarga el patrón de activación de ERK, un fenómeno que es característico de los procesos de activación de los macrófagos y opuesto a los fenómenos de proliferación. Este efecto también se produce de forma STAT1-dependiente. Además, este alargamiento de la activación de las ERK puede ser simulado por un inhibidor de la expresión de MKP-1, el GF109203X, que al igual que el IFN-γ inhibe la proliferación de los macrófagos.

Finalmente, y tal y como ya se había descrito, hemos observado que el tratamiento de los macrófagos con el IFN-γ inhibe la expresión de c-*myc* inducida por los factores de crecimiento, siendo también este efecto STAT1-dependiente. En un intento de correlacionar la inhibición de MKP-1, y el subsiguiente alargamiento del patrón de activación de las ERK, con la expresión de c-*myc*, estudiamos el efecto de los inhibidores PD98059 (inhibidor de la activación de ERK) y GF109203X (inhibidor de la expresión de MKP-1) sobre la expresión de c-*myc*. En este sentido, hemos observado que la actividad de ERK-1/2 no es necesaria para la inducción de c-*myc* por el M-CSF, aunque parece que sí lo sea para la inhibición de su expresión, puesto que un aumento de la actividad de ERK tras el tratamiento con GF109203X o con IFN-γ inhibe la expresión de c-*myc*.

Este fenómeno permite hipotetizar porque los factores de crecimiento como el M-CSF inducen de manera tan rápida pero a la vez tan corta la actividad de las quinasas ERK. En este aspecto, creemos que la actividad de ERK debe de ser necesaria para la señalización temprana de la proliferación, como lo demuestra el tratamiento con PD98059, pero que una vez ya está iniciada dicha señal, ERK debe de ser rápidamente inhibida mediante la actuación de la fosfatasa MKP-1 para permitir la expresión prolongada de c-*myc*, la cual es necesaria para el correcto desarrollo del ciclo celular.

IFN-γ inhibits macrophage proliferation via the prolongation of the activation pattern of ERK-1/2, which regulates c-*myc* expression

Abstract

Different cytokines or growth factors induce macrophages to proliferate, become activated, differentiate to specific macrophage populations, or die through apoptosis. Bone marrow-derived macrophages proliferate in response to their specific growth factor M-CSF. Several signaling pathways are required for the proliferative action of M-CSF, including c-*myc* expression. M-CSF also induces the activation of the ERK pathway during a very controlled period of time. Expression of the phosphatase MKP-1 shortly after the activation of ERK-1/2 leads to the prompt dephosphorylation and inactivation of these kinases. As a counterpart to proliferation, macrophages can become activated and participate in the development of an immune response. IFN-γ is the main cytokine involved in macrophage activation and it potently inhibits macrophage proliferation. The anti-proliferative effect of IFN-γ is STAT1-dependent. Moreover, this effect is not mediated by the IFN-γ-induced expression of the cdk inhibitor $p21^{Waf1}$, but by the inhibition of c-*myc* expression in response to growth factors. In the present work we demonstrate that IFN-γ inhibits the M-CSF-induced expression of MKP-1, leading to the prolongation of the activation pattern of ERK-1/2. By artificially inhibiting MKP-1 expression, the elongation of ERK activity is shown here to correlate with the down-regulation of the M-CSF-induced expression of c-*myc* and the subsequent antiproliferative effect of IFN-γ in macrophages.

Introduction

Macrophages perform essential functions in the organism by acting as regulators of homeostasis and as effector cells in infection, wounding and tumor growth (Celada and Nathan, 1994). Macrophages originate in the bone marrow and, through the blood stream, reach all the tissues in the organism. According to the local needs, resident macrophages either continue proliferating or become activated and perform their specialized functions (Boehm et al., 1997).

Macrophage colony-specific growth factor (M-CSF) is the major and only specific growth factor for this cell type (Stanley et al., 1997). The receptor for M-CSF is the product of the protooncogene *c-fms* (Vairo and Hamilton, 1991). The binding of M-CSF leads to the autophosphorylation of the receptor and the subsequent recruitment of several signal transducing molecules (Hamilton, 1997; Stanley et al., 1997; Vairo and Hamilton, 1991). One of the cascades activated by M-CSF is the Raf/MEK/extracellular-regulated kinase (ERK) pathway (Büscher et al., 1995; Jaworowski et al., 1996). Raf, a serine/threonine protein kinase, phosphorylates and activates the threonine/tyrosine protein kinase MEK-1, which, in turn, phosphorylates and activates ERKs 1 and 2 (Crews et al., 1992; Howe et al., 1992). These are proline-directed serine/threonine protein kinases, also named p44- and p42-mitogen-activated protein kinases (MAPKs), respectively (Seger and Krebs, 1995). Active ERKs phosphorylate and regulate several cellular proteins, including additional protein kinases, components of the cytoskeleton, phospholipase A_2 and transcription factors, such as Elk/TCF and c-Jun, which regulate the expression of immediate early genes (Seger and Krebs, 1995; Treisman, 1996). Tight control of the time-course of ERK activity is essential for the fate of several cellular processes (Marshall, 1995; Sarbassov et al., 1997; Tombes et al., 1998; Valledor et al., 2000b). The negative regulation of ERK activity is mainly exerted by members of a family of dual specificity tyrosine phosphatases, including MAPK phosphatase-1 (MKP-1). This phosphatase dephosphorylates both phosphothreonine and phosphotyrosine residues required for ERK-1/2 activity (Alessi et al., 1993; Sun et al., 1993).

As a counterpart of proliferation, macrophage activation is characterized by a series of biochemical and morphological modifications that allow these cells to perform their professional functions (Boehm et al., 1997; Celada and Nathan, 1994). Interferon-gamma (IFN-γ), a cytokine mainly secreted by activated T lymphocytes, acts as the major macrophage activator. IFN-γ induces the sequential activation by phosphorylation of Janus kinases (JAK)-1 and –2 and signal transducer and activator of transcription (STAT)-1. As a consequence, the latter undergoes dimerization and translocation to the nucleus, where it regulates gene transcription (Ihle and Kerr, 1995; Schindler and Darnell, 1995).

Previous work from our group and other investigators has shown that IFN-γ is a potent inhibitor of macrophage proliferation (Vairo et al., 1996; Xaus et al., 1999b). However, the mechanisms that account for the blockage of cell growth mediated by this cytokine are not understood. It has been described in several cell types that downregulation of c-*myc* in response to IFN-γ is responsible for the antiproliferative effect of this cytokine and over-expression of c-*myc* is able to bypass the inhibitory effect of IFN-γ treatment (Dey et al., 1999; Vairo et al., 1996). Other authors have attributed the antiproliferative effect of IFN-γ to the induction of the cdk inhibitor $p21^{Waf1}$ (Chin et al., 1996; Subramaniam and Johnson, 1997). However, several studies using $p21^{Waf1}$ antisense oligonucleotides or $p21^{Waf1}$ *knock-out* mice are controversial in regards to the essential role of $p21^{Waf1}$ as a mediator of the antiproliferative effects of IFN-γ (Chin et al., 1996; Hobeika et al., 1999; Subramaniam and Johnson, 1997; Vivo et al., 2001; Xaus et al., 1999).

In the present report we show that the inhibitory action of IFN-γ on both c-*myc* induction and macrophage proliferation is dependent on STAT1 activation. IFN-γ does not lead to direct phosphorylation of ERK-1/2 in bone marrow-derived macrophages. However, in a STAT-1-dependent manner, IFN-γ induced a prolongation of the time-course of ERK-1/2 activity initially stimulated by M-CSF, a phenomenon that has been associated with a loss of proliferation in other cellular systems. We have explored the mechanism by which IFN-γ extends ERK activity in macrophages and found that this cytokine potently inhibits the induction of the expression of the phosphatase MKP-1. The effect of IFN-γ on c-*myc* expression and macrophage proliferation can be mimicked by inhibiting MKP-1 expression artificially, thus suggesting that the prolongation of ERK-1/2 activity exerts a negative effect on c-*myc* expression. Indeed, the blockage of ERK activation with a selective inhibitor potentiates the induction of c-*myc* in response to M-CSF. The data presented here elucidate a novel mechanism by which IFN-γ regulates c-*myc* expression and exerts its anti-proliferative effect in macrophages.

Material and methods

Materials

Recombinant M-CSF was provided as a gift by DNAX (Palo Alto, CA). In some experiments, we used L-cell conditioned medium as the source of this growth factor. PD98059 and GF109203X were purchased from Calbiochem (San Diego, CA). IFN-γ was kindly donated by Genetech Inc. (South San Francisco, CA). All other products were of the best grade available and were obtained from Sigma Chemical (S. Louis, MO). Deionized wather further purified with a millipore Milli-Q system (Millipore, Bedford, MA) was used.

Cell Culture

Bone marrow-derived macrophages were obtained from six-to-ten-week-old Balb/c mice (Charles River Laboratories Inc., Wilmington, MA) as described (Celada et al., 1984). Macrophages were cultured in DMEM (Sigma), supplemented with 20% fetal bovine serum (FBS) (Sigma) and 30% L-cell conditioned medium as a source of M-CSF. All the experiments were performed once macrophages reached 80% confluency, normally after 6 days of culture. At this time, the cells were deprived of M-CSF for 18 hours and then treated with M-CSF in the presence or absence of IFN-γ. STAT1 *knock-out* mice were kindly provided by Dr. Schreiber (Washington University School of Medicine, St Louis, MO). $p21^{Waf1}$ *knock-out* mice were kindly donated by Dr. Philip Leder (Harvard Medical School, HHMI).

Proliferation assay

Cell proliferation was measured as previously described (Celada and Maki, 1992; Celada et al., 1996) with minor modifications. Quiescent cells (10^5) were incubated for 24 hours in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA) in 1 ml of medium with the indicated concentrations of M-CSF. The media was aspirated and replaced with 0.5 ml of media containing ^{3}H-thymidine (1 μCi/ml) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After 4-6 hours of incubation at 37ºC, the media was removed and the cells were fixed in ice-cold 70% methanol. After

three washes in ice-cold 10% trichloroacetic acid (TCA), the cells were solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter. Each point was performed in triplicate and the results were expressed as the mean ± SD.

In parallel experiments, we plated 10^6 macrophages in the presence or absence of M-CSF (1200 U/ml) and IFN-γ (300 U/ml) for 24 and 48 hours. Then, the number of surviving cells was measured by trypan blue exclusion with a hemacytometer. Again, each point was performed in triplicate, and the results were expressed as the mean ± SD.

RNA extraction and Northern Blot analysis

The cells were washed twice in cold PBS and extraction of total RNA was performed as described (Chomcynski and Sacchi, 1987). Total RNA samples (15-20 μg) were separated on 1.2% agarose gels containing formaldehyde and transferred to nylon membranes (Genescreen, NEN Life Science Products, Boston, MA). For MKP-1 mRNA detection, we obtained the full length cDNA fragment of MKP-1 following purification from a Hind III digestion of the plasmid pBluescript KS/MKP-1 (kindly provided by Dr. R. Bravo, Bristol-Myers Squibb Pharmac. Res. Inst., Princeton, NJ). The pMH117 plasmid corresponding to the mouse $p21^{Waf1}$ full-length cDNA cloned in pEx-lox was kindly provided by Dr. Massague (Sloan Kettering Institute, HHMI, New York, NY). A probe specific for c-*myc* was donated by Dr. Evan (ICRF, London, UK) To detect the L32 transcript, we used the EcoRI/HindIII fragment of pGEM1/L32 as a probe (Celada et al., 1989). In some experiments we also used a 18S rRNA probe as a control for loading ad transfer (Torczynski et al., 1983). All probes were labeled with α-^{32}P-dCTP (ICN). After hybridization at 65^{o}C, membranes were exposed to Kodak X-AR films (Kodak Company, Rochester, NY). The bands of interest were quantified with a Molecular Analyst System (Bio-Rad Labs., Richmond, CA).

Determination of ERK phosphorylation state by a gel mobility shift

The cells were washed twice in cold PBS and lysed on ice with a lysis solution containing 1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 250 mM

NaCl and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide and 1mM PMSF). Sodium orthovanadate (1 mM) was added when inhibition of the activity of tyrosine phosphatases was required. Insoluble material was removed by centrifugation at 13000 X g for 8 min at 4°C. Mobility shift assays were performed as described (Samuels et al., 1993). Briefly, cell lysates (60 μg) were heated at 95°C in Laemli SDS loading buffer and subjected to 7.5% SDS-PAGE to allow efficient separation of phosphorylated and dephosphorylated forms of ERKs. The samples were then electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham Corp., Arlington Heigths, IL). The blocking of the membrane was carried out in 5% non-fat dry milk in TBS-T for at least 1 hour at room temperature. Incubations with anti-ERK-1/2 primary antibody (1:10000) (kindly provided by Dr. M. J. Weber, University of Virginia, School of Medicine, Charlostesville, VA) and with peroxidase-conjugated anti-mouse IgG antibody (1:5000) (Cappel, ICN) were performed in TBS-T for 1 hour each at room temperature.

Determination of ERK activity by in-gel-kinase assay

The analysis of ERK activity was performed as described (Chao et al., 1992). 50 μg of total protein, obtained as described above, were separated by 12.5% SDS-PAGE containing 0.1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 hour at room temperature. The gel was then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol (buffer A) for 1 hour at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hour at room temperature and then renatured by incubating it with five changes of buffer A containing 0.04% Tween-20 for 16 hours at 4°C. To perform the phosphorylation assay, the gel was first equilibrated in 40 mM Hepes-NaOH (pH 7.4) containing 2 mM DTT, 0.1 mM EGTA, 15 mM $MgCl_2$, 300 μM sodium orthovanadate for 30 minutes at 25 °C and then incubated in the same solution but also containing 50 μM ATP and 100 μCi γ-^{32}P-ATP (ICN). The reaction was terminated by washing the gel with 5% TCA containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to X-ray films (Kodak) and quantitated with a Bio-Rad Molecular Analyst.

Results

The following studies were performed with bone marrow-derived macrophages because they constitute a homogeneous population of primary macrophages that can proliferate in response to M-CSF and become activated under the effect of IFN-γ (Celada et al., 1996; Xaus et al., 1999b). In contrast to other cells of the immune system, such as lymphocytes, macrophage activation is associated with an inhibition of cell proliferation (Valledor et al., 2000a; Xaus et al., 1999b). Macrophage proliferation in response to M-CSF was measured by ^{3}H-thymidine incorporation (Fig.1A) and by counting the increase on cell numbers (Fig. 1B). The treatment with IFN-γ inhibits M-CSF-dependent proliferation (Fig 1A, B). The effect of IFN-γ was dose-dependent and maximal at saturating concentrations of IFN-γ, which, in macrophages, corresponds to 300 U/ml (Celada et al., 1985) (Fig. 2).

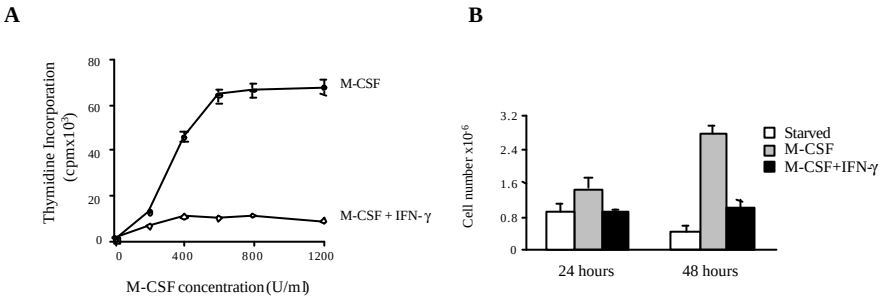


Figure 1: IFN-γ inhibits macrophage proliferation. A) BMDM were obtained after 7 days of culture in the presence of M-CSF. 10^5 quiescent macrophages were incubated with M-CSF in the presence or absence of 300 U/ml of IFN-γ for 24 hours. Proliferation was determined by ^{3}H-Thymidine incorporation as indicated in Material and methods. B) 10^6 quiescent macrophages were cultured in the presence or absence of 1200 U/ml M-CSF either alone or with IFN-γ (300 U/ml) for 24 or 48 hours. Proliferation was determined by counting the numbers of viable cells using an hemocytometer. C) 10^5 macrophages were cultured with M-CSF (1200 U/ml) in the presence of the indicated concentrations of IFN-γ for 24 hours and proliferation was determined by thymidine incorporation. In all cases, triplicates were performed for each experiment and three independent experiments were carried out. Here we represented the mean ± SD of one representative experiment each.

IFN-γ primarily leads to the activation of STAT1, which regulates the transcription of several genes (reviewed in Darnell, 1997; Stark et al., 1998).

Although the JAK/STAT pathway represents the major signaling cascade described for IFN-γ, some macrophage responses take place independently of STAT1 (Gil et al., 2001; Ramana et al., 2001). For this reason, we used macrophages derived from STAT-1 deficient mice in order to study whether the inhibition of macrophage proliferation was mediated by this pathway or not. As shown in figure 2, the inhibitory effect of IFN-γ is absent in STAT1 deficient cells, indicating that the growth arrest mediated by this cytokine is a STAT1-dependent mechanism.

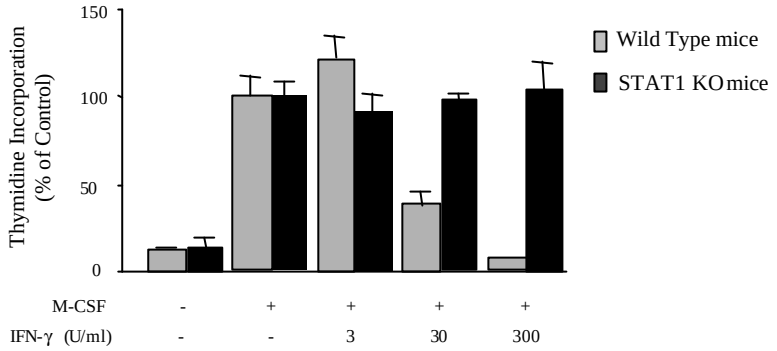


Figure 2: IFN-γ inhibits macrophage proliferation through a STAT1 dependent fashion. Macrophages were obtained from wild-type and STAT1 KO mice. 10^5 quiescent macrophages were incubated in 24-well plates in the presence of 1200 U/ml of M-CSF either alone or with the indicated concentrations of IFN-γ for 24 hours. Proliferation was determined by ^{3}H-thymidine incorporation as indicated above. Each point was done in triplicate and represented as the % of ^{3}H-thymidine incorporated by macrophages in the absence of IFN-γ (control). The graphic represents the mean ± SD of two representative experiments.

In order to determine the time frame of efficient IFN-γ anti-proliferative action, macrophages were stimulated with saturating concentrations of this cytokine at different times before and after the addition of the growth factor M-CSF (Fig. 3). The preincubation with IFN-γ or the addition of IFN-γ and M-CSF simultaneously lead to a total inhibition of macrophage proliferation in response to the growth factor. When IFN-γ was added a few minutes up to an hour after the start of the stimulation with M-CSF, there was still full inhibition of the incorporation of ^{3}H-thymidine. However, the capability of fully blocking macrophage growth was gradually lost when IFN-γ was added two or more hours after the stimulation with M-CSF. In conclusion, IFN-γ needs to regulate some of the events that take place within the first two hours of M-CSF signaling in order to fully inhibit macrophage proliferation.

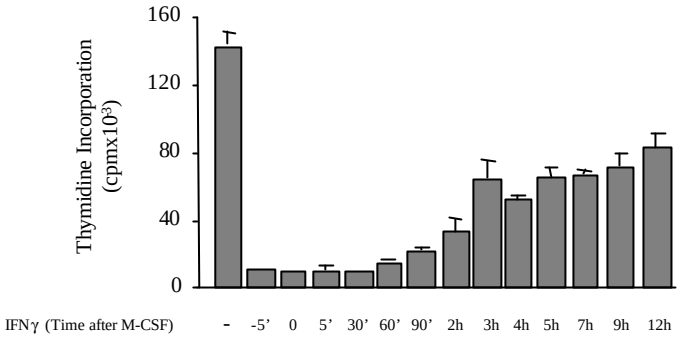


Figure 3: IFN-γ fully inhibits macrophage proliferation only when it is added before or shortly after M-CSF stimulation. 10^5 quiescent cells were incubated in 24-well plates in the presence of 1200 U/ml of M-CSF and treated with 300 U/ml of IFN-γ at the indicated times before or after M-CSF stimulation. Proliferation was determined 24 hours after M-CSF stimulation by ^{3}H-thymidine incorporation. Each point was done in triplicate and represented as the mean ± SD of two independent experiments.

In previous studies we demonstrated that IFN-γ induces the expression of $p21^{Waf1}$ in primary macrophages (Xaus et al., 1999b). $p21^{Waf1}$ belongs to the CIP/KIP family of inhibitors of cyclin-dependent kinases (cdk), thus constituting negative regulators of the cell cycle. In those studies we described an essential role for $p21^{Waf1}$ in the protection of macrophages from apoptosis induced by different stimuli. Time-course studies by northern blotting showed that $p21^{Waf1}$ mRNA expression is induced after 1 hour of treatment with IFN-γ (Fig. 4A). Maximal induction takes place after 3 hours of stimulation with this cytokine. Moreover, the induction of $p21^{Waf1}$ by IFN-γ is a STAT1 dependent mechanism (fig 4B). For this reason, we further studied the role of $p21^{Waf1}$ in the mediation of the blockage of proliferation induced by IFN-γ. However, while this protein plays an essential role in the growth arrest of several cell types, IFN-γ was still able to inhibit macrophage proliferation in macrophages derived from $p21^{Waf1}$ *knock-out* mice (Fig 4C). Taken together, these results indicate that IFN-γ blocks macrophage proliferation through a STAT1-dependent, $p21^{Waf1}$-independent mechanism.

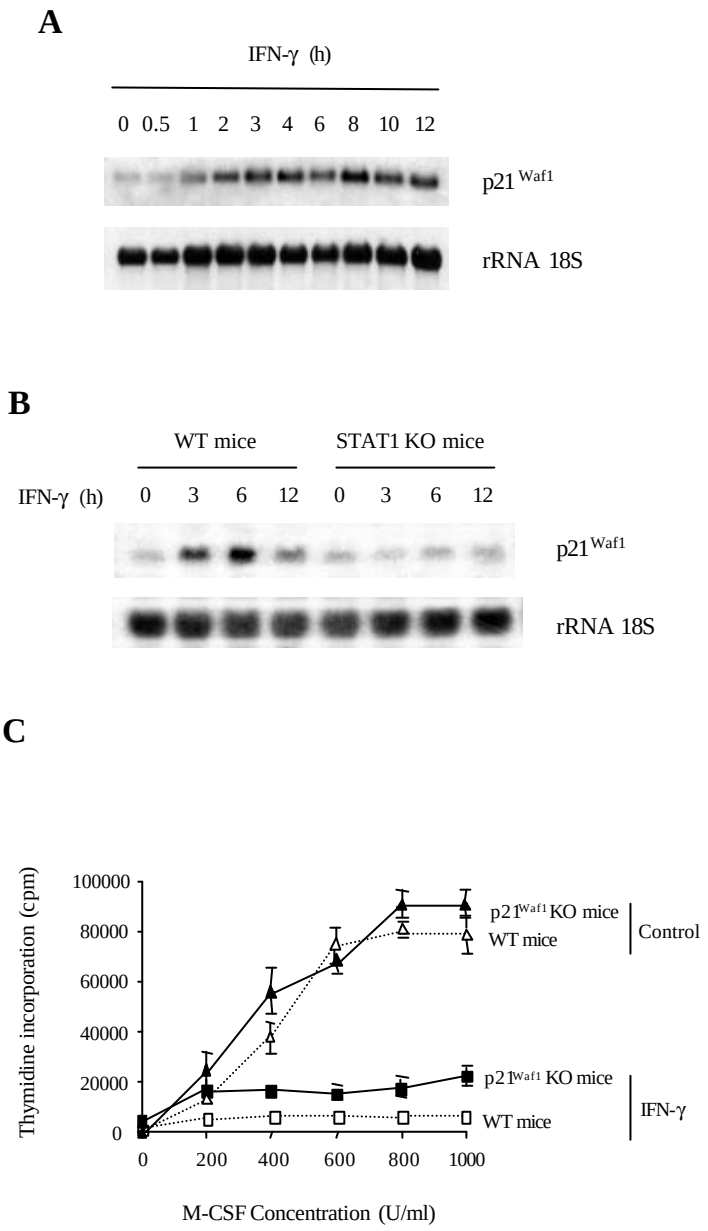


Figure 4: IFN-γ inhibits macrophage proliferation through a p21^{Waf1}-independent way. A) The expression of p21^{Waf1} was analyzed by northern blot (20 μg total RNA per lane) in macrophages treated with 300 U/ml of IFN-γ for the indicated periods of time. B) The expression of p21^{Waf1} induced by IFN-γ was analyzed by northern blotting in macrophages obtained from wild type and STAT1 KO mice. Cells were treated with 300 U/ml of IFN-γ for the indicated times. In both cases, the expression of the 18 S rRNA was used as a control for RNA loading and transfer. Similar results were obtained from three (A) or two (B) independent experiments. C) 10^5 macrophages were obtained from wild-type and p21^{Waf1} *knock-out* mice and incubated with the indicated amounts of M-CSF either alone or with 300 U/ml of IFN-γ for 24 hours. Proliferation was determined by ^{3}H-thymidine incorporation. Each point was done in triplicate and represented as the mean ± SD of three independent experiments.

One of the initial events in M-CSF signal transduction is the activation of the MEK/ERK pathway. Our previous work demonstrated two important facts. First, this cascade is essential for macrophage proliferation in response to M-CSF (Valledor et al., 1999; 2000b). And second, the time-course of ERK-1/2 activity differs between proliferative or activating signals. In brief, growth factors induced the activation of ERK-1/2 very quickly and transiently, while activating factors lead to a more delayed and prolonged pattern of ERK activation (Valledor et al., 2000b). In the present study, we used two different methods to detect ERK activation. One is based in the requirement of ERK-1/2 to be phosphorylated in order to become activated. Phosphorylated ERKs migrate slower than the non-phosphorylated form and can be detected by a mobility shift in an acrylamide gel. As shown in Fig. 5A, M-CSF triggered the phosphorylation of ERK-2. However, we could not detect any phosphorylation of this protein in response to IFN-γ. The second method was based on the capability of active ERK-1/2 to phosphorylate a substrate, myelin basic protein, polymerized in the same gel through which the samples are separated. Again, IFN-γ was not able to induce ERK-1/2 activation in comparison with M-CSF (Fig. 5B).

The same method was used to further investigate if IFN-γ modified the time-course of ERK phosphorylation induced by M-CSF (Fig. 5C). Strong ERK activation was detected 5-10 minutes after the start of M-CSF stimulation and it dropped significantly after 30 minutes of treatment. Interestingly, when IFN-γ was added simultaneously to M-CSF, ERK-1/2 activity still peaked at the same time, but a significantly higher amount of ERK-1/2 remained active even at three hours post-stimulation. Equivalent results were obtained when the cells were preincubated with IFN-γ and then treated with M-CSF (data not shown). Taken together, these results indicate that IFN-γ is not able to induce ERK phosphorylation and activation by its own, but leads to a prolongation of the pattern of ERK activity in response to M-CSF.

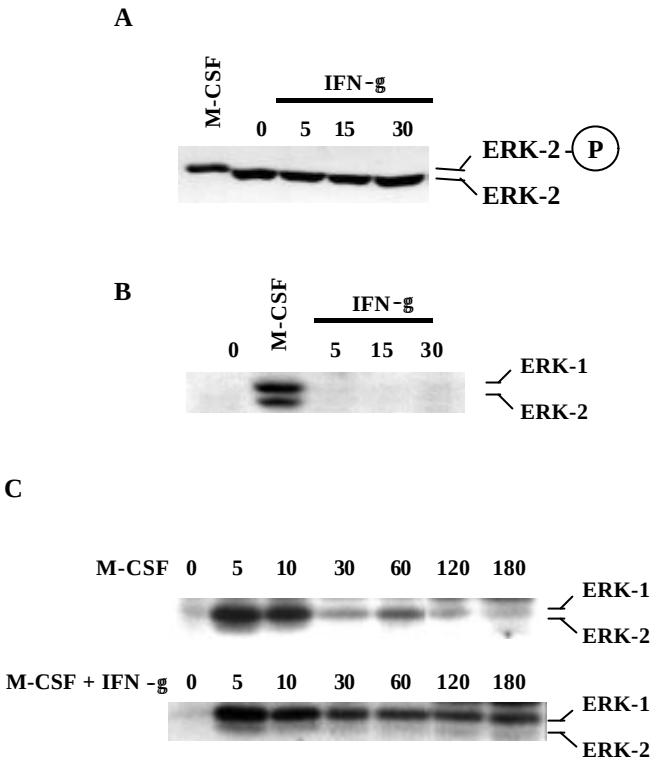


Figure 5. IFN-γ elongates the M-CSF-induced activation of extracellularly-regulated kinase (ERK)-1/2. Quiescent macrophages were stimulated with 300 U/ml of IFN-γ ,in the absence of M-CSF, for the indicated periods of time. Control cells were treated with M-CSF (1200 U/ml) for 5 minutes. The activation of the kinases ERK-1 and -2 was analyzed by mobility shift (A) or by an in gel-kinase assay (B) as described in material and methods. C). Quiescent macrophages were treated with M-CSF (1200 U/ml) for the indicated periods of time in the presence or absence of IFN-γ (300 U/ml). The activation of the kinases ERK-1 and -2 was analyzed by an in gel-kinase assay. Identical results were obtained from five independent experiments.

We further studied if this effect was STAT1-dependent. An in gel kinase assay was performed using wild type and STAT1 deficient macrophages treated with M-CSF and IFN-γ simultaneously (Fig. 6A). In cells deficients for STAT1, IFN-γ was not able to prolong the ERK-1/2 activity initially triggered by M-CSF. In order to investigate the mechanism that leads to the prolongation of ERK-1/2 activation by IFN-γ, we studied the effect of this cytokine on the expression of the phosphatase MKP-1, which is a crucial negative regulator of ERK activity. In control bone marrow-derived macrophages, the expression of MKP-1 mRNA was induced after 30 minutes of treatment with M-CSF, as measured by northern blotting (Fig. 6B). We understand that expression of MKP-1 represents a negative feedback mechanism that M-CSF triggers in order to tightly control the activity of ERK-1/2 and this may explain why ERK phosphorylation and activation takes place during a

short time frame (Valledor et al., 1999). Interestingly, IFN-γ blocked the induction of MKP-1 when added simultaneously to M-CSF, which correlates with the prolongation of ERK-1/2 activity observed in these conditions. The same result was obtained when the cells were first preincubated with IFN-γ and then stimulated with M-CSF (data not shown). Furthermore, we studied if this effect was STAT1-dependent. In STAT-1 *knock-out* cells, M-CSF was able to fully induce MKP-1 expression, however the blocking effect of IFN-γ was absent (Fig. 6B), correlating with the loss of prolongation of ERK activity (Fig. 6A). All these results suggest that IFN-γ blocks the expression of MKP-1 in a STAT1-dependent fashion and this blockage accounts for the prolongation of the time-course of ERK activity described here.

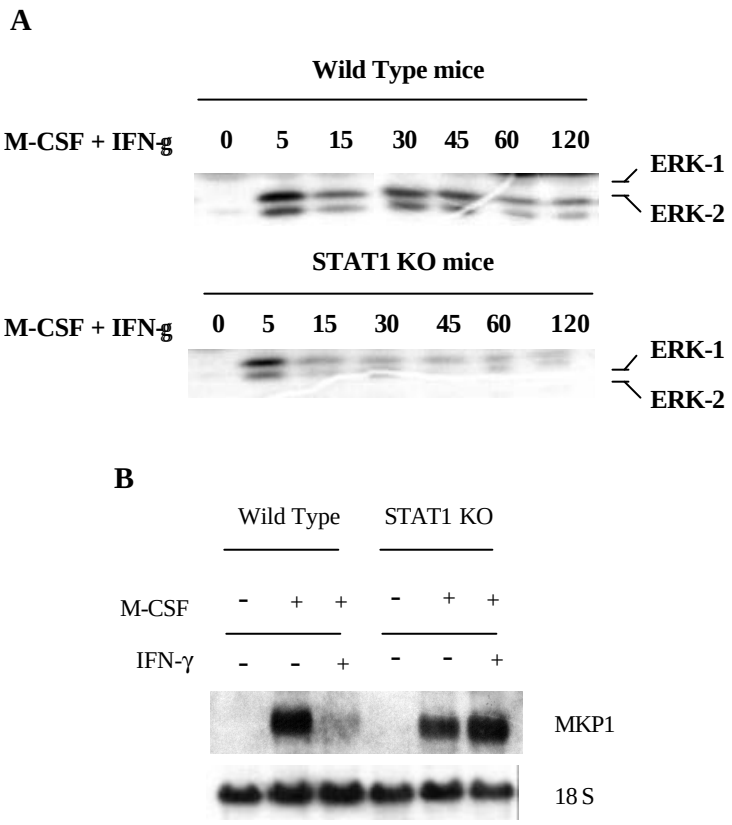


Figure 6. IFN-γ inhibits MKP-1 expression and elongates ERK activity through a STAT1-dependent mechanism. A) Quiescent macrophages from control and STAT1 *knock-out* mice were treated with M-CSF (1200 U/ml) for the indicated periods of times in the presence of IFN-γ (300 U/ml). The activation of the kinases ERK-1 and -2 was analyzed by an in gel-kinase assay. B). The expression of the phosphatase MKP-1 was determined by northern blotting (15 μg of total RNA per lane). Macrophages derived from control and STAT1 *knock-out* mice were stimulated with M-CSF (1200 U/ml) for 30 minutes in the presence or absence of 300 U/ml IFN-γ. 18 S rRNA expression was used to check for differences in gel loading and transfer. In both cases similar results were obtained from two independent experiments.

Based on the data shown so far, we hypothesized that the change in the pattern of ERK activity could represent a mechanism for the inhibition of macrophage proliferation. In order to explore this hypothesis, we mimicked the elongation of ERK activity through an independent way and then we studied its effect on macrophage proliferation. In our previous work, we have demonstrated that the induction of MKP-1 in macrophages treated with M-CSF is regulated by PKC (Valledor et al., 1999), and that GF109203X was able to inhibit the expression of MKP-1 in these cells. As shown in figures 7A and B, GF109203X blocked the expression of MKP-1 in a dose dependent manner. Interestingly, this inhibition of MKP-1 expression correlated with an elongation of ERK-1/2 activity after its initial triggering by M-CSF (Fig. 7C). Finally, the prolongation of ERK-1/2 activity under the effect of GF109203X correlated with an inhibition of M-CSF-induced proliferation (Fig. 7D). In conclusion, the blockage of MKP-1 expression by either IFN-γ or a synthetic compound translated into a prolongation of the M-CSF-induced ERK-1/2 activity and an inhibition of macrophage proliferation.

To elucidate the mechanism by which the elongation of ERK-1/2 activity influences macrophage proliferation, we explored its implications on the expression of the oncogene c-*myc*. Inhibition of c-*myc* expression has been implicated in the blockage of proliferation induced by IFN-γ (Dey et al., 1999; Vairo et al., 1996). M-CSF induces the expression of c-*myc* in quiescent cells (Fig. 8A) and the expression of this gene is detectable thereafter in all phases of the cell cycle (Roussel, 1997). Treatment of macrophages with IFN-γ inhibits the expression of c-*myc* in a STAT1-dependent way (Fig. 8A). Activation of ERK-1/2 is not necessary for the induction of c-*myc* in response to M-CSF, as observed by the treatment of macrophages with PD98059, a selective inhibitor of the MEK/ERK cascade, but PD98059 increased c-*myc* expression induced by M-CSF (Fig. 8B). Interestingly, in a similar way to what we observed with IFN-γ, GF109203X was able to elongate the kinetics of ERK-1/2 activity as shown above and to inhibit c-*myc* expression induced by M-CSF (Fig. 8C).

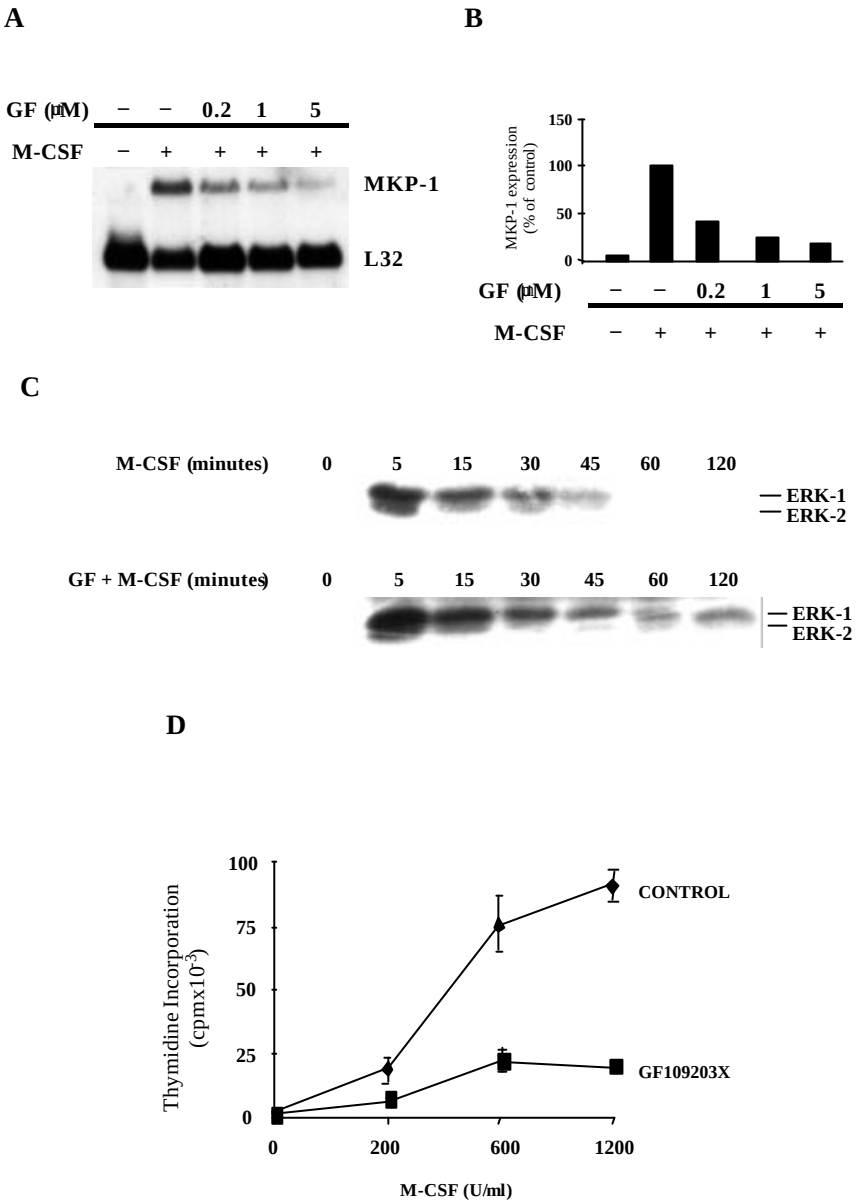


Figure 7. The inhibition of MKP-1 expression by GF109203X also leads to the elongation of ERK activity and the blockage of macrophage proliferation. A and B) Quiescent macrophages were either left untreated or pretreated with different concentrations of GF109203X (GF) for 1 hour and then stimulated with M-CSF (1200 U/ml) for 30 minutes. The expression of the phosphatase MKP-1 was determined by northern blotting (15 μg of total RNA per lane) (A) and represented as a percentage of maximal expression (B). C) Quiescent macrophages were treated with M-CSF (1200 U/ml) for the indicated periods of time in the presence or absence of GF (5 μM). The activation of the kinases ERK-1 and -2 was analyzed by an in gel-kinase assay. This figure shows one representative experiment out of three independent assays. D) 10^5 quiescent cells were left untreated or pretreated with GF109203X (5 μM) for 1 hour and then stimulated with the indicated amounts of M-CSF. Proliferation was determined 24 hours after M-CSF stimulation. Each point was done in triplicate and represented as the mean ± SD of three independent experiments.

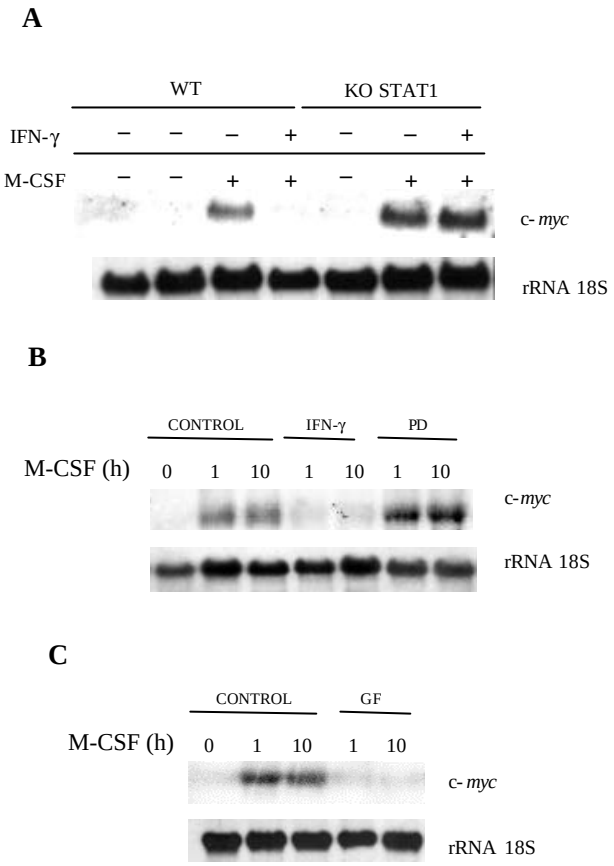


Figure 8. The inhibition of c-*myc* expression by IFN-γ is STAT1 and MEK/ERK-dependent. A) Quiescent macrophages from control and STAT1 *knock-out* mice were treated with M-CSF (1200 U/ml) for 1 hour in the presence or absence of IFN-γ (300 U/ml). B) Quiescent macrophages were pretreated or not with PD 98059 (PD; 50 μM) for 1 hour and then treated with M-CSF (1200 U/ml) for the indicated periods of time in the presence or absence of IFN-γ (300 U/ml). C) Quiescent macrophages were either left untreated or pretreated with 5 μM of GF109203X (GF) for 1 hour and then stimulated with M-CSF (1200 U/ml) for the indicated periods of time. In all three cases, the expression of the oncogene c-*myc* was determined by northern blotting (20 μg of total RNA per lane). 18S rRNA expression was used to check for differences in gel loading and transfer. Similar results were obtained in two independent experiments.

Discussion

Lymphocytes undergo a process of clonal expansion when they become activated. During such process, the cells become competent to perform their functions, including the production and secretion of cytokines and antibodies (Neuberger, 1997). In contrast, macrophages cannot simultaneously proliferate and become activated. In fact, macrophage activation and the subsequent

enhancement of their capability to perform specialized functions is linked to their growth arrest. We have previously demonstrated a role for the MEK/ERK pathway in the regulation of several macrophage functions (Valledor et al., 1999; 2000a; 2000b). Signaling through the MEK/ERK cascade is required both for macrophage proliferation and for the controlled production of cytokines during macrophage activation. Moreover, a clear correlation exists between the time-course of ERK-1/2 activity and the fact that bone marrow-derived macrophages commit towards proliferation or activation. A short and transient peak of ERK activity is common to all the proliferative signals studied so far, while activating agents induce a more delayed and sustained pattern of ERK activity (Valledor et al., 1999; 2000a; 2000b).

IFN-γ inhibits the proliferation of several cell types and STAT1 and IRF-1 are essential mediators of this effect (Bromberg et al., 1996; Chin et al., 1996; Taniguchi et al., 1997; Xaus et al., 1999b). In contrast to this general observation, recent studies have demonstrated that IFN-γ exerts a pro-proliferative action in some cellular types (Asao and Fu, 2000), including astrocytes (Rubio and Torres, 1999) and cells with impaired STAT1 signaling (Ramana et al., 2000; 2001). In our hands, however, IFN-γ potently inhibits the proliferation of bone marrow-derived macrophages. The pathway involved in the general growth inhibitory effect of IFN-γ is not fully understood. Inhibition of either cyclin D or c-*myc* expression, or induction of $p21^{Waf1}$ expression, are the most likely mechanisms for this action (Chin et al., 1996; Dey et al., 1999; Hobeika et al., 1999; Subramaniam and Johnson, 1997; Vairo et al., 1996; Vivo et al., 2001; Xaus et al., 1999b). The aim of this work was the study of the signals involved in the anti-proliferative effect of IFN-γ in bone marrow-derived macrophages.

Our group, in parallel to other investigators, has observed that $p21^{Waf1}$ is expressed in response to IFN-γ in a STAT1 dependent fashion (Chin et al., 1996; Xaus et al., 1999b). The fact that the anti-proliferative activity of IFN-γ is dependent on STAT1 expression opened the question of whether or not $p21^{Waf1}$ had a role in the mediation of the anti-proliferative effect of IFN-γ. However, IFN-γ was able to induce high levels of $p21^{Waf1}$ expression even when the cells were treated with IFN-γ two or more hours after the stimulation with M-CSF (data not shown), a situation that is demonstrated here to lead to a significant reduction of the capability of IFN-γ

to fully inhibit macrophage proliferation. Moreover, treatment with IFN-γ fully inhibited macrophage proliferation in mice deficient for $p21^{Waf1}$. These results indicate that there is no correlation between the induction of $p21^{Waf1}$ and the capability of IFN-γ to block macrophage proliferation. Other authors have also described $p21^{Waf1}$- independent cases of cell growth arrest by IFN-γ (Sharma et al., 1998; Vivo et al., 2001). On the other hand, we have recently observed p21-independent mechanisms for the blockage of macrophage proliferation by agents such as decorin (Xaus et al., 2001b) or cyclosporin A (submitted to publication). Nevertheless, experiments from our group and others seem to attribute to $p21^{Waf1}$ an indispensable role in the regulation of macrophage survival in response to several apoptotic stimuli (Xaus et al., 1999b; 2001b).

Since the kinetics of ERK-1/2 activity has been involved in the control of several processes in macrophages (Valledor et al., 1999; 2000a; 2000b) we decided to explore whether IFN-γ regulated this cascade in our cellular model. IFN-γ, in contrast to other activating factors such as LPS (Valledor et al., 2000a), did not induce ERK-1/2 activity by itself. IFN-γ did not inhibit ERK-1/2 activation by M-CSF either. However, IFN-γ was able to elongate the pattern of activation of ERK-1/2 in response to M-CSF. This effect was mediated by the inhibition of the expression of the phosphatase MKP-1 in a STAT1 dependent manner.

It has been recently suggested that terminal differentiation and senescence of a number of cellular models is related to the inhibition of proliferation by persistent activation of the ERK-1/2 pathway (Fanton et al., 2001; Lin et al., 1998). From the data shown here, a similar mechanism can be suggested for the IFN-γ-mediated blockage of macrophage proliferation. However, those models correlate an elongated ERK-1/2 activity with the expression of $p21^{Waf1}$ and the subsequent inhibition of cell growth. Even though our results do not sustain an essential role for $p21^{Waf1}$ in the inhibition of macrophage proliferation by IFN-γ, it might be possible that the elongation of ERK-1/2 activity is sufficient for the anti-proliferative effect independently of $p21^{Waf1}$ expression. In order to confirm our hypothesis we aimed for the artificial elongation of ERK-1/2 activity and then the analysis of its effect on macrophage proliferation. Since transfection of bone marrow-derived macrophages is very inefficient (Celada et al., 1996), we were not able to approach this problem by expressing a constitutive form of MEK or by blocking MKP-1 expression by an

antisense technology. Instead, we used GF109203X, a synthetic inhibitor of protein kinase Cε, which we had previously shown to block MKP-1 expression in our cellular model (Valledor et al., 1999; 2000b). GF109203X not only inhibited MKP-1 expression induced by M-CSF, but also elongated ERK activity and inhibited macrophage proliferation in strong parallelism with the effect of IFN-γ. Moreover, the anti-proliferative effect of GF109203X is not related to $p21^{Waf1}$ expression since treatment of macrophages with GF109203X in the presence or absence of M-CSF does not induce the expression levels of this cdk inhibitor (manuscript in preparation). Taken together, our data support the hypothesis that blockage of MKP-1 expression by IFN-γ accounts for the elongation of ERK activity and this effect correlates with the inhibition of macrophage proliferation.

Inhibition of the expression of the protooncogene c-*myc* has been extensively correlated with the antimitogenic effects of IFN-γ. For this reason, we further explored whether the elongation of ERK activity correlated with c-*myc* inhibition. The members of the *myc* oncoprotein family (comprising c-, N-, L-, and s-*myc*) are transcription factors of the basic region helix-loop-helix leuzine zipper class whose functions have been correlated with proliferation, apoptosis, and tumorigenesis in many cell types (for reviews see refs. DePinho et al., 1991; Henriksson and Luscher, 1996; Meichle et al., 1992). c-*myc* expression is a characteristic immediate early event in response to mitogenic stimulation of quiescent cells and is induced by a wide variety of mitogens, cytokines, and growth factors. Indeed, there is evidence that c-*myc* is required for cell cycle entry (Roussel et al., 1991) and that c-*myc* overexpression promotes proliferation at several levels (Karn et al., 1989; Sorrentino et al., 1986; Stern et al., 1986). Furthermore, the terminal differentiation of several cell types is accompanied by the down regulation of c-*myc* expression, and the ectopic overexpression of c-*myc* can inhibit or delay differentiation (Henriksson and Luscher, 1996). The ectopic expression of deregulated c-*myc* caused partial relaxation of the antiproliferative effect of IFN-γ (Vairo et al., 1996). However, the mechanisms that account for the IFN-γ- mediated inhibition of c-*myc* are still controversial. IFN-γ-induced double-stranded RNA (dsRNA)- activated protein kinase (PKR) (Meurs et al., 1990) was suggested to be a mediator of c-*myc* downregulation (Raveh et al., 1996). PKR is a cytoplasmic serine/threonine kinase that is largely ribosome-associated. It is expressed constitutively at low levels in a large variety of mammalian cells and induced several fold in response to IFNs.

Upon activation, PKR is autophosphorylated at multiple sites (reviewed in Clemens, 1987 and references therein) and then it phosphorylates the α subunit of the eukariotic peptide chain initiation factor eIF-2, thereby inhibiting protein translation (Hershey, 1991). Thus, IFN-γ through PKR activation, is able to regulate c-*myc* expression at a translational level. However, it has also been described that IFN-γ regulates the expression of the small protein Mad1 (Chin et al., 1996) or the protein p202 (Wang et al., 2000) thus modulating the binding capabilities of c-*myc* to the promoter of its target genes.

In this report we have provided evidence that IFN-γ is also able to regulate c-*myc* expression at a transcriptional or post-transcriptional level, since we observe a reduction in the c-*myc* mRNA levels after IFN-γ treatment. It has been described that ERK can mediate c-Myc phosphorylation on serine 62 (Gupta et al., 1993; Seth et al., 1992). The activation of c-Myc is necessary for macrophage proliferation in response to M-CSF (Langer et al., 1992). Our previous work also demonstrates that activation of the MEK/ERK cascade is also required for macrophage mitogenesis (Valledor et al., 1999). Blockage of ERK activation caused a growth arrest of macrophages at the G_1 phase of the cell cycle. In fact, the activation of this pathway is also necessary for the proliferation of several other cell types in response to specific growth factors or serum (Brondello et al., 1995; Pages et al., 1993; Robinson and Cobb, 1997). ERK may act as a positive regulator of cyclin D_1 expression (Lavoie et al., 1996), which is required for progression through the G_1 phase of the cell cycle in response to M-CSF (Roussel, 1997). Interestingly, we show here that the inhibition of ERK activation in bone marrow-derived macrophages increases the levels of c-*myc* mRNA after the stimulation with M-CSF. However, the blockage of ERK activity with the inhibitor PD98059 not only does not inhibit M-CSF-induced c-*myc* expression but increased it, suggesting that ERK activation may play an inhibitory rather than stimulatory effect on c-*myc* expression. On the other hand, the elongation of ERK activity in cells treated with GF109203X correlated with the inhibition of M-CSF-induced expression of c-*myc*. Such observation helps to explain the requirement of a short and transient pattern of activation of ERK-1/2 in order to trigger mitogenesis in response to growth factors (Valledor et al., 1999; 2000b). In this regard, a short and fast activation of ERK induced by M-CSF must be necessary to trigger an

initial proliferative signal, however it has to be immediately downregulated to allow the c-*myc* expression, which is inhibited by ERK activity.

The data presented here provides evidence for a direct correlation between a prolonged pattern of ERK activation and the inhibition of c-*myc* expression in response to IFN-γ. Our results are in accordance with the fact that terminal cell differentiation associated with prolonged ERK activation is also associated with a reduction in c-*myc* expression (Henriksson and Luscher, 1996). Thus we present a novel mechanism, involving the prolongation of ERK activity, for the inhibition of c-*myc* expression and proliferation in response to IFN-γ. Future studies will be addressed to determine the exact mechanism by which ERK-1/2 prolonged activity downregulates the transcription of c-*myc* or its mRNA stability.

Ciclosporina A y FK506 bloquean la proliferacion de los macrofagos inhibiendo la activación de ERK-1/2 a través de un mecanismo independiente de la calcineurina

La calcineurina, es una fosfatasa que juega un papel muy importante en la regulación de la activación de los linfocitos T. La calcineurina defosforila y activa al factor de transcripción NF-AT implicado en la expresión de citocinas como la IL-2, IL-4 o el IFN-γ. Estas citocinas son requeridas para la regulación de la respuesta inmunitaria. La ciclosporina A (CsA) y el FK506 son drogas inmunosupresoras ampliamente utilizadas para evitar el rechazo de órganos transplantados y en el tratamiento de ciertas enfermedades autoinmunes o inflamatorias. Tanto la CsA como el FK506 ejercen su principal efecto biológico a través de la inhibición de la actividad de la fosfatasa calcineurina en los linfocitos, y en consecuencia inhibiendo o modulando la progresión de la respuesta inmunológica.

A pesar de que los efectos de la CsA y FK506 han sido ampliamente estudiados en los linfocitos T, no se ha descrito el efecto que pueden causar estos inmunosupresores sobre otras células del sistema inmunitario, como puede ser el caso de los macrófagos. El objetivo de este trabajo fue por tanto analizar el papel de la calcineurina en la regulación de las funciones de los macrófagos y el efecto que ejercen estas drogas sobre dichas funciones en los macrófagos.

La CsA y el FK506, a concentraciones capaces de inhibir la actividad de la calcineurina no bloquean la proliferación de los macrófagos derivados de médula ósea. Sin embargo, cuando utilizamos altas concentraciones de estos inmunosupresores, tanto la CsA como el FK506 inhiben la activación de las quinasas ERK-1/2 necesarias para la proliferación, sugiriendo que este efecto de la CsA y el FK506 en los macrófagos no depende de la activación de la calcineurina. La ausencia de la implicación de la calcineurina en la proliferación de los macrófagos fue corroborada utilizando inhibidores de la calmodulina y quelantes de calcio, dos activadores necesarios para la actividad de esta fosfatasa, los cuales tampoco tuvieron ningún efecto inhibidor sobre la proliferación de los macrófagos inducida por el M-CSF. Además, hemos

descartado que el efecto inhibidor de la proliferación de la CsA y el FK506 a altas concentraciones sea debido a su actuación sobre las proteínas MDR, inhibidas en otros sistemas celulares a altas concentraciones de estas drogas.

Por otro lado, la CsA y el FK506 ejercen un efecto diferente en macrófagos activados con LPS. El FK506, pero no la CsA, reduce la expresión de citocinas (TNF-α, IL-1β) inducidas por el LPS. Además, mientras que el tratamiento de los macrófagos con CsA no afecta al patrón de activación de ERK-1/2 inducido por el LPS, a diferencia de lo observado tras el tratamiento con M-CSF, el FK506 bloquea parcialmente la activación de estas quinasas en ambos casos. Este efecto en el patrón de activación de ERK-1/2, se puede relacionar con el efecto de estas drogas sobre la expresión de la fosfatasa MKP-1. Mientras que el tratamiento de la CsA bloquea la expresión de MKP-1, el FK506 la superinduce. Además, la diferencia observada entre los efectos de la CsA sobre la actividad de ERK inducida por el LPS o por el M-CSF sugiere que las vías de activación de ERK por ambos estímulos son distintas. Mientras que la activación de ERK por el M-CSF se inhibe por CsA, la activación de ERK por el LPS no lo es.

En conclusión, nuestros resultados indican que la calcineurina no se encuentra involucrada en la biología del macrófago, por lo que respecta a la regulación de la proliferación y de la activación, y que la CsA y el FK506 bloquean a dosis altas la proliferación de los macrófagos a través de la inhibición de la activación de las ERK-1/2 por mecanismos distintos e independientes de la calcineurina.

Cyclosporin A and FK506 block M-CSF-dependant macrophage proliferation through a calcineurin-independent mechanism

Abstract

We determine the role of calcineurin on macrophage biology by using the inhibitors cyclosporin A (CsA) and FK506. We found that CsA or FK506 at a concentration that inhibit calcineurin do not block the proliferation of bone marrow macrophages. However, using higher concentrations, CsA or FK506 inhibited proliferation as well as the activation of the extracellular signal-regulated kinases (ERK) that are necessary for proliferation. The effect of CsA and FK506 is not related to calcineurin because the inhibition of calmodulin-dependent pathways did not modify the macrophage response to M-CSF. The effect of CsA and FK506 is not related to multidrug resistance (MDR) proteins. Concerning macrophage activation we found several differences between the calcineurin inhibitors used. FK506 but not CsA reduced the LPS-induced expression of cytokines, such as TNF-α or IL-1β. Also, while CsA do not affect LPS-induced ERK activation, FK506 partially inhibited such activation that correlates with the impairment of cytokine synthesis. Finally, these drugs also showed opposite effects on the induction of the phosphatase MKP-1, which inactivates by dephosphorylation ERK-1/2. While the treatment with CsA blocked the expression of MKP-1, FK506 superinduced it. In conclusion, our results indicate that calcineurin is not involved on macrophage biology and that CsA and FK506 block macrophage proliferation through ERK-1/2 inactivation.

Introduction

Calcineurin, a Ca^{2+}- and calmodulin-regulated protein phosphatase, plays a key role in the activation of T lymphocytes (Ho et al., 1996). In resting cells, NF-AT transcriptional factors are phosphorylated and retained in the cytoplasm. Activated calcineurin dephosphorylates conserved serine residues in the amino terminus of NF-AT proteins which, after removal of phosphate, are translocated into the nucleus to serve as subunits of transcription factor complexes in the promoters of cytokines such as IL-2, IL-4 or IFN-γ (Stankunas et al., 1999). These cytokines are required for the regulation of the immune response.

Cyclosporin A (CsA) and FK506 are chemically different immunosuppresive drugs widely used in the prevention of allogenic graft rejection and in the treatment of certain autoimmune diseases (Fathman and Myers, 1992; Kahan, 1993; Thomson, 1990). Both CsA and FK506 are known to exert their biological effect by binding to their cognate intracellular receptor immunophilins, cyclophilin and FK506-binding protein (FKBP), respectively. These complexes subsequently bind to and block the activation of a common target, the phosphatase calcineurin (Liu et al., 1991; Schreiber and Crabtree, 1992).

Macrophages have critical functions in the immune system. They behave as regulators of homeostasis and as effector cells in infection, wound healing and tumor growth (Celada and Nathan, 1993). Macrophages release a large number of molecules including cytokines such as TNF-α, IL-1β or IL-6 (Gordon, 1999). Although phosphatases play a critical role in cellular physiology, very little is known about the function of calcineurin in macrophages. In the present report we find that only at very high doses CsA and FK506 inhibited macrophage proliferation induced by physiological growth factors, such as M-CSF, GM-CSF or IL-3. We had previously shown that the activation of extracellular signal-regulated kinases (ERK)-1 and –2, two members of the mitogen-activated protein kinase (MAPK) superfamily, is required for the M-CSF-induced proliferation of bone marrow-derived macrophages (Valledor et al., 1999; 2000b). Here we show for the first time that both CsA and FK506 block the activation of ERK-1/2 in response to M-CSF, thus explaining their inhibitory effect on macrophage proliferation. However, the inhibitory action on the calcium/calmodulin-dependent phosphatase calcineurin is unlikely to mediate this effect since the macrophage response to M-CSF was not impaired by the use of either a calcium chelator or a calmodulin inhibitor. In conclusion, this study shows that CsA and FK506 disturb the M-CSF-induced activation of ERK-1/2 and the subsequent proliferation of macrophages. The marked differences between CsA and FK506 in the control of other functions, together with the fact that the macrophage response to M-CSF is independent of calcium/calmodulin-dependent pathways, suggests that molecules other than calcineurin may be mediating the effect of these drugs in macrophages.

Materials and Methods

Cells and reagents

Bone marrow-derived macrophages were obtained from six-to-ten-week-old Balb/c mice (Charles River Laboratories Inc., Wilmington, MA) as described (Celada et al., 1984). Macrophages were cultured in DMEM (Sigma Chemical Co., St. Louis, MO), supplemented with 20% FBS (Sigma) and 30% L-cell conditioned medium as a source of M-CSF. All the experiments were initiated when the macrophage cultures were 80% confluent, normally after five days of culture. At this stage, the cells were rendered quiescent by deprivating them of L-cell conditioned medium for 16-18 hours, and then subjected to different experimental conditions.

CsA was a kind gift from Novartis (Basel, Switzerland). FK506, BAPTA-AM, Verapamil and W-13 were purchased from Calbiochem (La Jolla, CA). CsA and FK506 were disolved in pure ethanol and dimethylsulfoxide (DMSO), respectively, following the manufacturer's recommendations. LPS was obtained from Sigma. For some experiments we used recombinant IFN-γ M-CSF, GM-CSF and IL-3 (DNAX, Palo Alto, CA).

Proliferation assay

Cell proliferation was measured as previously described (Celada and Maki, 1992; Celada et al., 1996) with minor modifications. Quiescent cells (10^5) were incubated for 24 hours in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA) in 1 ml of medium with the indicated concentrations of M-CSF. The media was aspirated and replaced with 0.5 ml of media containing ^{3}H-thymidine (1 μCi/ml) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After 4-6 hours of incubation at 37ºC, the media was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% trichloroacetic acid (TCA), the cells were solubilized in 1% SDS and 0.3 M NaOH at room temperature. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter. Each point was performed in triplicate and the results were expressed as the mean ± SD.

Detection of apoptosis by the analysis of chromatin fragmentation

Fragmentation of DNA due to internucleosomal cleavage was determined using a commercial ELISA kit (Cell Death Detection ELISA Kit plus, Boehringer Mannheim, Indianapolis, IN) as previously described (Xaus et al., 1999b). Each point was performed in triplicate and the results were expressed as the mean ± SD.

RNA extraction and Northern Blot analysis

The cells were washed twice in cold PBS. Total RNA was extracted by the acidic thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA samples (15 μg) were separated on 1.2% agarose gels containing formaldehyde and transferred to nylon membranes (Genescreen, NEN Life Science Products, Boston, MA). For MKP-1 mRNA detection, we obtained the full length cDNA fragment of MKP-1 following purification from a Hind III digestion of the plasmid pBSKS/MKP-1 (kindly provided by Dr. R. Bravo, Bristol-Myers Squibb Pharmac. Res. Inst., Princeton, NJ). The probe for calcineurin was obtained by RT-PCR (One stept RT-PCR kit, Clontech, Palo Alto, CA) using total RNA from macrophages and the following primers: forward, 5'-TATGACGCCTGTATGGATG CC-3' and reverse, 5'-GGAGCCAGTACGGATGCGGGG-3'. For TNFα mRNA detection, we used the EcoRI/HindIII fragment of pSP65/TNFα (kindly supplied by Dr. M. Nabholz, ISREC, Epalinges, Switzerland). To study the expression of IL-1β, we obtained a probe by digesting the construct pGEM1/IL-1β (kindly provided by Dr. R. Wilson, Glaxo Research and Dev. Limited, Greenford, U. K.) with EcoRI/PstI. As control for RNA loading and transfer, we used a 18 S rRNA probe (Torczynski et al., 1983). All the probes were labeled with ^{32}P-α-dCTP (ICN Pharmaceuticals, Costa Mesa, CA). After incubating in hybridization solution (20% Formamide, 5X Denhart's, 5X SSC, 10 mM EDTA, 1% SDS, 25 mM Na_2HPO_4, 25 mM NaH_2PO_4 and 0.2 mg/ml salmon sperm DNA) at 65°C, the membranes were washed and exposed to Kodak X-AR films (Kodak Company, Rochester, NY).

Western blot analysis

Western blot analysis was conducted as previously described (Valledor et al., 1999). 70 μg of protein from cell lysates were loaded per lane and separated on a 10 % SDS-PAGE. For ERK-1 expression we used a mouse monoclonal antibody against ERK-1 (Santa Cruz Biotechnology, Santa Cruz, CA). A mouse monoclonal antibody against β-actin (Sigma) was used as a control for protein loading and tranfer. Peroxidase-conjugated anti-mouse IgG (Cappel-Organon Teknik., Durnham, NC) was used as secondary antibodies. Incubations were performed for 1 hour at room temperature. ECL detection was performed and the membranes were exposed to X-ray films (Kodak).

Determination of ERK activity by in-gel-kinase assay

This assay was performed as previously described (Valledor et al., 2000b). Briefly, 50 μg of total protein were separated by 12.5% SDS-PAGE in the presence of 0.1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 hour at room temperature. The gel was then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-ME (buffer A) for 1 hour at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hour at room temperature and then renatured by incubating with five changes of buffer A containing 0.04% Tween-20 for 16 hours at 4ºC. To perform the phosphorylation assay, the gel was first equilibrated in 40 mM Hepes-NaOH (pH 7.4) containing 2 mM DTT, 0.1 mM EGTA, 15 mM $MgCl_2$, 300 μM sodium orthovanadate, and then incubated for 1 hour in the same solution containing 50 μM ATP and 100 μCi γ-^{32}P-ATP (ICN). The reaction was stopped by washing the gel with 5% TCA containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to X-ray films (Kodak).

Results

Phosphatases play a key role in the mechanism of proliferation induced by growth factors and cytokines. In this study we wanted to determine the role of

calcineurin on macrophage biology**.** In previous works, we have described the critical role of the phosphatase MKP-1 in the regulation of macrophage activities (Valledor et al., 1999; 2000a). Analysis by northern blotting of quiescent macrophages stimulated with 1200 U/ml M-CSF for 30 minutes showed that MKP-1 expression is inducible by growth factors such as M-CSF (Fig. 1), as we and others have previously described (Noguchi et al., 1993; Valledor et al., 1999). Using the probe for calcineurin a band of 3.6 Kb corresponding to the size of the mRNA of this phosphatase (Guerini and Klee, 1989; Song et al., 1993) was observed (Fig. 1). By contrast with MKP-1, calcineurin showed a constitutive expression that this not modified after M-CSF treatment (Fig. 1). No modifications of calcineurin expression were observed after LPS or IFN-γ treatment (data not shown).

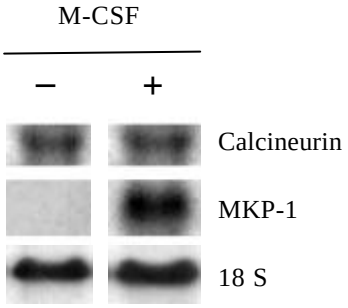


Figure 1. The expression of the phosphatase calcineurin in macrophages is constitutive. Quiescent bone marrow-derived macrophages were treated with 1.200 U/ml of M-CSF for 30 minutes and the phosphatases were detected by Northern blotting (15 μg of total RNA per lane). 18 S rRNA expression was used to check for differences in gel loading and transfer. Similar results were obtained from two independent experiments.

In a first attempt to determine the role of calcineurin on macrophage proliferation we used the inhibitors CsA and FK506. Quiescent bone marrow macrophages were stimulated with different concentrations of the macrophage specific growth factor M-CSF in the presence or absence of CsA or FK506 (Fig. 2). M-CSF induced macrophage proliferation in a dose-dependent manner as measured by thymidine incorporation or cell counting (Celada et al., 1996; Xaus et al., 1999b). At the concentration of 0.1 μg/ml of CsA or 0.1 μM of FK506 that inhibit T lymphocytes activation (Ho et al., 1996), none of the inhibitors affect macrophage proliferation (Fig. 2). However, at higher concentrations, either CsA or FK506 inhibit thymidine incorporation (Fig. 2A) and reduce the number of cells that growth in the presence of M-CSF (Fig. 2B). Similar results on proliferation were found when recombinant GM-CSF or IL-3 was used as growth factors (Fig. 3).

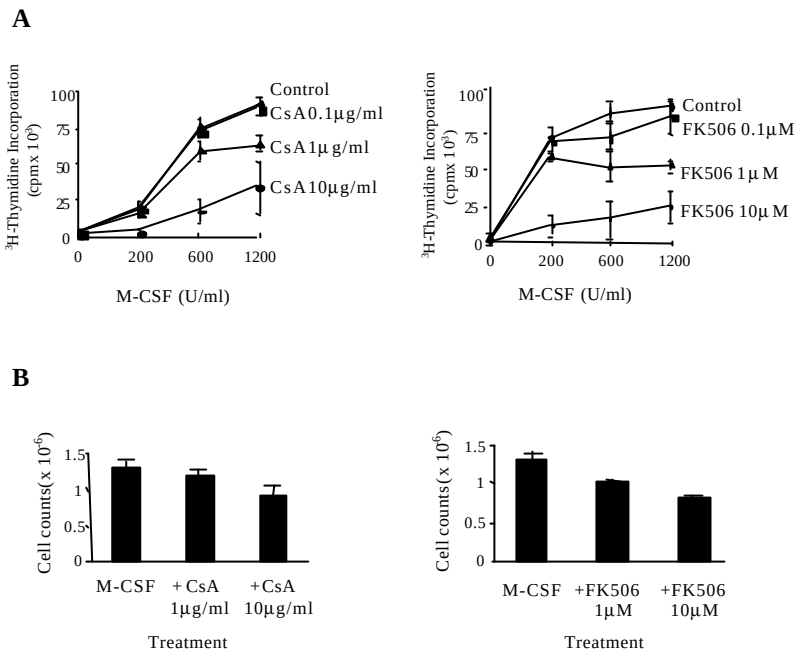


Figure 2. Effect of calcineurin inhibitors on M-CSF-dependent macrophage proliferation. Quiescent macrophages were incubated with the indicated concentrations of M-CSF in the presence or absence of different doses of CsA (left) or FK506 (right). In all the cases control cells were incubated with the corresponding vehicle in the absence of the drug. Proliferation was measured by ^{3}H-thymidime incorporation (A) or by viable cell counting using trypan blue (B) after culture the cells with saturating amounts M-CSF (1.200 U/ml) in the presence or absence of different concentrations of CsA or FK506. Mean values ± SD was obtained from triplicates. Similar results were obtained from three independent experiments.

The cell counting by tripan blue exclusion (Fig. 2 and 3) already showed that cell death or a reduced viability was not important in macrophages treated at high doses of these drugs. However, we wanted to assess whether the inhibition of macrophage proliferation could be due to the induction of apoptosis. For this reason, we studied the fragmentation of chromatin, which is an indicator of programmed cell death. CsA by itself did not induce detectable levels of apoptosis in macrophages (Fig. 4A). In a dose-dependent manner, CsA protected partially from the apoptosis induced by the absence of M-CSF (Fig. 4A).

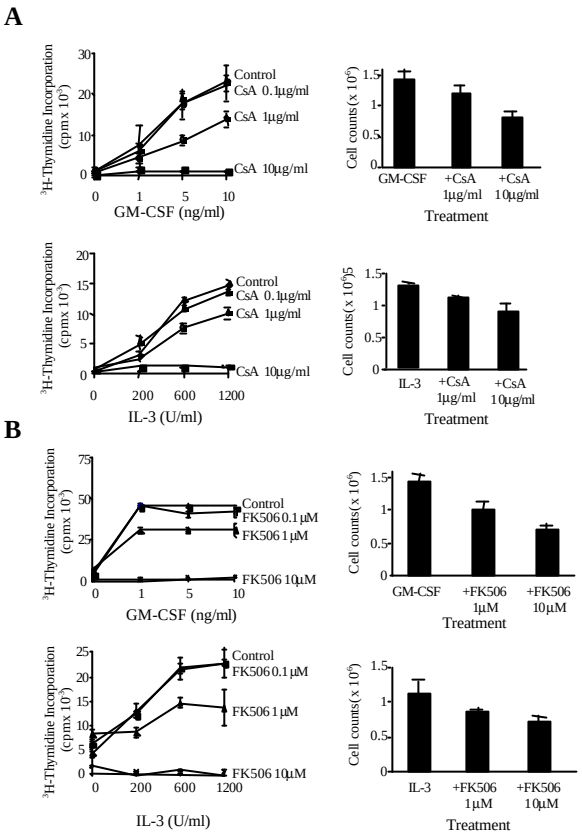


Figure 3. Effect of CsA and FK506 on GM-CSF- or IL-3-dependent macrophage proliferation. Quiescent macrophages were incubated with the indicated concentrations of GM-CSF or IL-3 in the presence or absence of different doses of CsA (A) or FK506 (B). Control cells were incubated with the corresponding vehicle in the absence of the drug. At the left, ^{3}H-thymidime incorporation was assayed as a measure of macrophage proliferation. Mean values ± SD was obtained from triplicates. At the right, cell numbers were determined after treating the cells with saturating amounts of each growth factor in the presence or absence of different concentrations of CsA or FK506. Similar results were obtained from three independent experiments.

These results indicate that the inhibition of macrophage proliferation by CsA is not caused by an increase in cell death. FK506, at the highest concentration used in the proliferation assays, induced low but significant levels of apoptosis in bone marrow macrophages cultured in the presence of M-CSF (Fig. 4B). However, the amount of apoptosis induced by FK506 is not sufficient to account for the global effect observed on macrophage proliferation at that concentration. Besides, this drug did not reduce the apoptosis of M-CSF-deprived macrophages (Fig. 4B).

Thus, although we used high doses of these drugs, the viability of the cells was not compromised and the results that we observed could not be attributed to toxic effects of CsA nor FK506.

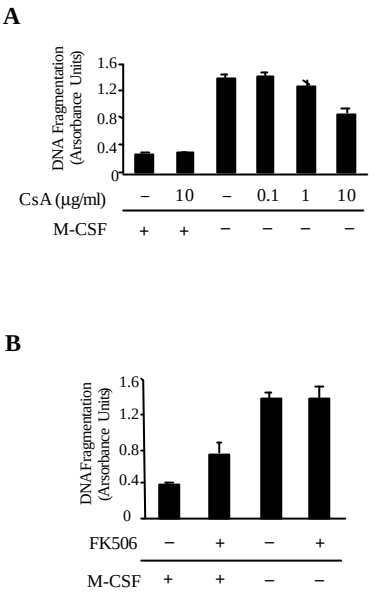


Figure 4. Effect of CsA and FK506 on macrophage apoptosis. Quiescent macrophages were split in two and half of the cells were incubated for 36 hours with M-CSF (1200 U/ml) in the presence or absence of CsA (10 μg/ml) (A) or FK506 (10 μM) (B). The other half were cultured in media containing either the indicated doses of CsA (A) or FK506 (10 μM) (B), but no M-CSF. Apoptosis was determined using an ELISA technique that detect cytoplasmic histone-associated DNA fragments. Each experiment was performed in triplicate and the results were expressed as the mean ± SD. Similar results were obtained from three independent experiments.

In previous studies we have shown that inhibition of ERK-1/2 kinases blocks macrophage proliferation in response to M-CSF, GM-CSF or IL-3. Therefore, the activation of the kinases ERK-1/2 is critical for the proliferation of bone marrow macrophages (Valledor et al., 1999; 2000b). For this reason, we determined the effect of both CsA and FK506 on the activation of this pathway. As we showed previously, M-CSF induces a transient and acute peak of ERK activation as analyzed by an in-gel kinase assay using MBP as substrate (Valledor et al., 1999; 2000a) (Fig. 5A). Interestingly, CsA at a concentration that inhibits macrophage proliferation (10 μg/ml) inhibited the activation of ERK-1/2 induced by M-CSF (Fig. 5A). Thus, the effect of this calcineurin inhibitor on the ERK kinases activation correlates with their negative effect on macrophage proliferation.

In previous studies, we have also shown that MKP-1 is induced by M-CSF independently of the ERK activation and mediates the deactivation of the ERK cascade (Valledor et al., 1999; 2000a). Therefore, we investigated the effect of CsA on the expression of this phosphatase. The treatment with CsA blocked the induction of the phosphatase mediated by M-CSF (Fig. 5B). An inhibition of the

MKP-1 expression was usually related to an elongation of the ERK activity induced by M-CSF due to a defect on ERK deactivation pathway (Valledor et al., 1999; 2000a). Since this is not the case of CsA treatment, we wanted to determine whether the effect of CsA is at ERK activity or, in contrast, directly to ERK expression levels. Western blot analysis showed that the treatment with CsA did not modify the levels of ERK expression in macrophages (Fig. 5C).

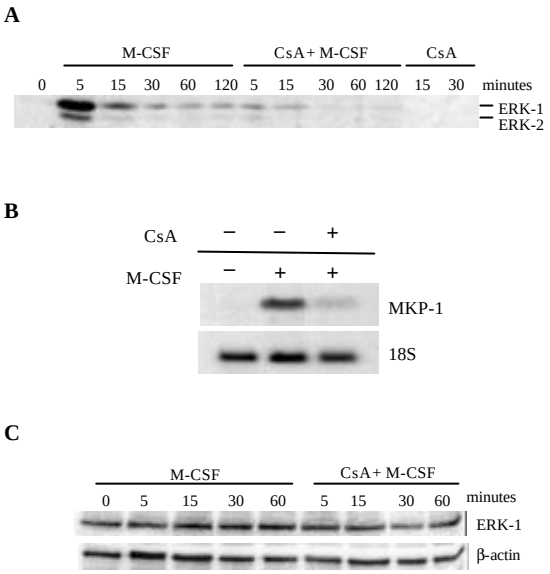


Figure 5. CsA inhibits the activation of extracellular-regulated kinase (ERK)-1/2. A) Quiescent macrophages were preincubated for 1 hour with CsA (10 μg/ml) or with vehicle. The cells were then treated with M-CSF (1200 U/ml) for the indicated times. The activation of the kinases ERK-1 and -2 was analyzed by an in gel-kinase assay as described in material and methods. B). The expression of the phosphatase MKP-1 was determined by northern blotting (15 μg of total RNA per lane) in macrophages pretreated for 1 hour with CsA (10 μg/ml) and then stimulated with M-CSF (1200 U/ml) for 30 minutes. 18 S rRNA expression was used to check for differences in gel loading and transfer. C) The levels of ERK protein were checked by western blotting (70 μg protein per lane) in macrophages stimulated with 1200 U/ml of M-CSF for the indicated times in presence or absence of CsA. The amount of β-actin was analyzed as a control for protein loading and transfer. Identical results were obtained from two independent experiments

FK506 also inhibited M-CSF-induced ERK activity at a concentration able to block macrophage proliferation (Fig. 6A) and, similar to CsA, FK506 alone was not able to induce ERK activity (data not shown). However, when we analyze the effect of FK506 on the MKP-1 expression striking differences were observed between the activity of the both calcineurin inhibitors. In relation to CsA, FK506 had an opposite

effect on the regulation of MKP-1 expression. This drug induced by itself the expression of MKP-1 and enhanced the induction of this phosphatase mediated by M-CSF (Fig. 6B). Again, the FK506 treatment did not modify the expression levels of ERK in macrophages (Fig. 6C).

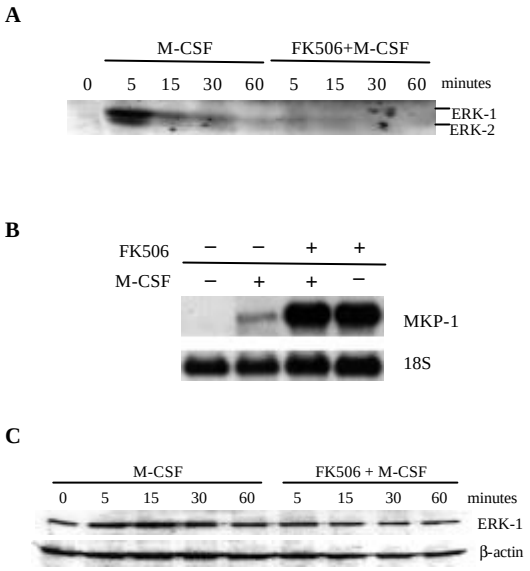


Figure 6. FK506 inhibits the activation of extracellular-regulated kinase (ERK)-1/2. A) Quiescent macrophages were preincubated for 1 hour with FK506 (10 μM) or with vehicle. The cells were then treated with M-CSF (1200 U/ml) for the indicated times. The activation of the kinases ERK-1 and -2 was analyzed by an in gel-kinase assay as described in material and methods. B). The expression of the phosphatase MKP-1 was determined by northern blotting (15 μg of total RNA per lane) in macrophages pretreated for 1 hour with FK506 (10 μM) and then stimulated with M-CSF (1200 U/ml) for 30 minutes. 18 S rRNA expression was used to check for differences in gel loading and transfer. C) The levels of ERK protein were checked by western blotting (70 μg protein per lane) in macrophages stimulated with 1200 U/ml of M-CSF for the indicated times in presence or absence of FK506. The amount of β-actin was analyzed as a control for protein loading and transfer. Similar results were obtained from three independent experiments.

So far we found that macrophage proliferation is only affected by CsA or FK506 at higher doses than the required to inhibit calcineurin function (Ho et al., 1996) and there are differences between CsA and FK506 on apoptosis and MKP-1 induction by M-CSF. All this argues about the role of calcineurin on macrophage proliferation. The calcineurin phosphatase is expressed constitutively in macrophages and requires to be activated by the calcium/calmodulin complex. To determine the role of calcium/calmodulin on macrophage proliferation we treated these cells with the calcium chelator BAPTA-AM that renders calmodulin inactive.

Concentrations up to 1 μM of this compound did not inhibit macrophage proliferation in response to M-CSF (Fig 7A). In order to corroborate these results, we tried to block this pathway using another approach through the inhibition of the calmodulin activity using the specific inhibitor W13. Macrophage treatment with W-13 does not affect proliferation (Fig. 7B). Moreover, the treatment of macrophages with W13 did not affect the M-CSF-induction of the MKP-1 phosphatase or the activation of ERK-1/2 kinases (Fig. 7C and D). As previously described (Vairo and Hamilton, 1991), we have not been able to detect mobilization of intracellular calcium during macrophage response to M-CSF (data not shown). All these data showed that although in most cellular models CsA and FK506 inhibit the activation of the calcium/calmodulin-dependent phosphatase calcineurin this common target cannot account for the effects of CsA and FK506 on macrophage proliferation.

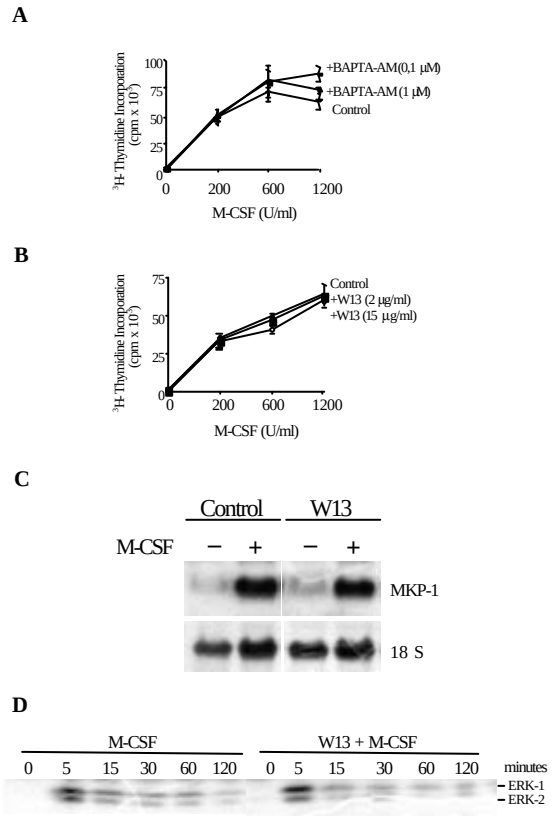


Figure 7. BAPTA-AM and W13 do not alter M-CSF-dependent proliferation of macrophages. Quiescent macrophages were preincubated for 1 hour with the indicated concentrations of the calcium chelator BAPTA-AM (A) or the calmodulin inhibitor W13 (B) and then stimulated with different doses of M-CSF. Macrophage proliferation was determined by the measure of ^{3}H-thymidime incorporation. Mean values ± SD was obtained from triplicates. C). The expression of MKP-1 was determined after treating macrophages with M-CSF (1.200 U/ml) for 30 minutes in the presence of W13 (15 μg/ml) Similar results were obtained from two independent experiments. D) The M-CSF-dependent ERK-1/2 activation was determined in the presence of W13 (15 μg/ml) by in-gel-kinase assay in macrophages treated with 1.200 U/ml of M-CSF for the indicated times and as described in material and methods. Similar results were obtained from two independent experiments.

Other molecules than calcineurin may account for the biological effects of CsA and FK506 in macrophages. Because these drugs at high doses are inhibitors of the multidrug resistance (MDR) proteins (Ford and Hait, 1990), we analyzed the role of these proteins on macrophage proliferation. The role verapamil, an inhibitor of the MDR effects (Gottesman and Pastan, 1993), was tested on M-CSF-dependent macrophage proliferation. No effect was observed when we used concentrations of verapamil up to 2 μg/ml (Fig. 8A), that are higher than the concentration reported to block MDR proteins (Tsuruo et al., 1981). Also, no effects of verapamil were found on macrophage apoptosis (Fig. 8B). Finally, verapamil also was unable to block M-CSF-dependent ERK activation (data not shown) or induction of MKP-1 phosphatase (Fig 8C). These data suggest that the effect of CsA or FK506 does not seem to be mediated through inhibition of the MDR proteins.

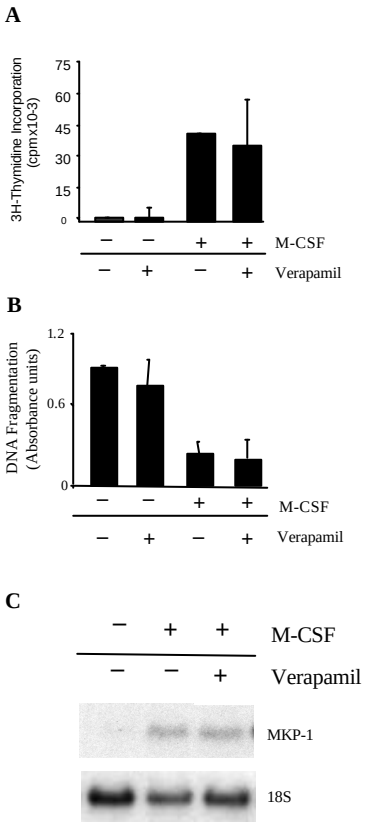


Figure 8. Multidrug resistance (MDR) proteins are not mediators of the effect of Csa and FK506 on macrophages. A) Quiescent macrophages were cultured in presence or absence of 1200 U/ml of M-CSF for 24 hours with or without 2 μg/ml verapamil. ^{3}H-thymidime incorporation was assayed as a measure of macrophage proliferation. Mean values ± SD was obtained from triplicates. B) Quiescent macrophages were split in two and half of the cells were incubated for 36 hours with M-CSF (1.200 U/ml) in the presence or absence of Verapamil (2 μg/ml) The other half were cultured in media containing verapamil but no M-CSF. Apoptosis was determined using an ELISA technique. Mean values ± SD was obtained from triplicates. Identical results were obtained from two independent experiments. C) The expression of MKP-1 was determined after treating macrophages with M-CSF (1.200 U/ml) for 30 minutes in the presence of Verapamil (2 μg/ml). Similar results were obtained from three independent experiments.

The lack of parallelism between CsA and FK506 in the control of a number of functions related to macrophage proliferation and survival opened the question of whether these differences would be also evident during the control of macrophage activation. Again, we had previously shown that the activation of the ERK cascade was necessary for the correct synthesis of cytokines in LPS-stimulated macrophages (Valledor et al., 2000a; 2000b). CsA inhibited LPS-induced MKP-1 expression (Fig. 9A) as we observed with M-CSF (Fig. 5B). However, when we analyzed the activation of the ERK cascade by LPS, in contrast to what we have described for the macrophage response to M-CSF (Fig. 5A), CsA did not inhibit the LPS-mediated activation of ERK-1/2 (Fig. 9C). As we described, when the MKP-1 expression is inhibited, we observed some prolongation of the duration of ERK activity (Fig. 9C). Again, FK506 showed a different pattern of the ERK pathway regulation. FK506, like in response to M-CSF (Fig. 6B), enhanced the LPS-induction of MKP-1 expression (Fig. 9C) and inhibited ERK activation (Fig. 9D).

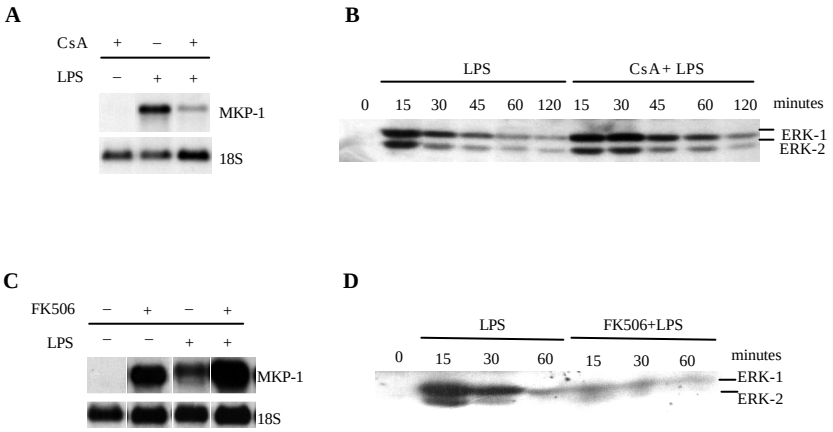


Figure 9. ERK activation by LPS is modulated differently by CsA and FK506. Bone marrow macrophages were pretreated with CsA (10 μg/ml) (A and B) or FK506 (10 μM) (C and D) and then stimulated with LPS (100 ng/ml) for the indicated times. The expression of MKP-1 (A and C) was analyzed by northern blot. ERK activity was measured by an in gel kinase assay (B and D). Similar results were obtained from three independent experiments.

To determine which are the consequences of this differential regulation of the ERK pathway in the LPS-induced macrophage activation, we analyze the effect of both drugs on LPS-induced cytokine expression. Analysis by northern blotting showed differences on the expression pattern of TNF-α and IL-1β induced by LPS

in presence of CsA or FK506 (Fig.10). CsA did not inhibit the LPS-induced synthesis of TNF-α and IL-1β (Fig. 10A). Interestingly, the levels of induction of these cytokines were reproducibly higher in the CsA-treated macrophages. In contrast, the treatment with FK506 was able to induce the expression of TNF-α and IL1β by itself (Fig. 10B). However, this drug reduced the capability of LPS to further increase cytokine synthesis. The effects observed on cytokine expression were consistent with the effect of both inhibitors on ERK activation induced by LPS.

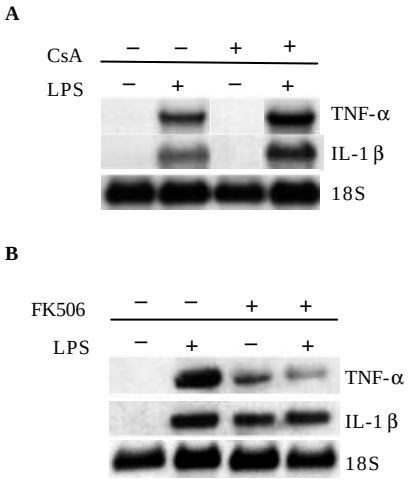


Figure 10. Cytokine expression induced by LPS is modulated differently by CsA and FK506. Bone marrow macrophages were pretreated for 1 hour with CsA (10 μg/ml) (A) or FK506 (10 μM) (B) and then stimulated with LPS (100 ng/ml) for 12 hours. The expression of IL-1-β and TNF-α was analyzed by northern blot (15 μg total RNA per lane). The expression of the 18 S rRNA was used as a control for RNA loading and transfer. Similar results were obtained from three independent experiments.

Discussion

The phosphorylation-dephosphorylation of signal transduction proteins plays a critical role in the behaviour of the cells in response to different growth factors or cytokines. We showed previously that the dual specificity tyrosine/serine phosphatase MKP-1 plays a crucial role in the regulation of macrophage decisions towards proliferation versus activation (Valledor et al., 1999; 2000a; 2000b). However, there are several other phosphatases that may play critic physiologycal roles in macrophages. Using bone marrow-derived macrophages as cellular model we were able to detect the expression of calcineurin and although several publications described a variety of effects on macrophages by blocking this phosphatase with CsA or FK506 (Losa Garcia et al., 1996), very little is known on macrophage proliferation. The concentrations of calcineurin inhibitors needed to block specific aspects of the biology of monocytic/macrophagic cells are higher

than the ones required to inhibit the activities of other cells of the immune system, including T lymphocytes (reviewed in Losa Garcia et al., 1996).

In this report we have shown that calcineurin inhibitors CsA and FK506 at concentrations sufficient to inhibit calcineurin (Ho et al., 1996), do not block the proliferation of bone marrow macrophages. However, we observed, by the first time, that using higher doses of both calcineurin inhibitors the M-CSF-, GM-CSF- or IL-3-dependant proliferation was inhibited. The arrest of proliferation is not related to an increase of apoptosis induced, at least, by CsA, and does not seem that the slight increase of apoptosis induced by FK506 treatment was sufficient to explain the effect in macrophage proliferation. Moreover, the experiments of cell counting by trypan blue exclusion confirm the lack of toxicity of both drugs inasmuch of the high doses used.

Although it is assumed that CsA and FK506 induce their biological effects through the inhibition of a common target, the phosphatase calcineurin, we have no evidence that the actions observed in macrophages are mediated through this mechanism. Neither a calcium chelator nor the inhibition of calmodulin-dependent pathways altered the macrophage response to M-CSF. Although calmodulin has been involved in the regulation of cell cycle (Cooper et al., 1994) this has not been confirm in all the cellular models (Taules et al., 1998). Instead of that, our results and the differences observed between CsA and FK506 activities, suggest that, in macrophages, CsA and FK506 act on different targets and independently of the phosphatase calcineurin. In this regard, we explore the role of MDR proteins that are inhibited at high doses of CsA and FK506 (Ford and Hait, 1990). However, the inhibition of MDR proteins with verapamil does not affect macrophage proliferation excluding a role of MDR proteins on the observed effect of both calcineurin inhibitors on macrophages.

There are other targets for the effects of calcineurin inhibitors. Peptidyl-prolyl cis-trans isomerase (PPIase), which is essential for the refolding of certain proteins, is identical to cyclophilin, the intracellular binding molecule for CsA (de Hoog et al., 2000). Since binding of CsA inhibits PPIase activity (de Hoog et al., 2000), CsA also appears to exert its effect by inhibiting the isomerase-dependent refolding of proteins. Inhibition of PPIase activity may prevent certain proteins from

assuming full activity. Besides, although cyclophilin and FKBP12 are the bests characterized immunophilins, other related members are thought to be expressed less abundantly in a number of cell types (reviewed in Takahashi et al., 1989). Moreover, new interactions of these molecules with other components of the signal transduction network have been recently described (Cameron et al., 1995; Coss et al., 1998; Huse et al., 1999; Jin and Burakoff, 1993; Schreiber, 1991; Stancato et al., 1994), thus opening the possibility that CsA and FK506 may act on targets other than calcineurin *in vivo*.

We have demonstrated for the first time that both CsA and FK506 are able to exert a negative effect on the activation of the ERK pathway during the macrophage response to M-CSF. Although we could not present any data regarding the target that CsA or FK506 are using to inhibit macrophage proliferation, our results indicate that their effect is mediated by disturbing the ERK pathway. In this sense, recently it has been described that a novel immunophillin could bind and regulate Raf-1, a MAPK kinase upstream of the ERK pathway (Jin and Burakoff, 1993; Stancato et al., 1994). Interestingly, in distinct cell types, CsA has been also shown to inhibit the activation of other members of the MAPK superfamily, including c-Jun N-terminal kinase (JNK) and p38 (Ishizuka et al., 1998; Lian et al., 2001; Matsuda et al., 1998; Werlen et al., 1998).

The treatment with calmodulin inhibitors has been related to the expression of the cyclin inhibitor $p21^{waf1}$ (Sugano et al., 1998; Winter et al., 1998). However, studies from our group using macrophages from $p21^{Waf1}$ KO mice showed that $p21^{Waf1}$ is not necessary to inhibit macrophage proliferation neither by other stimuli (Bosch et al., 1998; Xaus et al., 1999b) nor by CsA or FK506 (unpublished results).

Both drugs, CsA and FK506, block the M-CSF-induced ERK activation, however, it seems that they use different mechanisms. CsA may act directly inhibiting a target upstream of ERK activation in response to M-CSF but not LPS, since it does not affect ERK expression and inhibits MKP-1 that deactivates ERK. By contrast, FK506 increases the MKP-1 expression that could account for the inhibition of the ERK activation induced by both M-CSF and LPS. Winter et al. (1998) also observed an increased expression of MKP-1 in response to FK506

treatment. This hypothesis is supported by the differences observed on the effect of both calcineurin inhibitors in the M-CSF- and LPS-induced ERK activity.

In previous studies of our group, we demonstrated that ERK activation and its regulation by MKP-1 is important for the expression of cytokines induced by LPS (Valledor et al., 2000a; 2000b). In the present work we observed that this correlation between ERK activation and cytokine expression also exist. An elongation of ERK activity induced by CsA correlates with an increase on cytokine expression, while the ERK inhibition induced by FK506 implies a reduction in the expression of both TNF-α and IL-1β induced by LPS.

The data shown in this report help to understand how CsA and FK506 modulate the contribution of macrophages to the immune response and why these drugs may lead to different side effects. Importantly, although these compounds did not have a drastic effect on the synthesis of cytokines during macrophage activation, both drugs impaired macrophage proliferation, which may have relevant consequences by its own right. Macrophages are produced in the bone marrow and, through the blood, reach the different tissues in the organism, where they can continue proliferating in response to specific growth factors. In a local site of inflammation, and under the effect of IFN-γ secreted by T lymphocytes, macrophages become activated and are protected against apoptosis (Xaus et al., 1999b). However, in the presence of CsA or FK506, the production of IFN-γ by T cells is impaired (Ho et al., 1996) and in parallel, these drugs disrupt the proliferation of primary macrophages, as shown here. With a decreased macrophagic population it is very likely that those processes dependent on macrophage function, such as antigen presentation, are seriously compromised.

La decorina inhibe la proliferación de los macrófagos inducida por el M-CSF y potencia su supervivencia a través de la expresión de $p27^{Kip1}$ y $p21^{Waf1}$

La decorina es un pequeño proteoglicano que se encuentra formando parte de la matriz extracelular de la mayoría de los tejidos de los mamíferos. La importancia biológica de esta molécula no está bien definida, aunque diferentes estudios indican que la decorina interviene en la fibrilogénesis del colágeno y que podría jugar un papel como inhibidor del crecimiento de células tumorales. Ambos efectos asociados a la decorina han llevado a este compuesto a ser utilizado en diversos ensayos clínicos dirigidos al tratamiento de algunos tumores sólidos y de enfermedades fibróticas. Sin embargo, hasta la fecha poco se conoce del efecto de la decorina sobre células normales.

En este estudio quisimos estudiar cual era el efecto de la decorina sobre los macrófagos. En este sentido, hemos demostrado que la decorina inhibe la proliferación de los macrófagos inducida por el M-CSF, mediante un bloqueo del ciclo celular a nivel de la fase G_1 sin afectar a la viabilidad de los macrófagos. La decorina no sólo no induce apoptosis en los macrófagos, sino que además, la decorina rescata a los macrófagos de la inducción de apoptosis producida por la falta de factores de crecimiento.

Al analizar a nivel molecular el mecanismo por el cual la decorina podía ejercer su efecto antiproliferativo observamos que la incubación de los macrófagos con decorina induce la expresión de dos inhibidores del ciclo celular, $p21^{Waf1}$ y $p27^{Kip1}$. Para estudiar el papel de cada uno de estos inhibidores inducidos por la decorina, utilizamos macrófagos procedentes de ratones *knock-out* de $p21^{Waf1}$ y $p27^{Kip1}$. Así, hemos establecido que la inhibición de la proliferación mediada por la decorina está relacionada con la expresión de $p27^{Kip1}$, pero no de $p21^{Waf1}$, ya que en macrófagos deficientes de estos inhibidores, la decorina no es capaz de inhibir la proliferación sólo en aquellos macrófagos procedentes de los ratones KO de $p27^{Kip1}$. Mientras que la expresión de $p21^{Waf1}$ es necesaria para proteger a los macrófagos de la apoptosis inducida por falta de factores de crecimiento, tal y como demuestra la ausencia de protección en los macrófagos que carecen de $p21^{Waf1}$.

Por otro lado, la decorina inhibe la expresión de la fosfatasa MKP-1 e induce un alargamiento de la actividad ERK, característica de algunos procesos de activación, como el producido por el principal activador endógeno de los macrófagos, el IFN-γ, tal y como ya hemos mencionado en otros trabajos. Sin embargo, el efecto de la decorina en los macrófagos no se debe a la interacción de este proteoglicano con el receptor del IFN-γ ni con el receptor del factor de crecimiento epidérmico (EGF) tal y como han sugerido otros autores al estudiar el efecto antiproliferativo de la decorina en células tumorales.

Además, utilizando otros componentes de la matriz extracelular como la fibronectina, hemos demostrado que la adhesión de los macrófagos a la matriz extracelular (ya sea a la decorina o a la fibronectina) parece ser suficiente para algunos de los procesos observados, mientras que para otros se requiere algún tipo de señalización específica por parte de la decorina. Así, la adhesión a la matriz es suficiente para el efecto antiproliferativo de la decorina y, por tanto, de la inducción de $p27^{Kip1}$, la inhibición de MKP-1 y el alargamiento de la activación de las ERK-1/2. Sin embargo, el efecto antiapoptótico de la decorina, el cual esta mediado por la expresión de $p21^{Waf1}$, no puede ser simulado por la adhesión a la fibronectina, por lo que se trata de un efecto específico de este proteoglicano. En la actualidad se desconoce el receptor de la decorina, aunque nuestros resultados con anticuerpos bloqueantes sugieren que se trata de un miembro de la familia de las integrinas β1 independiente.

Decorin inhibits M-CSF-proliferation of macrophages and enhances cell survival through induction of $p27^{Kip1}$ and $p21^{Waf1}$

Abstract

Decorin is a small proteoglycan that is ubiquitous in the extracellular matrix of mamalian tissues. It has been extensively demonstrated that decorin inhibits tumor cell growth, however no data has been reported on the effects of decorin in normal cells. Using non-transformed macrophages from bone marrow, we found that decorin inhibits M-CSF-dependent proliferation by inducing blockage at the G_1 phase of the cell cycle without afecting cell viability. Besides, decorin rescues macrophages from the induction of apoptosis after growth factor withdrawal. Decorin induces the expression of the cdk-inhibitors $p21^{Waf1}$ and $p27^{Kip1}$. Using macrophages from mice where these genes have been disrupted, we show that inhibition of proliferation mediated by decorin is related to $p27^{Kip1}$ expression, whereas $p21^{Waf1}$ expression is necessary to protect macrophages from apoptosis. Decorin also inhibits M-CSF-dependent expression of MKP-1 and extends the kinetics of ERK-activity, which is characteristic when macrophages become activated instead of proliferating. The effect of decorin on macrophages is not due to its interaction with EGF or IFN-γ receptors. Furthermore, decorin increases macrophage adhesion to the extracellular matrix, and this may be partially responsible for the expression of $p27^{Kip1}$ and the modification of ERK-activity, but not for the increased cell survival.

Introduction

Stimulated monocytes and macrophages secrete a diverse set of mediators that influence cellular immune functions and inflammation. These mediators include pro- and anti-inflammatory cytokines, prostaglandins, leukotriens, and reactive oxygen metabolites (Nathan, 1987). At the inflammatory sites, proteoglycans are both secreted by activated mononuclear leukocytes and released as a result of extracellular matrix (ECM) degradation. Thus, proteoglycans, which are major constituents of the ECM, are another class of molecules produced by monocytes and macrophages (Laskin et al., 1991; Uhlin-Hansen et al., 1992) that are potential modulators of the immune response.

Decorin belongs to a family of small leucine-rich proteoglycans (Iozzo, 1996; Iozzo and Murdoch, 1996) and it is found in the extracellular matrix of several of tissues such as skin (Choi et al., 1989; Nakamura et al., 1983), cartilage (Poole et al., 1986; Rosenberg et al., 1985)) and bone (Fisher et al., 1983). The biological importance of these molecules is unclear. *In vitro* binding studies have shown that some of them interact with several types of collagen (Bidanset et al., 1992; Schonherr et al., 1995) and act as important regulators of collagen fibrillogenesis. In support of this hypothesis, a decorin-deficient mouse was found to have fragile skin with an abnormal organization of collagen fibers (Danielson et al., 1997). Decorin may also affect the production of other extracellular matrix components by regulating the activity of TGF-β (Hildebrand et al., 1994; Yamaguchi et al., 1990). Additionally, decorin can modulate the interactions of matrix molecules (e.g. fibronectin) with cells (Lewandowska et al., 1987; Schmidt et al., 1987; Winnemoller et al., 1991). These observations suggest that decorin, perhaps other proteoglycans regulate at several levels the production and assembly of the extracellular matrix and hence the remodeling of connective tissue.

Different observations have revealed that decorin is involved in the control of cell proliferation. The forced expression of decorin in CHO cells leads to a decreased growth rate, lower saturation density, and altered morphology (Yamaguchi and Ruoslahti, 1988). It has been suggested that decorin causes these effects by sequestering TGF-β, an autocrine growth stimulator for these cells (Yamaguchi et al., 1990). Besides, decorin is markedly upregulated during quiescence in human diploid fibroblasts (Coppock et al., 1993; Mauviel et al., 1995) and its expression is strongly supressed upon viral transformation with SV40 (Coppock et al., 1993). Recently, an anti-oncogenic role for decorin has been reported (Santra et al., 2000). Decorin is rarely expressed by malignant epithelial cells from a wide variety of human tumors, including colon, pancreas, prostate and breast carcinomas (Iozzo and Cohen, 1993). In human colon carcinoma cells, the *de novo* expression of decorin reverted the cells to a normal phenotype: the cells lost anchorage-independent growth, failed to generate tumors in *scid* mice, and became arrested in the G_1 phase of the cell cycle (Santra et al., 1995). When decorin expression was abrogated by decorin-specific antisense oligodeoxynucleotides treat the cell reentered the cell cycle (Santra et al., 1995).

This growth arrest induced by decorin was associated with a marked expression of $p21^{Waf1}$. Moreover, decorin inhibits proliferation in tumor cells with different histogenetic backgrounds through the induction of $p21^{Waf1}$ and it has been described that decorin interacts with components of the epidermal growth factor (EGF) receptor family expressed by these cells (Moscatello et al., 1998; Santra et al., 2000).

Besides the anti-oncogenic role of decorin, a protective role of decorin in fibrotic diseases has been observed (Giri et al., 1997; Isaka et al., 1996). However, there is no data regarding the effects of decorin on normal cells. We have analyzed the role of decorin in the control of macrophage proliferation. We have used primary bone marrow-derived macrophages cultures, which are a homogeneous population that respond to physiological proliferative or activating stimuli (Celada et al., 1996). Decorin inhibits M-CSF-dependent proliferation of macrophages and induces the expression of $p21^{Waf1}$ and, in contrast with other cellular models, also $p27^{Kip1}$. Moreover, decorin increases both the adhesion of these cells and their resistance to die after growth factor withdrawal. The effects of decorin in macrophages are not mediated through interaction with EGF or IFN-γ receptors.

Materials and Methods

Reagents

Recombinant purified decorin was a generous gift from E. Ruoslahti (The Burnham Institute, La Jolla, CA, USA). Histone H1 was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Fibronectin, vitronectin, poly-2-hidroxyethyl methacrylate, collagen and laminin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-thymidine was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). 4, 6-diamidino-2-phenylindole (DAPI) and the RGD peptide were purchased from Calbiochem (La Jolla, CA, USA). All the other products were of the best grade available and were purchased from Sigma. Deionized water further purified with a Millipore Milli-Q system (Bedford, MA, USA) was used.

Cell culture

Bone marrow-derived macrophages were isolated from six-week old balb/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) as described (Celada et al., 1984). The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 30% L-cell conditioned media as a source of M-CSF. The cells were incubated at 37ºC in a humidified 5% CO_2 environment. After 7 days of culture an homogeneous population of adherent macrophages was obtained (>99% Mac-1^+). The purity of the culture was checked regularly by flow cytometry using anti-Mac-1 antibodies (BD Pharmingen, Heidelberg, Germany). To render the cells quiescent, when the macrophages were 80% confluent they were deprived of L-cell conditioned medium for 18 hours before carrying out the experiment. Bone marrow-derived macrophages from *knock-out* mice were isolated in the same conditions. The $p21^{Waf1}$ and $p27^{Kip1}$ *knock-out* mice were obtained from Dr. J. Roberts from HHMI, Seattle, WA and IFN-γR *knock-out* mice were a kind gift by Dr. M.Modolell from the Max Plank Institute, Freiburg, Germany.

Antibodies and constructs

The analysis of $p21^{Waf1}$ and $p27^{Kip1}$ expresion by Western blotting was performed with monoclonal anti-mouse $p21^{Waf1}$ and $p27^{Kip1}$ antibodies (BD Pharmingen, Heidelberg, Germany). Antibodies to cdk-4 and cyclin D_1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-mouse IgG (Cappel, Turnhout, Belgium) was used as secondary antibody. A primary antibody against mouse β-actin was used as loading control and purchased from Sigma. The antibody against cdk-2 (M-2) used for the analysis of cdk-2 activity and the polyclonal anti-mouse β1 integrin antibody were obtained from Santa Cruz.

The pMH117 plasmid corresponds to the mouse $p21^{Waf1}$ full-length cDNA cloned in pEx-lox and was kindly provided by Dr. J. Massague (Sloan Kettering Institute, HHMI, New York, NY, USA). The MKP-1 probe was obtained from Dr. R. Bravo (Bristol-Myers Squibb, Princeton, NJ). The probe for the 18S rRNA was obtained as described (Torczynski et al., 1983).

Proliferation assay

Cell proliferation was measured as previously described (Celada and Maki, 1992) with minor modifications. The cells were deprived of M-CSF for 18 hours and then 10^5 macrophages were incubated for 24 hours in 24-well plates (3424 MARK II; Costar Corp., Cambridge, MA, USA) in 1 ml of complete medium in the presence or absence of the indicated reagents. In some cases, the plates were pre-coated with the indicated extracellular matrix proteins. After this period of time, the media was removed and replaced with 0.5 ml of media containing [^{3}H]-thymidine (1 μCi/ml). After six additional hours of incubation at 37ºC, the media was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% trichloroacetic acid, the cells were solubilized in 1% SDS, 0.3 N NaOH. Radioactivity was counted by liquid scintillation using a 1500 Tri-Carb Packard scintillation counter. Each point was performed in triplicates and the results were expressed as the mean ± SD.

In parallel experiments, $1x10^6$ cells were plated in 35 mm cell culture dishes and after 24-48 hours of culture in the indicated conditions, the viable cells were collected and counted by trypan blue exclusion using a hemocytometer. Again, each experiment was performed three times and the results were expressed as the mean ± SD.

Apoptosis assay

Low molecular apoptotic DNA caused by internucleosomal cleavage was measured as described (Xaus et al., 2000b), using an ELISA technique based on the detection of histone-associated DNA fragments (Cell Death Detection ELISA plus, Roche Diagnostics, Mannheim, Germany). Each point was performed in triplicate, and the result was expressed as the mean ± SD.

Analysis of DNA content with DAPI

10^6 cells were previously subjected to a specific treatment and then the analysis of DNA content was analyzed as described previously (Xaus et al., 1999b). 12,000 cells were counted for each histogram, and cell cycle distributions

were analyzed with the Multicycle program (Phoenix Flow Systems, Inc.; San Diego, CA, USA)

Adhesion analysis

Cell adhesion to the substrate was analyzed by cristal violet staining. Flat-bottomed ELISA plates were pre-coated in 50 µl/well PBS containing the indicated amount of each matrix protein, or BSA as a control, over-night at 4ºC or for 2 hours at 37ºC. After coating, the wells were blocked with PBS and 1.5% BSA for 1 hour at 37ºC. Then, 10,000 cells/well were cultured for only 30-60 minutes due to the high capacity of macrophages to attach themselves at any surface. The cells were washed twice in PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. After 3 washes by immersion with bidistilled water, the cells were stained in a solution of 0.1 % cristal violet in bidistilled water for 20 minutes. After 3 new washes, the plates were dried at 37ºC, developed by adding 0.1 M HCl for 5 minutes and quantified using an ELISA reader at 630 nm. Each sample was analyzed in triplicate and the results were represented as the mean ± SD.

Protein extraction and Western blot analysis

Western blot analysis were performed as previously described (Xaus et al., 1999b). 100 µg of cell lysates were loaded per lane. The analysis of $p21^{Waf1}$ and $p27^{Kip1}$ expresion was performed with monoclonal anti-mouse $p21^{Waf1}$ and $p27^{Kip1}$ antibodies (BD Pharmingen). Antibodies to cdk-4 and cyclin D_1 were obtained from Santa Cruz Biotechnology. A primary antibody against mouse β-actin was used as loading control and purchased from Sigma. Peroxidase-conjugated anti-mouse IgG was used as secondary antibody. All antibody incubations were performed for 1 hour at room temperature.

Determination of ERK activity by in-gel-kinase assay

50 µg of total protein were separated by 12.5 % SDS-PAGE in the presence of 0.1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 hour at room temperature. The gel was

then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol (buffer A) for 1 hour at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hour at room temperature and then renatured by incubating with five changes of buffer A containing 0.04% Tween-20 for 16 hours at 4ºC. In order to perform the phosphorylation assay, the gel was first equilibrated in 40 mM Hepes-NaOH (pH 7.4) containing 2 μM DTT, 0.1 mM EGTA, 15 mM $MgCl_2$, 300 μM sodium orthovanadate for 30 minutes at 25ºC and then incubated for 1 hour in the same solution containing 50 μM ATP and 100 μCi ^{32}P-γ-ATP (ICN Pharmaceuticals, Costa Mesa, CA, USA). The reaction was stopped by washing the gel with 5% trichloroacetic acid containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to X-ray films (Kodak) and quantified using a Bio-Rad Molecular Analyst system (Bio-Rad Labs., Richmond, CA).

Northern blot analysis

Total cellular RNA (20 μg), extracted with the acid guanidinium thiocyanate-phenol-chloroform method was separated in 1% agarose with 5 mM MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0/ 1 M formaldehyde buffer, transferred overnight to a GeneScreen membrane (Life Science Products, Boston, MA) and fixed by UV irradiation (150 mJ). All the probes were labeled with ^{32}P-α-dCTP (ICN) using the oligolabeling kit method (Amersham Pharmacia). After incubating the membranes for 18 hours at 65ºC in hybridization solution (20% formamide, 5X Denhart's, 5X SSC, 10 mM EDTA, 1% SDS, 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 0.2 mg/ml salmon sperm DNA and 10^6 cpm/ml of ^{32}P-labeled probe), they were exposed to Kodak films.

Cdk-2 activity

Quiescent macrophages were cultured in 60 mm plates pre-coated with 10 μg/ml BSA or decorin and stimulated with 1000 U/ml M-CSF for the indicated times. The analysis of cdk-2 activity was performed as described elsewhere, without modifications (Xaus et al., 1999b).

Results

In the present work, we used macrophages obtained from bone marrow cultures, since they represent an homogeneous population of macrophages that require M-CSF to proliferate and survive. Under the effect of M-CSF, macrophages proliferate in a dose dependent manner. When decorin (10 μg/ml) was pre-coated to the plates, macrophage proliferation was inhibited (Fig. 1A). This effect was dose-dependent, and macrophage proliferation was completely inhibited at a concentration of 100 μg/ml of decorin (Fig. 1B). It is important to note that the indicated concentrations of decorin correspond to the concentration of the pre-coating solution and that we are not able to quantify the amount of decorin adsorved to the plate after pre-coating, but other proteins bound under the same conditions less than 10-20%. Decorin also inhibits macrophage proliferation in the presence of either recombinant M-CSF or GM-CSF proteins (Fig. 1C).

The inhibitory effect of decorin was comfirmed by flow cytometry (Fig. 1D) and by cell counting (Fig. 1E). The distribution of the DNA content of cells stained with DAPI showed that macrophages treated with decorin are blocked mainly at the G_1 phase of the cell cycle (73 %) whereas macrophages growing in normal conditions showed a distribution corresponding to an active proliferating population (51% cells at G_1 phase) (Fig. 1C). Moreover, the inhibition of proliferation was not due to a lower cell viability since we did not detect any subdiploid peak corresponding to apoptotic cells (Fig. 1C) or a decrease in the cell number after 48 hours of culture in the presence of 100 μg/ml of decorin (Fig. 1E).

It has been reported that decorin inhibits proliferation through the expression of $p21^{Waf1}$ in certain tumor cellular models (Moscatello et al., 1998; Santra et al., 1997). Therefore, we analyzed the expression of this cdk inhibitor in macrophages treated with decorin. Western blot analysis showed that decorin induced the expression of $p21^{Waf1}$ in a time- and dose-dependent manner (Fig. 2A). However, and in contrast with other cellular models, decorin also induced in macrophages the expression of another cdk inhibitor, $p27^{Kip1}$ (Fig. 2A). No differences were observed in the expression of cyclin D_1, E, cdk-2 and cdk-4 protein analyzed by western blot (data not shown).

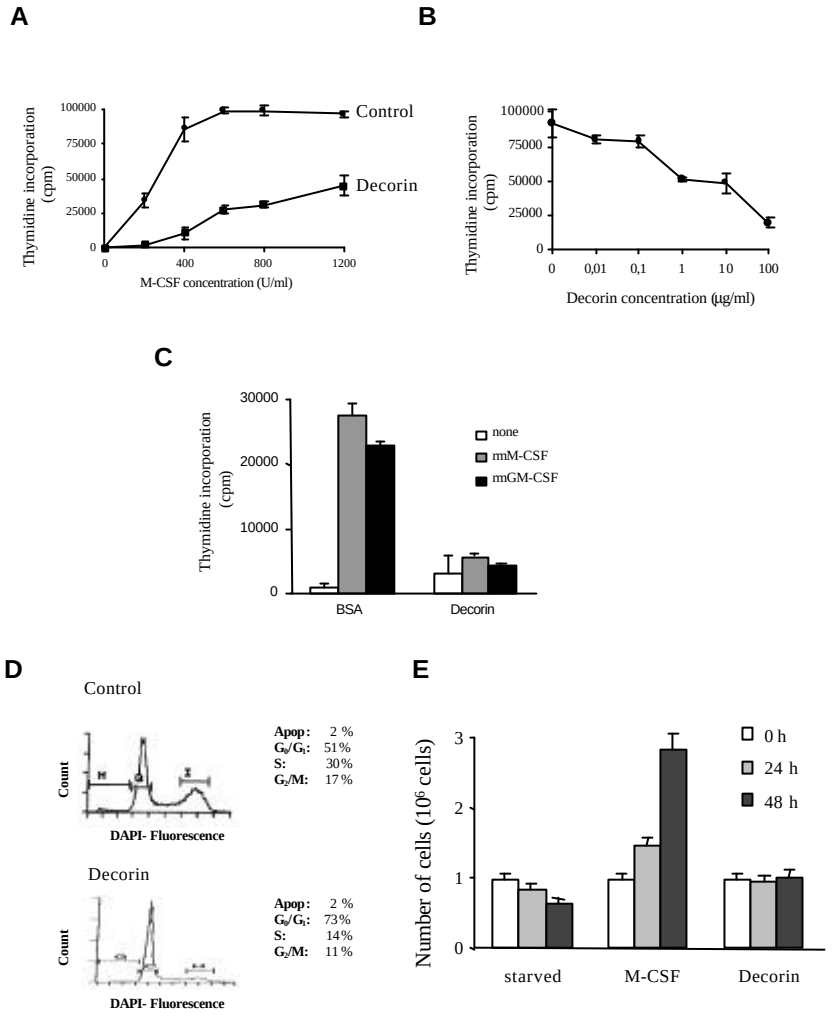


Figure 1. Decorin inhibits the M-CSF-dependent proliferation of bone marrow-derived macrophages. A) BMDM were obtained after 7 days of culture in the presence of M-CSF. 10^5 macrophages were incubated in the presence of the indicated amounts of M-CSF in 24-well plates pre-coated with BSA (10 µg/ml) (Control) or with decorin (10 µg/ml). Proliferation was determined as described in *Materials and methods*. B) Decorin inhibits macrophage proliferation in a dose-dependent manner. 10^5 macrophages were incubated in 24-well plates pre-coated with the indicated amounts of decorin in the presence of 1000 U/ml of M-CSF. In both cases (A and B), each determination was made in triplicate and the values represented correspond to the mean ± SD of one representative of three independent experiments. C) Decorin also blocks proliferation of macrophages induced by recombinant M-CSF (2 ng/ml) or GM-CSF (10 ng/ml). D) Decorin blocks the cell cycle at G_1 phase.10^6 macrophages were cultured in the presence of 1000 U/ml of M-CSF in 35 mm Petri dishes pre-coated with BSA or with 100 µg/ml decorin for 24 hours. DNA content was measured by DAPI staining and flow cytometry. Cell cycle distribution was analyzed using the Multicycle program (Phoenix Flow Systems). E) Counting of viable cells cultured in 100 µg/ml decorin pre-coated plates for 24-48 hours. The cells were counted by trypan blue exclusion using an hemocytometer. Each point was performed in triplicate, and the results were represented as the mean ± SD.

Moreover, the analysis of cdk-2 activity measured as *in vitro* phosphorylation of histone H-1 showed that treatment of macrophages with decorin inhibits cdk-2 activity (Fig. 2B). In order to characterize the involvement of each of these two molecules in the inhibitory effect of decorin, we used macrophages obtained from mice where these genes have been disrupted by homologous recombination. Decorin inhibited proliferation in macrophages from $p21^{Waf1}$ KO mice but no effect was observed in macrophages from $p27^{Kip1}$ KO mice (Fig. 2C). Thus, although decorin in macrophages induced, the expression of both $p21^{Waf1}$ and $p27^{Kip1}$, only $p27^{Kip1}$ was involved in the inhibition of proliferation by decorin, in contrast with what has been reported in tumor cells (Santra et al., 1997).

Due to these differences between primary macrophages and tumor cells, and since it has been described that decorin inhibits tumor cell growth through its interaction with the EGF receptor (Moscatello et al., 1998; Santra et al., 2000), the activation of the ERK pathway and the expression of $p21^{Waf1}$, we analyzed the involvement of this pathway in our model. Both IFN-γ and decorin inhibit M-CSF-dependent macrophage proliferation (Fig. 3A) and induce $p21^{Waf1}$ mRNA expression (Fig 3B). However, no induction of $p21^{Waf1}$ or inhibition of macrophage proliferation was observed after the treatment with 100 ng/ml of EGF, thus suggesting that this growth factor and its receptor do not modulate macrophage proliferation. We also analyzed the effect of decorin and EGF on the activation of the ERK pathway. In-gel kinase assays showed that the treatment of macrophages with M-CSF induced the activation of ERK-1 and -2 with a maximal activation after 5 minutes, but this activation decreased quickly and basal levels were reached after 15-30 minutes. In contrast, the treatment with decorin or EGF alone did not induce ERK activation. However, decorin, but not EGF, modified and extended the kinetics of ERK activity induced by M-CSF (Fig. 3C). The extension of the ERK activity in response to decorin correlated with the inhibition of the MKP-1 expression (Fig. 3D), a phosphatase responsible for ERK dephosphorylation and inactivation (Sun et al., 1993).

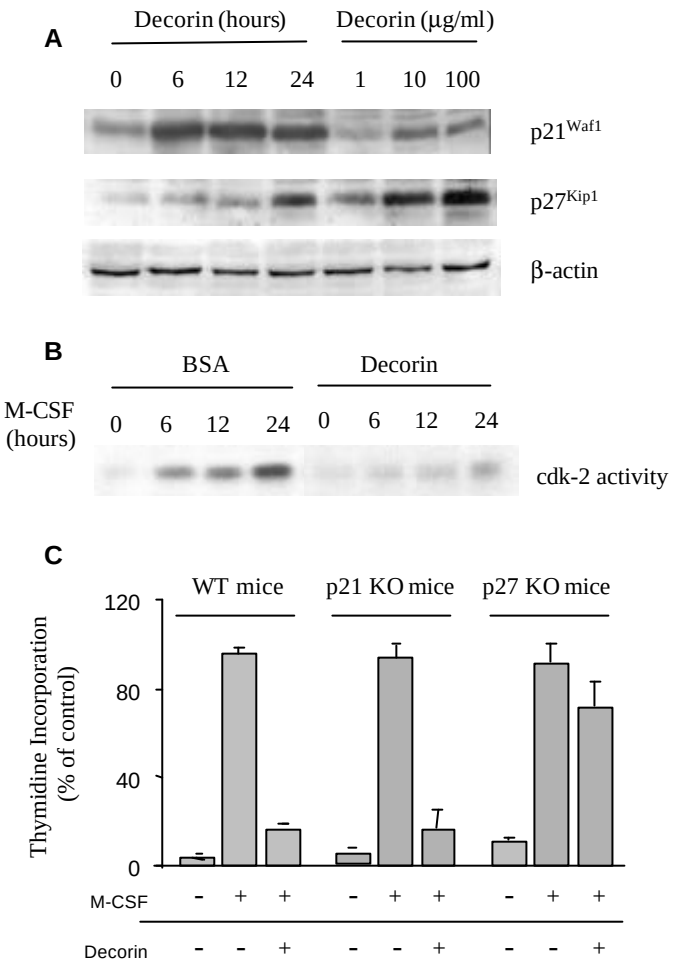


Figure 2: Decorin inhibits macrophage proliferation through p27^{Kip1} expression. A) Decorin induces the expression of both p21^{Waf1} and p27^{Kip1}. The expression of p21^{Waf1} and p27^{Kip1} after treatment with decorin was analyzed by Western blotting. Macrophages were cultured in 100 µg/ml decorin pre-coated surface for the indicated times or for 24 hours at the indicated concentrations of decorin. 100 µg of total protein were loaded per lane. p21^{Waf1} and p27^{Kip1} expression was analyzed using monoclonal antibodies as described in *Materials and methods*. The expression of β-actin was used as a control of sample loading and transfer efficiency. This is representative of four independent experiments. B) Decorin inhibits cdk-2 activity. The cdk-2 activity induced by M-CSF in macrophages cultured on plates pre-coated with BSA or decorin was measured as Histone H_1 phosphorilation *in vitro* at the indicated times after M-CSF stimulation. C) Decorin did not inhibit M-CSF-dependent proliferation of BMDM from p27^{Kip1} *knock-out* mice. After 7 days of culture, a total of 10^5 macrophages from wild-type, p27^{Kip1} or p21^{Waf1} *knock-out* mice were cultured for 24 hours in BSA or 10 µg/ml pre-coated plates in the presence of 1000 U/ml of M-CSF. Proliferation was determined by ^{3}H-Thymidine incorporation. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of two independent experiments.

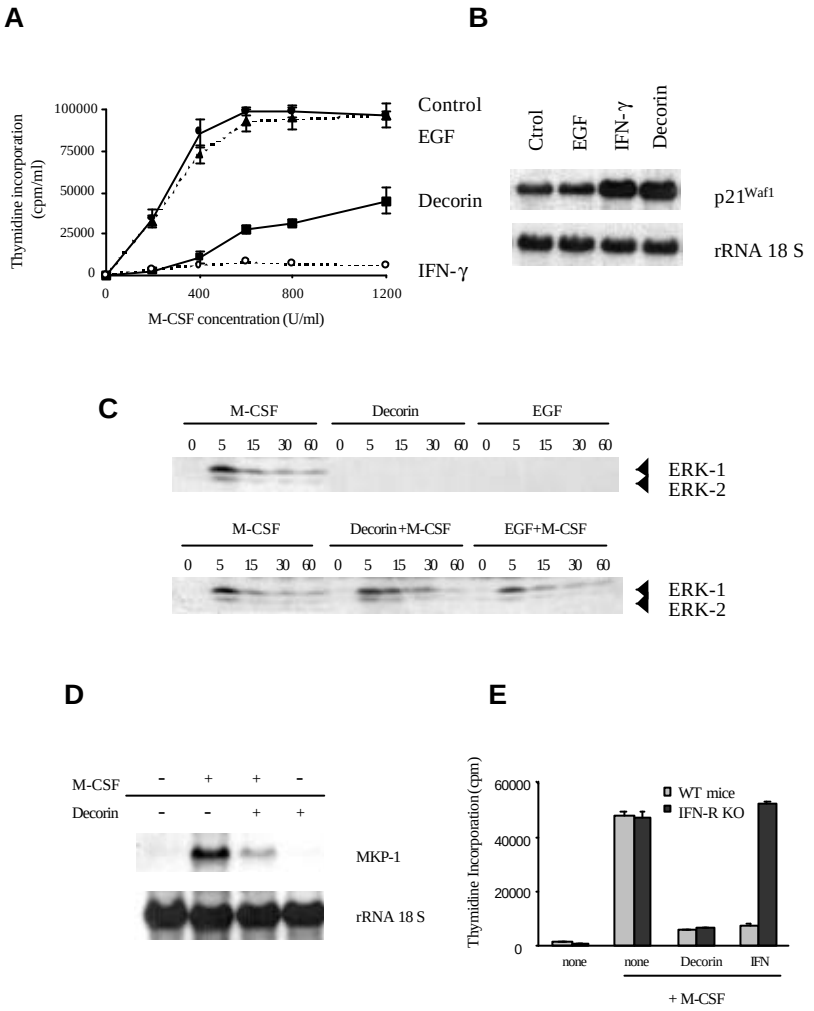


Figure 3: The inhibitory effect of decorin is independent of the EGF or IFN-g receptors. A) Decorin and IFN-γ but not EGF, inhibit macrophage proliferation. Macrophages were either treated with 20 nM EGF, 300 U/ml IFN-γ 10 μg/ml decorin or remained untreated for 24 hours in the presence of 1000 U/ml M-CSF, and proliferation was determined as indicated in *Materials and methods*. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of one representative of three independent experiments. B) Decorin and IFN-γ but not EGF, induce the expression of $p21^{Waf1}$ mRNA in BMDM. Macrophages were treated for three hours with either 20 nM EGF, 300 U/ml IFN-γ 10μg/ml decorin or remained untreated. $p21^{Waf1}$ expression was determined by Northern blotting. C) Decorin, but not EGF, elongates the M-CSF-induced activation of ERK. Quiescent macrophages were stimulated with one or a combination of the following 1000 U/ml M-CSF, 20 nM EGF or 10 μg/ml decorin for the indicated times. ERK activity was determined by in-gel-kinase assay. D) Decorin inhibits MKP-1 expression induced by M-CSF. Quiescent macrophages cultured on plates pre-coated with BSA or 10 μg/ml decorin were stimulated with M-CSF for 30 minutes. The expression of MKP-1 was analyzed by Northern blotting. The levels of the 18S rRNA transcript were used as a loading and transfer control. E) Decorin inhibits the proliferation of macrophages from IFNγ receptor KO mice. 10^5 macrophages from control and IFN-γ receptor KO mice were cultured in the presence of 1000 U/ml M-CSF and treated with 300 U/ml IFN-γ or 100 μg/ml decorin. Proliferation was determined after 24 hours by ^{3}H-Thymidine uptake. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of two independent experiments

The differences observed between the treatment with decorin and EGF suggest that the inhibition of macrophage proliferation induced by decorin could not be mediated through its interaction with the EGF receptor, since this growth factor does not seem to modulate macrophage proliferation in BMDM. However, IFN-γ mimics most of the effects of decorin, i.e., it inhibits macrophage proliferation, induces the expression of $p21^{Waf1}$ and also inhibits MKP-1 and extends ERK activity (Comalada et al., 2002c). Therefore, we tested whether the action of decorin through the activation of the IFN-γ receptor could explain the inhibition of proliferation. As expected, IFN-γ does not inhibit the proliferaton of macrophages obtained from IFN-γ receptor KO mice. However, decorin still inhibited the proliferation of macrophages from these mice (Fig. 3E), thus showing that the effects of decorin are independent of the IFN-γ receptor.

Macrophages growing *in vitro* adhere to the surface of the plates and it has been described in several cellular models that this adhesion is important for the proliferation and viability of the cells (Giancotti, 1997; Ruoslahti and Reed, 1994). Since decorin is a component of the extracellular matrix, we decided to test how this proteoglycan affects macrophage adhesion and if this was important in modulating their proliferation and viability.

We observed that decorin enhanced the adhesion of macrophages to the surface of plastic dishes (Fig. 4A). Moreover, macrophages growing in decorin pre-coated dishes showed a higher degree of spreading than non-treated macrophages, and their ameboid morphology changed to a more complex morphology with pseudopoda ramification (Fig. 4B).

Macrophages bind with different affinities to several proteins present in the ECM. We found that BMDM showed a higher level of binding to plates pre-coated with fibronectin and vitronectin, whereas laminin reduced their adhesion (Fig. 5A). We then tested how macrophage adhesion affected their proliferation. Using an inhibitor of cellular adhesion, Poly(2-Hidroxyethil Methacrylate) (PHM), that blocked macrophage adherence to the dishes (Fig. 5A), we showed that these cells need to adhere to proliferate (Fig. 5B). Surprisingly, a strong adhesion could also decrease macrophage proliferation. Macrophages growing on a fibronectin surface, to which they attach strongly, proliferate less than macrophages growing on control BSA-

pre-coated surface (Fig. 5B). Macrophages cultured on a surface to which they attach slightly (i.e., laminin-coated surface) showed a higher level of proliferation than control cells. Similar results were obtained using vitronectin or collagen I (data not shown). We could not discard that the different effects on proliferation were due to signaling through different integrin receptors. However, our results suggest that, although macrophage anchorage is necessary to proliferate, the proliferation of macrophages could be modulated by their degree of adhesion.

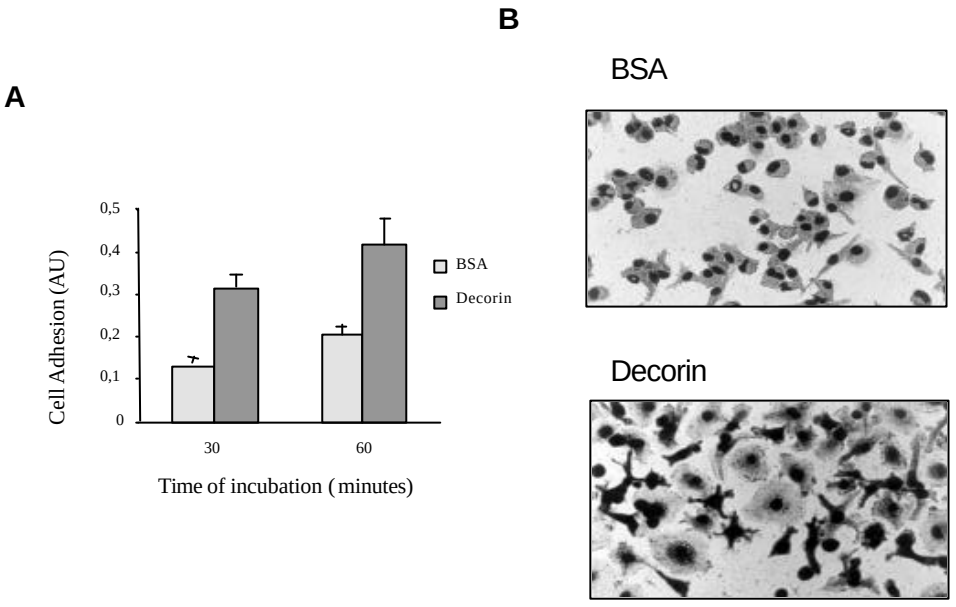


Figure 4: Decorin increases macrophage adhesion. A) 10,000 cells were cultured on plates pre-coated with BSA or 10 μg/ml decorin for only 30 and 60 minutes. After several washes, adhesion of macrophages was analyzed by cristal violet staining as indicated in *Materials and methods*. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of five independent experiments. B) Photographs of BMDM attached to BSA- (control) or decorin-coated surfaces using a phase contrast microscope with an objetive of 40X.

So far, our results showed that both decorin and fibronectin enhance macrophage adhesion and inhibit M-CSF-dependent proliferation. In order to determine if the effect of decorin on macrophage proliferation is mediated by its effect on adhesion, we checked the mechanism by which adhesion to fibronectin modulates macrophage proliferation.

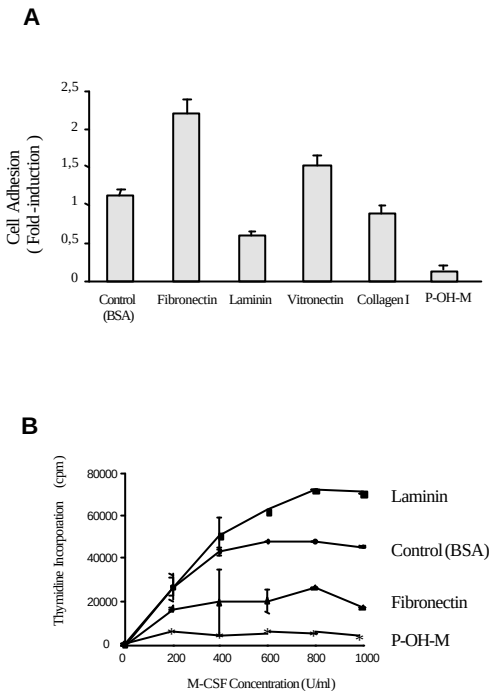


Figure 5: The adhesion of macrophages modulate their proliferation. A) Macrophages adhere preferably to fibronectin or vitronectin pre-coated surfaces. 10,000 cells were cultured for 60 minutes on plates pre-coated with 10 μg/ml BSA (Control), 10 μg/ml of the indicated components of the extracellular matrix, or 25 μg/ml of Poly (2-Hidroxyethyl Methacrylate) (P-OH-M), an inhibitor of cell adhesion. Macrophage adhesion was analyzed by cristal violet staining. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of three independent experiments. B) Adhesion modulates macrophage proliferation. Macrophages adhered to plates pre-coated with 10 μg/ml BSA, laminin or fibronectin, or 25 μg/ml PHM were stimulated with the indicated concentrations of M-CSF and their proliferation was analyzed by ^{3}H-Thymidine incorporation after 24 hours. Each point was made by triplicate, and the values represented correspond to the mean ± SD of two independent experiments.

When the expression of several components of the machinery that regulate progression through the G_1 phase of the cell cycle was analyzed, we found that both decorin and fibronectin did not modify the expression of either Cyclin D_1, cdk4 (Fig. 6A), cdk-2 or cyclin E protein expression (data not shown). However, whereas decorin induced the expression of both cdk-inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, fibronectin only induced $p27^{Kip1}$ expression (Fig. 6A). Similar to decorin, fibronectin also inhibited cdk-2 activity (data not shown). The adhesion of macrophages to a fibronectin-coated surface also extended ERK activity in response to M-CSF (Fig. 6B) by inhibiting MKP-1 expression (Fig. 6C). Also, the inhibitory effect of fibronectin on macrophage proliferation was abolished in macrophages from $p27^{Kip1}$ KO mice but not in those from $p21^{Waf1}$ KO mice (Fig 6D). This suggested that an increased macrophage adhesion probably inhibited macrophage proliferation, and this correlated with an extended ERK activity and the induction of $p27^{Kip1}$.

Since fibronectin inhibited proliferation in a similar way than decorin, and macrophages could produce fibronectin, we checked whether the effects of decorin

on macrophage adhesion and proliferation were only mediated by the release of fibronectin induced by decorin.

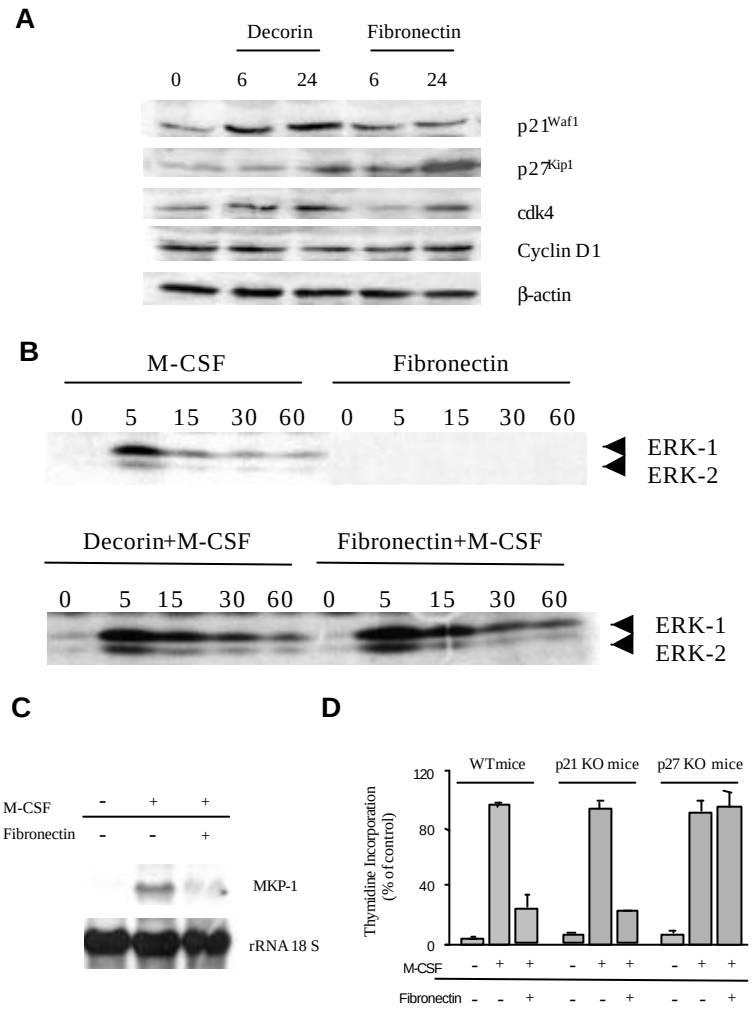


Figure 6: Fibronectin also inhibits macrophage proliferation through $p27^{Kip1}$ expression. A) Fibronectin induces $p27^{Kip1}$ but not $p21^{Waf1}$ expression. The expression of these components of the cell cycle machinery was analyzed by Western blotting. Macrophages were cultured on plates pre-coated with 10 μg/ml decorin or fibronectin for 6 or 24 hours. 100 μg of total protein were loaded per lane. The expression of β-actin was used as a sample loading and transfer efficiency control. This is the result of three independent experiments. B) Decorin and fibronectin elongate the activation of ERK induced by M-CSF. Quiescent macrophages were grown on plates pre-coated with 10 μg/ml fibronectin or 10 μg/ml decorin and were then treated with or without 1000 U/ml M-CSF for the indicated times. ERK activity was determined by in-gel-kinase assay. C) Fibronectin also inhibits MKP-1 expression induced by M-CSF. Quiescent macrophages cultured on plates pre-coated with BSA or 10 μg/ml fibronectin were stimulated with M-CSF for 30 minutes. The expression of MKP-1 was analyzed by Northern blotting. The levels of the 18S rRNA transcript were used as a loading and transfer control. D) Fibronectin did not inhibit M-CSF-dependent proliferation of BMDM from $p27^{Kip1}$ *knock-out* mice. After 7 days of culture, a total of 10^5 macrophages from wild-type, $p27^{Kip1}$ or $p21^{Waf1}$ *knock-out* mice were cultured for 24 hours on plates pre-coated with BSA or 10 μg/ml fibronectin in the presence of 1000 U/ml of M-CSF. Proliferation was determined by ^{3}H-Thymidine incorporation. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of two independent experiments.

Decorin modulates the interactions of matrix molecules, such as fibronectin, with the cells (Lewandowska et al., 1987; Schmidt et al., 1987). Therefore, we analyzed the effect of decorin on macrophage adhesion to fibronectin. The treatment of macrophages with decorin enhanced their adhesion to a fibronectin-coated surface but did not modify their adhesion to laminin-coated surfaces (Fig. 7). Since adhesion analysis are performed for only 30-60 minutes, it is unlikely that the effect of decorin will be mediated through an increase of fibronectin secretion induced by decorin. Moreover, northern blot analysis of fibronectin expression in macrophages demonstrated that decorin did not induce or increase fibronectin expression induced by M-CSF in macrophages (data not shown). This suggests that decorin binds to fibronectin through a different domain than the one recognized by macrophages and also that decorin and fibronectin are probably recognized by different receptors on macrophages.

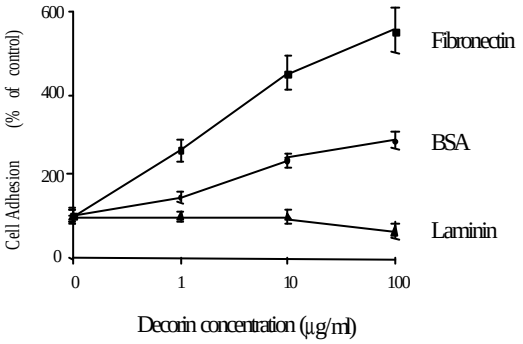


Figure 7: Decorin enhances macrophage adhesion to fibronectin but not to laminin. 10^4 macrophages were cultured on plates pre-coated with 10 µg/ml fibronectin or laminin together with the indicated amounts of decorin. After 60 minutes, adhesion of BMDM was determined by cristal violet staining as described in *Materials and methods*. Each determination was made by triplicate, and the values showed correspond to the mean ± SD of five independent experiments.

To definitively discard that the effects of decorin could be mediated by the secretion of fibronectin or through fibronectin receptors, we blocked the fibronectin signal pathway using anti-β1 polyclonal antibodies. We observed that anti-β1 antibodies reduced fibronectin-induced adhesion whereas no effect was observed on decorin adhesion (Fig. 8A). Adhesion of macrophages was almost completely blocked using an RGD peptide, which is a non-specific integrin inhibitor. Interestingly, the effect of anti-β1 antibodies on adhesion again correlated inversely with macrophage proliferation. Anti-β1 antibodies blocked the inhibitory effect of fibronectin on macrophage proliferation but had no effect on decorin inhibition (Fig. 8B). In the presence of RGD peptides, macrophages do not proliferate.

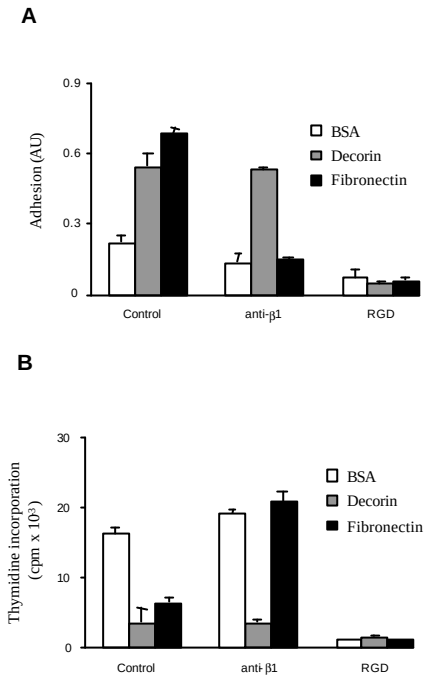


Figure 8: Fibronectin, but not decorin, mediates its effects through b1-integrin receptors. A) Adhesion of macrophages to fibronectin is mediated by a β1-integrin. 10,000 cells, previously stimulated with 1 mM RGD peptides or 5 μg/ml anti-β1 antibodies for 1 hour, were cultured for 60 minutes on plates pre-coated with 10 μg/ml BSA (Control) or 10 μg/ml of decorin or fibronectin. Macrophage adhesion was analyzed by cristal violet staining. Each determination was made by triplicate, and the values represented correspond to the mean ± SD of two independent experiments. B) The effect of fibronectin, but not that of decorin, on macrophage proliferation was inhibited by anti-β1 antibodies. Macrophages cultured as in A) were stimulated with 1000 U/ml of M-CSF and their proliferation was analyzed by ^{3}H-Thymidine incorporation after 24 hours. Each point was made by triplicate, and the values represented correspond to the mean ± SD of three independent experiments.

Also, attachment to the extracellular matrix may modulate cell viabilitty (Sun et al., 1993). Previously, we had reported that the expression of $p21^{Waf1}$ together with a blockage of the cell cycle protected macrophages from apoptosis (Xaus et al., 1999b). Since decorin increased macrophage adhesion to the ECM, induced $p21^{Waf1}$ expression and inhibited proliferation, we decided to further explore the role of decorin in the control of macrophage survival. From the experiments described in Figure 1, we concluded that decorin did not induce apoptosis in BMDM. Instead, decorin protected macrophages from apoptosis induced by growth factor withdrawal (Fig. 9). In contrast with its effect on macrophage proliferation, the increase in adhesion induced by decorin was not the mediator of this event, since the culture of macrophages on a fibronectin-coated surface did not protect macrophages from apoptosis induced by M-CSF starvation (Fig. 9). In a reminiscence of our previous observations with IFN-γ, to this effect was caused by the expression of $p21^{Waf1}$. Decorin did not inhibit apoptosis in macrophages from $p21^{Waf1}$ KO mice, whereas no differences were found in macrophages from $p27^{Kip1}$ KO mice (Fig. 9).

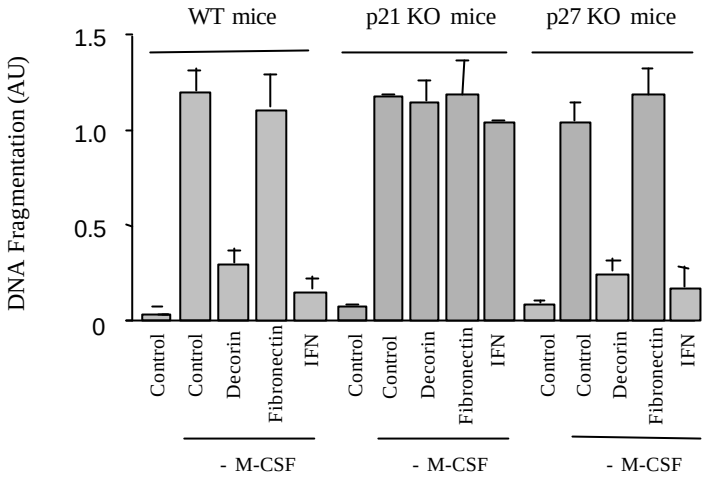


Figure 9: Decorin, but not fibronectin, protects macrophages from apoptosis induced by M-CSF starvation. Macrophages from wild-type, $p21^{Waf1}$ KO and $p27^{Kip1}$ KO mice were cultured on plates pre-coated with BSA, decorin or fibronectin were treated with 300 U/ml IFN-γ. The cells were then cultured in the absence of M-CSF for 24 hours. Apoptosis induced by M-CSF starvation was measured by ELISA (Roche), a method that detects fragmented histone-associated low molecular DNA. That is characteristic of the apoptotic process. Each point was made by triplicate, and the values are represented as the mean ± SD of two independent experiments.

Discussion

Macrophages are derived from undifferentiated stem cells in the bone marrow and through the blood they reach the different tissues where, in most cases, they undergo apoptosis. In the presence of specific growth factors or cytokines, macrophages proliferate, become activated or differentiate. In order to carry out their functional activities, macrophages have to become activated. After interacting with IFN-γ, a cytokine released by activated T lymphocytes, macrophages undergo biochemical, morphological and functional modifications that allow them to perform their functional activity (Celada and Nathan, 1994). IFN-γ also blocks their proliferation and protects them from apoptosis (Xaus et al., 1999b).

At the inflammatory sites, different proteoglycans (such as decorin) are secreted by macrophages (Uhlin-Hansen et al., 1993). Therefore, we wanted to

know its effect on bone marrow-derived macrophages, a homogeneous population of non-transformed cells (Celada et al., 1996). Decorin inhibits M-CSF-dependent proliferation of macrophages and inhibits apoptosis induced by growth factor withdrawal. After the treatment with decorin we detected the induction of both cdk inhibitors ($p21^{Waf1}$ and $p27^{Kip1}$). Using mice with these genes disrupted by homologous recombination, we have found that $p27^{Kip1}$ is responsible for the antiproliferative effect of decorin whereas $p21^{Waf1}$ is required to induce protection against apoptosis.

The inhibition of M-CSF-dependent proliferation in macrophage by decorin confirms previous observations in several tumor cells (Santra et al., 1997). However, the mechanism of inhibition is different to what has been described because in tumor cells the growth-supressive properties of decorin require a funcional cdk inhibitor $p21^{Waf1}$ (Santra et al., 1997). Our observation is in accordance with the fact that fibrillar collagen, a molecule that interacts closely with decorin both *in vitro* (Vogel and Trotter, 1987) and *in vivo* (Danielson et al., 1997), inhibits smooth muscle cell proliferation by inducing the expression of $p27^{Kip1}$ (Koyama et al., 1996). Besides, we have observed that binding of macrophages to fibronectin, another protein of the extracellular matrix that binds to decorin, also inhibits macrophage proliferation through the induction of $p27^{Kip1}$.

We have found other differences compared to tumor cells. It has also been reported that decorin causes a rapid phosphorylation of the EGF receptor and a concurrent activation of the ERK signal pathway, which leads to a protracted induction of endogenous $p21^{Waf1}$ and, ultimately, cell cycle arrest (Moscatello et al., 1998; Santra et al., 2000). Although we did not detect a direct activation of the ERK pathway by decorin, the latter extended the ERK activity induced by M-CSF. In previous works we have described that this elongation is important for the regulation of macrophage proliferation and activation (Valledor et al., 1999; 2000b). Besides, we have observed that decorin does not interact with EGF or IFN-γ receptors to inhibit macrophage proliferation.

An increasing number of observations indicate that proteoglycans can regulate cell proliferation through interaction with various growth factors (Ruoslahti, 1989). The expression of decorin in CHO cells has a dramatic effect on their

morphology and growth properties (Yamaguchi and Ruoslahti, 1988). This effect is partly caused by the ability of decorin to bind to TGF-β which stimulates the growth of those cells (Yamaguchi et al., 1990). Current studies indicate that the nature of the local extracellular matrix can modulate cell responses to a given signal in several ways, such as by modifying the affinity of the ligand for its cognate receptor or by influencing on proteolytic processing and internalization (Iozzo and Murdoch, 1996). We believe these could not be the mechanisms that explain the antiproliferative effect of decorin. Decorin binds to TGF-β and in previous studies we have shown that TGF-β increases the proliferation of macrophages (Celada and Maki, 1992). However, the blockage of TGF-β signaling using antibodies specific for this cytokine abolishes the enhancement of proliferation induced by TGF-β without modifying the normal proliferative capacity of macrophages (Celada and Maki, 1992). Moreover, decorin does not seem to act by sequestering growth factors required by macrophages, such as M-CSF, since decorin also inhibits the proliferation of RAW 264.7, a macrophagic cell line that is growth independent of M-CSF (data not shown). Moreover, the fact that decorin also inhibits the proliferation of macrophages using recombinant M-CSF suggests that no other growth factors or mitogenic derivatives (i.e. proteoglycan-M-CSF) present in the L-cell conditioned media should mediate the effects of decorin. Also, the inhibition of GM-CSF-induced proliferation by decorin suggests that the effect of decorin is a general mechanism of proliferation.

The growth of adherent cells such as fibroblasts or macrophages requires signals not only from growth factor receptors but also from integrins (Howe et al., 1998; Ruoslahti and Reed, 1994; Zhu et al., 1996). That is also true for macrophages, since the total inhibition of their adhesion blocks macrophage proliferation. However, we thereby show for the first time that the level of this adhesion has a strong effect on the modulation of the level of macrophage proliferation. A strong attachment induced by decorin or fibronectin is enough to reduce macrophage proliferation, whereas a slight adhesion such as that induced by laminin increases proliferation. The use of anti-β1 antibodies and RGD peptides comfirm this hypotesis. Macrophages adhere to plastic dishes mainly through integrin receptors, since RGD peptides almost totally block this adhesion. Moreover, macrophages need to adhere in order to proliferate; in this regard, RGD and P-OH-M inhibit both macrophage adhesion and proliferation. Besides, the

studies using anti-β1 antibodies and RGD suggest that the effect of decorin is mediated through a non-β1 integrin receptor whereas fibronectin uses a β1-integrin receptor. The fact that decorin has a synergic effect on macrophage adhesion to fibronectin also suggests that decorin uses a different receptor than fibronectin. However, more studies are necessary in order to determine which receptor is used by decorin in macrophages.

A few examples have been described for the G_1-phase blockage mediated by cellular adhesion. This effect is caused by the increase of the expression of $p27^{Kip1}$, which inhibits cyclinE-cdk2 kinase activity (Chen et al., 1997; Jiang et al., 2000; Koyama et al., 1996). Cell to cell contact induce the cells to stop proliferating during normal organ development. More recently it has become clear that such contact-mediated growth arrest is caused by the up-regulation of $p27^{Kip1}$. This is shown in the $p27^{Kip1}$ KO mice, which are generally bigger and have a significantly expanded hematopoietic progenitor pool (Johnson et al., 1998; Nakayama et al., 1996). We have observed that the inhibitory effect on macrophage proliferation induced by adhesion to some extracellular matrix components (ie. decorin or fibronectin) is also mediated through the expression of $p27^{Kip1}$.

Besides, the signals mediated by the extracellular matrix are important for cell survival. A laminin-rich extracellular matrix is a potential survival factor for differentiated mammary alveolar epithelial cells both *in vivo* and *in vitro* (Finlay et al., 2000). It has been suggested that the laminin-rich extracellular matrix acts through α6β1-integrin receptors to affect cell survival, partly by stabilizing PI-3 kinase-dependent survival responses to insulin (or IGF1) (Boudreau et al., 1995; Farrelly et al., 1999). Our results with decorin are very interesting and confirm previous results from our group (Xaus et al., 1999b). Decorin protects macrophages from apoptosis induced by M-CSF starvation. In this case, the effect of decorin is not mediated by the increase in adhesion. As we have described elsewhere (Xaus et al., 1999b), the induction of $p21^{Waf1}$ and the blockage of the cell cycle induced by decorin (or IFN-γ) is responsible for the protective effect against apoptosis. The results obtained using $p21^{Waf1}$ KO mice confirm this conclussion.

Our results could have physiological relevance. Although the concentration of decorin pre-coating solution used in our studies (10-100 μg/ml) could seem slightly higher than that estimated to occur in collagenous matrices (5-12.5 μg/ml) found *in vivo* (Santra et al., 2000), the amount of decorin adsorved to the plate should be significatively lower. Macrophages play a critical role during inflammation. From blood, macrophages reach the inflammatory foci and remain there until inflammation disappears (Bellingan et al., 1996). In the tissues, macrophages need to survive in the absence of growth factors. Whereas stimulated Th1 cells remain at the inflammatory loci and produce IFN-γ macrophages are protected against apoptosis (Xaus et al., 1999b). In addition to IFN-γ, the elements that form the extracellular matrix, such as decorin, could also protect against apoptosis. An example of this situation may be found in the formation of granulomas that can be appear in the course of certain inflammatory responses. The macrophages in these granulomas secrete decorin (Asakura et al., 1996; Limper et al., 1994). Each granuloma can be viewed as a small spherical organ made of a variety of differentiated macrophages whose function is to limit the expansion of, and allow the eventual destruction of, extravascular bacteria (Adams, 1976; Williams and Williams, 1983). Decorin may favor macrophage survival and accumulation in granulomas, thus leading to bacterial elimination. Our observations on IFN-γ and decorin could provide an explanation for long-term living macrophages within the tissues.

La decorina revierte el efecto represor del TGF-b producido de forma autocrina en los macrófagos activados

Los factores de crecimiento y diferentes citocinas inducen a los macrófagos a proliferar, activarse, diferenciarse o a morir mediante apoptosis. En los sitios de inflamación los proteoglicanos, entre otros productos, son secretados por los monocitos y los macrófagos y modulan la respuesta immunológica. El IFN-γ, el cual es liberado por los linfocitos T activados o las células NK, es el activador más potente de los macrófagos. En trabajos anteriores, hemos visto que el IFN-γ bloquea la proliferación de los macrófagos y protege de la apoptosis permitiendo a los macrófagos sobrevivir en los lugares de inflamación a pesar de las condiciones desfavorables. La decorina, de manera similar al IFN-γ, también inhibe la proliferación de los macrófagos y los protege de la apoptosis inducida por la falta de factores de crecimiento.

Por este motivo, en este trabajo quisimos analizar el efecto de la decorina sobre la activación de los macrófagos inducida por el IFN-γ o bien por el LPS. Hemos demostrado que la decorina incrementa la activación de los macrófagos. Así, la decorina potencia la expresión de los genes IAα e IAβ del MHC de clase II inducidos por el IFN-γ. De igual modo, la decorina aumenta la expresión de la óxido nítrico sintetasa (iNOS) y de citocinas como el TNF-α, la IL-1β y la IL-6, inducidas tanto por el IFN-γ como por el LPS.

En el trabajo anterior habíamos demostrado que los efectos antiproliferativos de la decorina estaban mediados por el aumento de la adhesión de los macrófagos a la matriz extracelular inducida por la decorina. Esto nos condujo a analizar el papel de la adhesión de los macrófagos a la matriz extracelular como responsable directo del aumento de activación observada en los macrófagos tras la estimulación con decorina. Mediante la utilización de diferentes proteínas de la matriz extracelular, hemos observado una correlación negativa entre adhesión y proliferación. Sin embargo, el efecto sobre la activación no se debe a un aumento de la adhesión celular ya que otras proteínas de la matriz extracelular como la fibronectica, que también induce un aumento de la adhesión igual que la decorina, no aumentan la activación de los macrófagos.

Por otro lado se ha descrito que la decorina es capaz de unirse al TGF-β y modular (ya sea aumentando o inhibiendo) la actividad de esta citocina. Debido a que el TGF-β es un potente inhibidor de la activación de los macrófagos decidimos analizar el efecto represor de la decorina sobre el TGF-β.

El TGF-β, es una citocina producida por los macrófagos de manera autocrina y es la responsable de la regulación negativa de la activación. El TGF-β antagoniza muchos de los procesos inducidos tanto por el IFN-γ como por el LPS. El tratamiento de los macrófagos con decorina permite secuestrar al TGF-β producido de forma endógena, inhibiendo la interacción del TGF-β con su receptor específico y de esta manera aumentar la activación de los macrófagos. Estos resultados han sido confirmados tanto por estudios de unión con TGF-β radiomarcado (*Scatchard*) como utilizando anticuerpos bloqueantes del TGF-β. El tratamiento de los macrófagos con estos anticuerpos potencia la activación de forma similar a la que se observa con la decorina.

Por tanto, este mecanismo general de potenciación de la activación mediada por la decorina demuestra que los macrófagos se encuentran reprimidos de forma basal mediante la producción de TGF-β, el cual impide el desencadenamiento de una respuesta estimuladora de los macrófagos en condiciones no patológicas o en ausencia del estímulo suficiente, puesto que dicha activación no justificada podría ser dañina para el organismo. En condiciones patológicas o en infecciones donde el sistema inmunológico debe actuar, se produce un incremento de los niveles de IFN-γ o de productos bacterianos capaces de decantar la balanza hacia un proceso inflamatorio. Los proteoglicanos de la matriz extracelular como la decorina, sin lugar a dudas, contribuyen a modular y regular más finamente este equilibrio entre mecanismos activadores e inhibidores de los procesos inflamatorios.

Decorin reverses the repressive effect of the autocrine produced TGF-b on macrophage activation

Abstract

Different cytokines or growth factors cause macrophages to proliferate, become activated, differentiate, or die through apoptosis. The extracellular matrix protein decorin, like IFN-γ, which is the major macrophage activator, inhibits proliferation and protects macrophages from the induction of apoptosis. Decorin enhances the IFN-γ -induced expression of the IAα and IAβ MHC class II genes. Also, decorin increases the IFNγ – or the LPS-induced expression of inducible- nitric oxide synthase (iNOS), TNF-α, IL-1β and IL-6 genes. Using different extracellular matrix proteins, we have found a negative correlation between adhesion and proliferation. However, the effects of decorin on macrophage activation seems not to be mediated through its effect on adhesion or proliferation. Instead, decorin accomplished this by abolishing the binding of TGF-β to macrophages, as shown by Scatchard analysis of labeled ^{125}I-TGF-β , which in absence of decorin showed a Kd of 0.11±0.03 nM and around 5000 receptors per cell. This was confirmed when we treated macrophages with antibodies to block the endogenously produced TGF-β , which enhanced macrophage activation in a way similar to that of decorin. This general mechanism of activation enhancement mediated by decorin demonstrates that macrophages are under negative regulation that can be reverted by proteins of the extracellular matrix.

Introduction

Macrophages play an important role in the immune response. They are produced in the bone marrow and through the blood reach different tissues. Most of the macrophages will die through apoptosis. However, in the presence of different cytokines or growth factors, macrophages proliferate, differentiate into several cell types (kupffer cells, Langerhans, microglia...) or become activated to develop their functions. At the inflammatory loci, macrophages phagocyte bacterias, remove cell debris, release several mediators, present antigens to T lymphocytes, and contribute to the resolution of the inflammation (Celada and Nathan, 1994).

Interferon gamma (IFN-γ), which is released by activated T lymphocytes or NK cells, is the most potent activator of macrophages, inducing the expression of more than 300 genes (Boehm et al., 1997). We found that IFN-γ also blocks macrophage proliferation and protects against apoptosis (Xaus et al., 1999b). This allows macrophages to survive at the inflammatory loci when IFN-γ is present, and explains the key role that T lymphocytes play in the delayed hypersensitivity (Gudmundsson and Hunninghake, 1997; Kaufman, 1995).

At the inflammatory locis, proteoglycans are secreted by monocytes (Laskin et al., 1991; Uhlin-Hansen et al., 1992) and macrophages (Uhlin-Hansen et al., 1993) and modulate the immune response. Decorin and other related molecules are considered to form a family called small leucine-rich proteoglycans that are found in the extracellular matrix (ECM) of a variety of tissues (Iozzo, 1997; Iozzo and Murdoch, 1996). Although, the biological importance of these molecules is unclear, several observations suggest that decorin and, perhaps, other proteoglycans regulate the remodeling of connective tissue. In particular, *in vitro* binding studies have shown that decorin interacts with several types of collagen (Bidanset et al., 1992; Schonherr et al., 1995), and it is believed to be an important regulator of collagen fibrillogenesis (Danielson et al., 1997). Decorin may also affect the production of other ECM components (Hildebrand et al., 1994; Yamaguchi et al., 1990). Additionally, decorin can modulate the interactions of matrix molecules such as fibronectin with cells (Lewandowska et al., 1987; Schmidt et al., 1987; Winnemoller et al., 1991).

Recently, we observed that decorin, like IFN-γ, inhibits proliferation of macrophages and enhances cell survival through the expression of $p27^{Kip1}$ and $p21^{Waf1}$ respectively (Xaus et al., 2001b). Since we have found that activation by IFNγ or LPS inhibits macrophage proliferation (Valledor et al., 2000b; Xaus et al., 2000a), we wanted to determine the effect of decorin on macrophage activation. In the study we report here, we have used primary cultures of bone marrow-derived macrophages, which are a homogeneous population of cells that respond to physiological proliferative or activating stimuli (Celada et al., 1996). Decorin enhances both LPS and IFN-γ-induced activation as measured by the capacity to increase MHC class II, iNOS and cytokine expression. This decorin effect is due to

the ability of this proteoglycan to block the binding of the autocrine produced TGF-β on the cell surface of macrophages.

Materials and Methods

Reagents

Recombinant purified decorin was a generous gift from Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA, USA). LPS, BSA, collagen I, vitronectin, laminin and fibronectin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-Thymidine, TGF-β and [^{125}I]-TGF-β were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). IFN-γ was a generous gift of Genentech, Inc. (South San Francisco, CA). All the other products were of the best grade available and were purchased from Sigma. Deionized water was used that had been further purified with a Millipore Milli-Q system (Bedford, MA, USA).

Cell culture

Bone marrow-derived macrophages were isolated from six-week old balb/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) as previously described (Celada et al., 1996). The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 30% L-cell conditioned media as a source of M-CSF. The cells were incubated at 37º C in a humidified 5% CO_2 atmosphere. After 7 days of culture a homogeneous population of adherent macrophages was obtained (>99% Mac-1^{+}).

In some circumstances, macrophages were cultured on a pre-coated plate using different components of the extracellular matrix (ECM) or BSA as a control. To pre-coat the plates, the plates were incubated over-night at 4ºC with a PBS solution of the indicated concentration of each ECM-component. After coating, the plates were blocked with PBS 10 μg/ml BSA for 1 hour at 37ºC, then the blocking solution was removed and the cells cultured with normal complete media.

Antibodies and constructs

For the analysis of IA surface expresion by flow cytometry, we used purified monoclonal anti-mouse $IA^{d,b}$ antibodies (BD Pharmingen, San Diego, CA). FITC-conjugated anti-mouse IgG (Cappel, Turnhout, Belgium) was used as secondary antibody. An irrelevant primary antibody was used as control and purchased from Sigma. For Western Blot analysis, we used a rabbit antibody against mouse iNOS (M-19, Santa Cruz Biotechnology, Santa Cruz, CA) and as a control, a mouse anti-β-actin antibody (Sigma). Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Cappel) were used as secondary antibodies. Blocking policlonal antibodies against TGF-β were obtained from Promega (Madison, WI). The same antibodies were used for the analysis of TGF-β expression by western blotting.

The cDNA probes for IA-α and IA-β used for northern blot were a kind gift from P. Cosson (Basel Institute für Immunobiologie, Basel, Switzerland). A rat-inducible NO synthase (iNOS) cDNA fragment (Cuevas et al., 1996) was used to detect of IFN-γ- and LPS-induced iNOS expression. For TNF-α mRNA detection we used a cDNA probe obtained from Dr. M. Nabholz (Institud Suisse de Recherches Experimentals sur le Cancer, Epalinges, Switzerland). To study the expression of IL-1β, we obtained a probe by digesting the construct pGEM1/IL-1β (kindly provided by Dr. R. Wilson, Glaxo Research and Development Limited, Greenford, UK) with EcoRI/PstI. The IL-6 cDNA probe was a generous gift from Dr. S. Rohatgi (Center for Blood Research, Boston, MA, USA). The probe for the 18S rRNA was obtained as described elsewhere (Torczynski et al., 1987).

Cell surface staining

Cell surface staining was done using specific antibodies and assessed using cytofluorometry analysis (Xaus et al., 1999a) with mouse monoclonal antibody anti-mouse IA^{db} (1 μg/10^6 cells). A non-related antibody was used as a control for non-specificity. The cells were then washed by centrifugation through a FBS cushion. Stained cell suspensions were analyzed using an Epics XL flow cytometer (Coulter, Hialeah, FL).

Proliferation and adhesion analysis

Cell proliferation was analyzed by ^{3}H-Thymidine incorporation and cell adhesion to the substrate was analyzed by crystal violet as previously described (Xaus et al., 2001b) with no modifications. Each sample was analyzed in triplicate and the results were represented as the mean ± SD.

Scatchard and TGF-β binding analysis

To analyze the binding of TGF-β to macrophages and the capacity of decorin to modulate it, we cultured 10^6 cells/well in 12-well plates pre-coated with 10 μg/ml BSA or decorin. After this, we wash them with Krebs Ringer Hepes (KRH; 128 mM NaCl, 5 mM KCl, 5 mM Mg SO_4, 1.3 mM $CaCl_2$, 50 mM Hepes) (pH 7.4). For the binding analysis we incubated cells with the indicated amounts of iodinated-TGF-β. For the Scatchard analysis, ^{125}I-TGF-β (100 pM) binding was competed with increasing amounts of cold TGF-β. The cells were incubated on a rotating platform for 3 hours at 4ºC. After that, cells were washed and crosslinked for 15 minutes at 4ºC with 0.5 ml of dioctyl sodium sulfosuccinate (DSS) solution (6 μg/ml in KRH). After two washes with STE (0.25 M Sucrose, 10 mM Tris-HCl, 1 mM EDTA), proteins were solubilized for 40 minutes at 4ºC with 200 μl of 0.5% Triton-Tris-HCl-EDTA and protease inhibitors. The supernatants were then transfer to test tubes and boiled for 1 minute. Samples were counted using a Packard gamma counter. Each point was performed in triplicates and the results were expressed as the mean ± SD.

Northern blot analysis

Northern blot analysis was performed as previously described (Xaus et al., 2001b) using 20 μg of total cellular RNA per lane. To check for differences in RNA loading, we analyzed the expression of the 18 S rRNA transcript. All probes were labeled with ^{32}P-α-dCTP (ICN Pharmaceuticals, Costa Mesa, CA, USA) with the oligolabeling kit method (Amersham Pharmacia). The bands of interest were quantified with a Molecular Analyst system (Bio-Rad Labs. Richmond, CA).

Protein extraction and Western blot analysis

Western blot analysis was conducted as previously described (Xaus et al., 1999b). 100 μg of protein from cell lysates were loaded per lane and separated on a 7.5% SDS-PAGE. For iNOS immunoblotting, we used a rabbit antibody against mouse iNOS (M-19, Santa Cruz Biotechnology) and as a control, a mouse anti-β-actin antibody (Sigma). For the anlysis of TGF-β expression we used a policlonal antibody directed to biologically active human TGF-β (Promega). Peroxidase-conjugated anti-rabbit and anti-mouse IgG (Cappel) was used as secondary antibodies. Incubations were performed for 1 hour at room temperature. ECL detection was performed (Amershanm Pharmacia) and the membranes were exposed to X-ray films (Amersham).

Results

Because we demonstrated previously that decorin inhibits the M-CSF-dependent proliferation of macrophages (Xaus et al., 2001b) and that the proliferative state of macrophages is able to modulate macrophage activity (Xaus et al., 1999b; 2000a) we wanted to determine the effects of decorin on macrophage activation. For this purpose we used macrophages obtained from bone marrow cultures, since they represent a homogeneous, non-transformed population of macrophages that can be activated *in vitro*, to induce proliferation, differentiation or apoptosis.

First we analyzed the effect of decorin on the MHC class II expression induced by IFN-γ that is the main macrophage activator (Boehm et al., 1997). Macrophages were cultured on plates pre-coated with 10 μg/ml decorin or with 10 μg/ml BSA as a control. It is important to note that the indicated concentrations of decorin correspond to the concentration of the pre-coating solution and that we are not able to quantify the amount of decorin adsorbed to the plate after pre-coating, but other proteins bound under the same conditions less than 10-20%. Once they attached to the plates, cells were stimulated with sub-saturating amounts of IFN-γ (10 U/ml) and the MHC class II expression analyzed by flow cytometry after 24-48 hours. Under these conditions, decorin induces a significant increase in MHC II

protein surface expression compared with macrophages treated only with IFN-γ (Fig. 1A and B). The increase in IA protein surface expression correlates, as measured by Northern blotting, with an increase on IAα and IAβ mRNA expression (Fig. 1C).

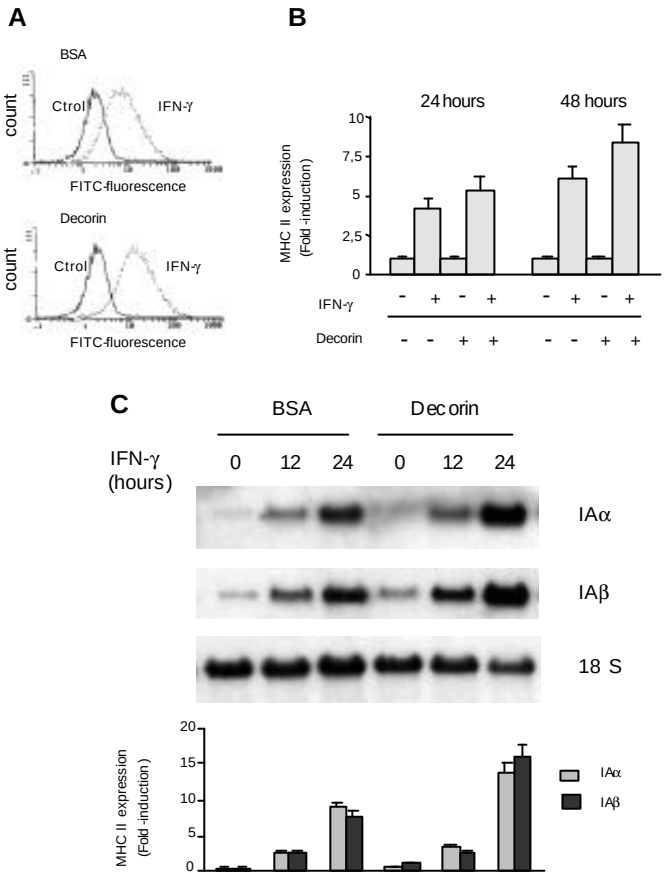


Figure 1: Decorin increases IFN-γ-induced MHC class II expression. A) Decorin increases IFNγ-induced MHC class II surface expression The expression of IA molecules on the cell surface was analyzed by flow cytometry using monoclonal specific antibodies. Macrophages were cultured in 10 μg/ml pre-coated BSA (BSA) or decorin (decorin) surface for 24 hours in presence (dot histogram) or absence (line histogram) of sub-saturating amounts of IFN-γ (10 U/ml). Cell surface staining was performed using mouse anti-$IA^{b,d}$ antibodies. A FITC-conjugated anti-mouse IgG was used as secondary antibody. B) Quantitation of IA surface expression. Macrophages were cultured as indicated and the expression of surface IA molecules was analyzed by flow cytometry as indicated above and quantitated using Immuno-4 software. The values represented correspond to the mean ± SD of three independent experiments. C) Decorin increases IFN-γ-induced MHC class II mRNA expression.The expression of IAα and IAβ mRNA was analyzed by Northern Blotting. Macrophages were cultured in 10 μg/ml pre-coated BSA or decorin surface and stimulated with sub-saturating amounts of IFN-γ (10 U/ml) for the indicated times. 20 μg of total RNA was used per lane. An 18S probe was used for loading and transfer control. Quantification of the bands of interest was performed by densitometry. Quantitation values represent the mean±SD of two representative experiments.

The effect of decorin on IFN-γ-induced activation is not specific for MHC II genes since decorin also increased the iNOS and cytokine mRNA expression induced by IFN-γ. Decorin slightly increased the levels of iNOS mRNA induced by 10 U/ml IFN-γ at 6 hours and elongated the expression kinetics of this enzyme in macrophages (Fig. 2A).

Subsaturating amounts of IFN-γ induced low-levels of TNF-α, IL-1β or IL-6 mRNA expression, which were only visible after over-exposing the film. Culture of macrophages on a 10 μg/ml decorin pre-coated plates is enough to induce maximal expression of TNF-α and IL-1β mRNA (Fig. 2B). The addition of 10 U/ml IFN-γ did not increase this expression. Concerning IL-6, decorin alone was not able to induce its expression, but subsaturating amounts of IFN-γ did increases IL-6 expression (Fig. 2B).

Because decorin is able to enhance several aspects of the macrophage activation induced by the endogenous activator IFN-γ we wanted to investigate the effect of some components of the bacterial wall such as LPS that could modulate several functions of macrophages. In this regard, we observed that under subsaturating amounts of LPS (1 ng/ml), decorin elongated the expression of iNOS (Fig. 2C) and enhances the expression of IL-6 (Fig. 2D). Thus, decorin enhanced the macrophage activation induced by both, endogenous and exogenous activators.

Since LPS and IFN-γ activate macrophages via different pathways, we tried to identify a possible common mechanism used by decorin to enhance both pathways. As we observed previously, decorin inhibited macrophage proliferation and enhances macrophage adhesion (Xaus et al., 2001b). We have also demonstrated that the proliferative estate of macrophages could modulate the activation capabilities of these cells (Xaus et al., 1999b; 2000a). Moreover, it has been described that cellular adhesion and integrin signaling is also a potent modulator of macrophage activities (Berton and Lowell, 1999; Jun et al., 1995). To explore the possible consequences of the increased adhesion and the anti-proliferative effect on the enhanced activation induced by decorin, we used other components of the ECM that modify macrophage adhesion.

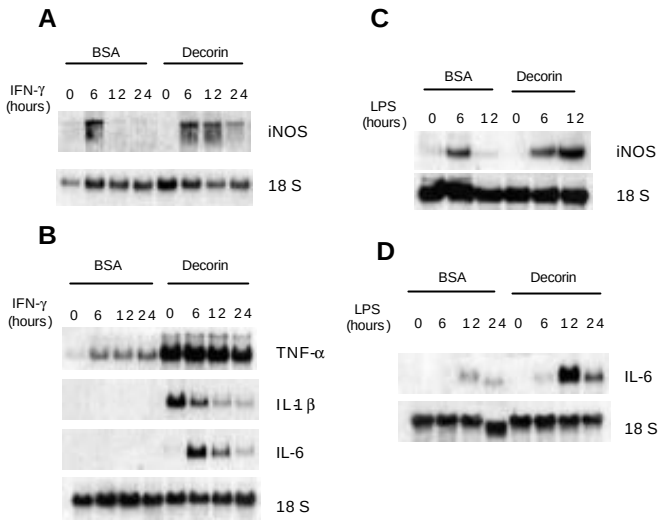


Figure 2: Deconin enhances IFN-γ- and LPS-dependent macrophage activation. Cells were cultured in pre-coated plates with BSA (10 μg/ml) or decorin (10 μg/ml) and treated with sub-saturating amounts of IFN-γ (10 U/ml) or LPS (1 ng/ml) for the indicated times. iNOS (A and C) or cytokine (B and D) (TNF-α, IL-1β, or IL-6) mRNA expression were analyzed by northern blotting as previously described. The amount of loaded RNA was corrected by ribosomal 18S RNA gene expression. The amount of the IFN-γ-induced expression of IL-1β and IL-6 could be seen after overexpossion of membranes. These results are representative of at least three independent experiments.

Macrophages cultured in plates treated with different ECM proteins, showed different degrees of adhesion. While decorin, vitronectin and fibronectin increased the adhesion of macrophages in relation to the plates treated with BSA, laminin and collagen I decreased their adhesion (Fig. 3). When we tested how macrophage adhesion affected their proliferation, we have found a negative correlation between the degree of adhesion and proliferation (Fig. 3). Specifically, macrophages growing on a fibronectin or decorin surfaces, to which they attached strongly, proliferated less than macrophages growing on a BSA-pre-coated surface (Fig. 3). By contrast, macrophages cultured on a surface to which they attach slightly, such laminin, showed more proliferation than control cells.

In contrast to decorin, fibronectin did not increase the MHC class II mRNA expression induced by IFN-γ (Fig 4A). Fibronectin was also not able to induce the basal expression of TNF-α or IL-1β nor did it to enhance their expression and the

expression of IL-6 in response to subsaturating amounts of IFN-γ neither (Fig 4B). More differences between decorin and fibronectin were observed when we analyzed the effect of fibronectin upon LPS activation of macrophages. In particular, fibronectin did not increase iNOS and citokine expression in response to subsaturating amounts of LPS, but instead reduced them. In addition, the mRNA levels of iNOS induced by LPS (1 ng/ml) (Fig. 4 C) and the levels of the cytokines tested, TNF-α, IL-1β and IL-6 (Fig. 4 D) were reduced in macrophages cultured on fibronectin-pre-coated plates.

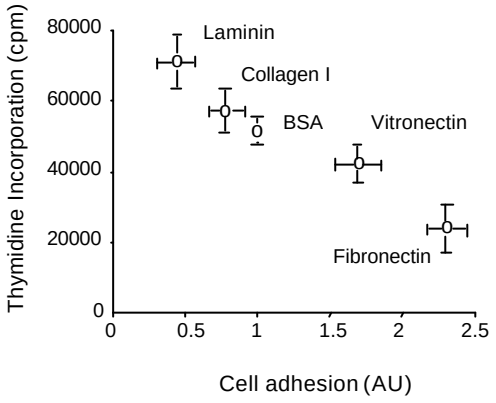


Figure 3: Negative correlation between adhesion and proliferation. Proliferation was determined by ^{3}H-Thymidine incorporation in cells cultured in 10 μg/ml of the indicated ECM proteins or BSA pre-coated plates for 24 hours. Each point was performed in triplicate, and the results were represented as the mean ± SD. For adhesion 10,000 cells were cultured on plates pre-coated with 10 μg/ml of the indicated ECM proteins or BSA for 30 minutes. After several washes, adhesion of macrophages was analyzed by cristal violet staining as indicated in *Materials and Methods*. Each determination was made in triplicate, and the values represented correspond to the mean ± SD of five independent experiments.

So far our results suggest that the anti-proliferative or adhesion-inducing effects of decorin on macrophages are not sufficient to explain its ability to enhance macrophage activation. For this reason we investigated other possible mechanisms. For example, it has been suggested previously that decorin binds TGF-β (Takeuchi et al., 1994; Yamaguchi and Ruoslahti, 1988). However, results do not agree regarding whether the decorin-TGF-β complex has a positive or negative effect on the interaction of TGF-β with its receptor on the cell surface (Takeuchi et al., 1994; Yamaguchi and Ruoslahti, 1988). Due to the repressive effect of TGF-β on both LPS- and IFN-γ-dependent activation (Letterio and Roberts, 1998), we decided to explore this hypothesis.

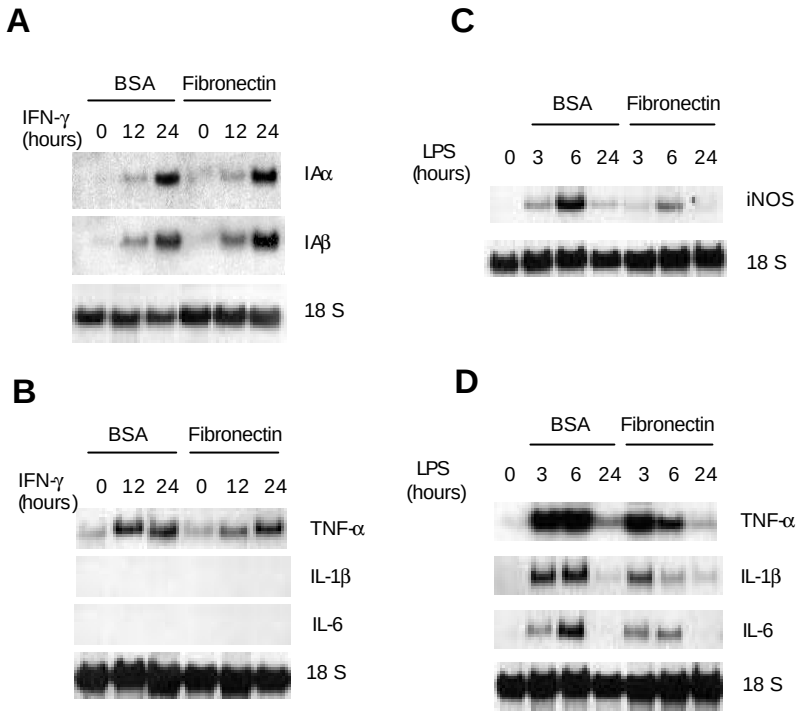


Figure 4: Fibronectin does not modulate IFN-g- or LPS-induced macrophage activation. Bone marrow-derived macrophages were cultured in pre-coated plates with BSA (10 μg/ml) or fibronectin (10 μg/ml) and treated with sub-saturating amounts of IFN-γ (10 U/ml) or LPS (1 ng/ml) for the indicated times. MHC class II (IAα and IAβ) (A), cytokine (TNF-α, IL-1β, or IL-6) (B and D) or iNOS (C) mRNA expression were analyzed by northern blotting. 20 μg of total RNA was carried per lane. The amount of loaded RNA was corrected by ribosomal 18S RNA gene expression. The specific bands corresponding to IFN-γ-induced expression of IL-1β and IL-6 could be seen after over-expossion of the membranes.

Figure 5 shows that ^{125}I-recombinant TGF-β binds to macrophages in a specific and saturable manner at 4ºC. In these experiments, 96-98% of the total binding was specific, since it was blocked in the presence of a 100-fold excess of unlabeled TGF-β. Binding was also homogeneous, non-cooperative, and of moderately high affinity (Fig. 5A). Further, the TGF-β binding was dose-dependent and saturable at 250 pM (Fig 5A). Scatchard analisis showed that macrophages are able to bind TGF-β with a Kd of 0.116±0.03 nM, and the number of receptors on the macrophage surface was calculated to be 4793±813 receptors/cell, which is similar to that described for other cell types (reviewed in Massague et al., 1990) (Fig 5B). However, the culture of macrophages on a 10 μg/ml decorin-precoated

plate abolished the binding of TGF-β to macrophages even at the highest ^{125}I-TGF-β level tested (500 pM) (Fig 5). Collectively, these binding experiments showed that TGF-β binds to the macrophage surface and that decorin inhibits this cytokine, preventing it from interacting with its receptor in macrophages.

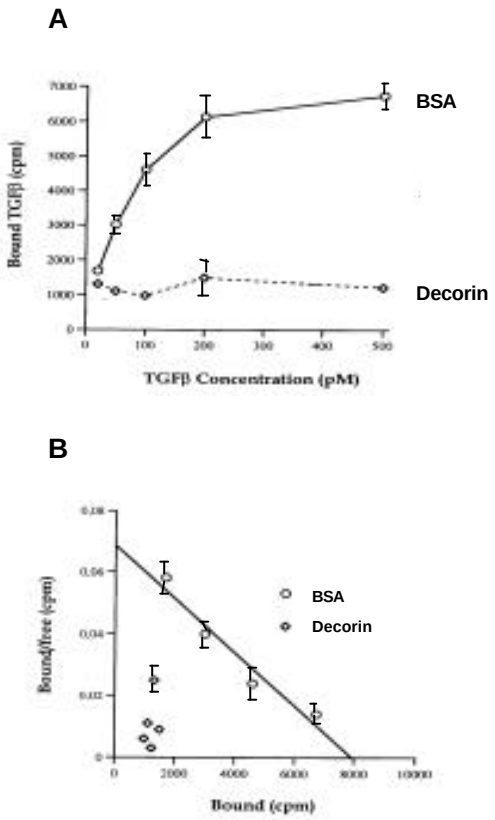


Figure 5: Decorin binds TGF-b and inhibits TGF-b binding to its receptor. A) Saturating curves of ^{125}I-TGF binding to macrophages cultured on a 10μg/ml BSA or decorin pre-coated surface. Binding experiments were performed as described in material and methods. Each point was made by triplicate and represented as mean ± SD. B) Scatchard analysis of ^{125}I-TGF-β in presence of increasing concentrations of unlabeled TGF-β in macrophages cultured on a 10 μg/ml BSA or decorin pre-coated surface. Each determination was made by triplicate and was represented as mean±SD. The scatchard showed represents one representative of three independent experiments.

Next, we explored whether decorin was able to suppress the inhibitory capability of exogenous TGF-β to block macrophage activation. Treatment of macrophages with 10 ng/ml TGF-β was sufficient to block completelly the IFN-γ–induced MHC class II mRNA expression. However, when macrophages were cultured in the presence of decorin, TGF-β was not able to block MHC II expression (Fig. 6A). Similar results were obtained when we analyzed the effect of TGF-β and decorin on iNOS or IL-6 mRNA expression induced by LPS (Fig. 6B).

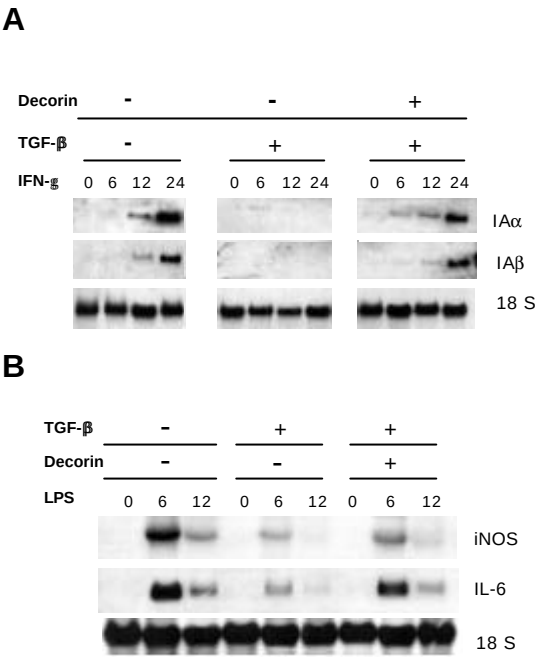


Figure 6: Decorin blocks the immunosupresive effect of TGF-β on macrophage activation. A) TGF-β suppresses the IFN-γ-induced MHC II expression and this effect is reverted by decorin. Macrophages were cultured in BSA or decorin pre-coated plates. Once attached, cells were stimulated with 1 ng/ml TGF-β for 30 minutes and then activated with sub-saturating amounts of IFN-γ for 6, 12 and 24 hours. The expression of IA mRNAs was analyzed by northern blotting. B) TGF-β blocks the LPS-induced iNOS and IL-6 expression and this effect is inhibited by decorin. Macrophages were cultured in 10 μg/ml BSA or decorin pre-coated plates. Then, cells were stimulated with 1 ng/ml TGF-β for 30 minutes and then activated with sub-saturating amounts of LPS for the indicated times. The expression of iNOS and IL-6 mRNAs were analyzed by northern blotting. In both panels the amount of loaded RNA was corrected by ribosomal 18S RNA gene expression.

Our results demonstrated that decorin was able to block the binding of TGF-β to the corresponding receptor, thereby reversing its inhibitory effects on macrophage activation. However, these results did not explain why decorin is able to enhance macrophage activation in the absence of exogenous TGF-β. This explanation was obtained when IFN-γ or LPS-activated macrophages were treated with blocking antibodies against TGF-β. Under these conditions, like decorin, anti-TGF-β antibodies were able to increase iNOS protein expression induced by subsaturating amounts of IFN-γ (Fig 7A) or LPS (Fig 7B). Anti-TGF-β and decorin had no effect on iNOS expression in the absence of any macrophage activator.

Moreover, western blot analyses showed that both IFN-γ and LPS at subsaturating amounts induced the expression of TGF-β in macrophages (Fig. 7C). Since decorin did not modify this expression, the blocking effect of decorin is due to TGF-β sequestration and inhibition of TGF-β binding to its receptor. Therefore, our results suggest that decorin increased macrophage activation by inhibiting the endogenous TGF-β produced by IFN-γ or LPS in macrophages.

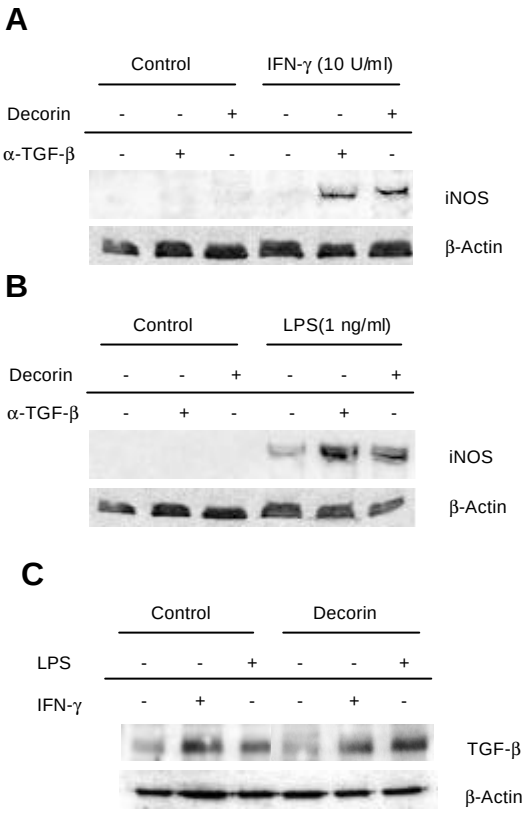


Figure 7: Decorin effects on macrophage activation are due to sequestration of endogenous TGF-β . $3x10^6$ macrophages were cultured in 10 μg/ml BSA or decorin pre-coated plates. After attachment to the plate, BSA-adhered cells were treated or not with 1 μg/ml anti-TGF-β policlonal antibodies for 30 minutes. After that, BSA- and decorin-pre-coated plates were stimulated or not with subsaturating amounts of IFN-γ (A) or LPS (B) for 6 hours and the expression of iNOS was analyzed by western blotting using mouse monoclonal anti-iNOS antibodies. A peroxidase-conjugated anti-mouse IgG was used as secondary antibody. The expression of β-actin was used as a control of sample loading and transfer efficiency. Each experiment was performed twice. C) Expression of TGF-β after treatment with subsaturating amounts of IFN-γ (10U/ml) or LPS (1 ng/ml) for 6 hours was analyzed by western blotting as indicated in *Material and methods*.

Discussion

Under different external stimuli cells are able to proliferate, to become activated and carry out their function, to remain quiescent, or to die through apoptosis. Although these events were initially considered as independent, recent reports have shown a cross-talk between the proliferative capacity of the cells and their activation or susceptibility to die through apoptosis. In these studies as cellular model we used bone marrow-derived macrophages, because they are nontransformed cells that respond to a variety of stimuli inducing, either proliferation, activation, differentiation or apoptosis. To carry out their functional activities, macrophages have to become activated. After interacting with IFN-γ, a cytokine released by activated T lymphocytes, the macrophages undergo biochemical and morphological modifications that allow them to perform their

functional activity (Boehm et al., 1997). Although IFN-γ is the major macrophage activator, other molecules such as LPS, the main component of the wall of gram-negative bacteria, can induce some aspects of macrophage activation.

Like other immune cells, macrophages are produced in large excess and only the small amount of cells required to develop a functional activity survive. We have found that IFN-γ not only activates macrophages but also prevents to the induction of apoptosis through the expression of the cdk inhibitor $p21^{Waf\,1}$ and the arrest of the cell cycling at the G_1/S boundary (Xaus et al., 1999b). For some functional activities, like the IFN-γ-induction of MHC II genes, the cell cycle plays a critical role (Xaus et al., 2000a).

In this context we were interested to determine the role of ECM proteins on the macrophage behavior. In a previous study, we observed that decorin, like IFN-γ blocks macrophage proliferation and protects against apoptosis through the induction of $p21^{Waf\,1}$ (Xaus et al., 2001b). In the present study, we have found that decorin enhances both IFN-γ and LPS- mediated activation. This enhancing effect was originally linked to the capacity of decorin to increase cell adhesion, on the basis of the fact that fibronectin, another ECM protein that induces cell adhesion, also inhibits macrophage proliferation. However, we found that decorin enhances the activation mediated by both LPS and IFN-γ, whereas fibronectin does not.

Because the adhesion induced by decorin did not seem to be the mechanism sufficient to increase macrophage activation, we analyzed the repressive effect of decorin on TGF-β. This cytokine is produced by macrophages in an autocrine manner and downregulates activation (Assoian et al., 1987; Grotendorst et al., 1989). TGF-β has been observed to antagonize IFN-γ-driven processes of macrophage activation such as the production of H_2O_2, NO, the up-regulation of iNOS, the release of TNF-α, or the IFN-γ-induced killing of intracellular microorganisms (Bermudez, 1993; Bogdan et al., 1992; Ding et al., 1990; Silva et al., 1991; Tsunawaki et al., 1988; Vodovotz et al., 1993). Also, TGF-β shows an inhibitory effect on IFN-γ-induced MHC class II gene (Czarniecki et al., 1988), and this is mediated by the conserved proximal promoter elements (Reimold et al., 1993). The repressive effect of TGF-β on IFN-γ is based on the crosstalk between

the molecules involved in the signal transduction pathways. The TGF-β/SMAD signaling cascades are inhibited by the IFN-γ/STAT pathways and viceversa (Pitts et al., 2001; Ulloa et al., 1999). In addition, TGF-β also inhibits in macrophages LPS-induced activation. TGF-β inhibits LPS-induced iNOS expression (Werner et al., 2000) or reduces the expression of pro-inflammatory cytokines during septic shock (Imai et al., 2000).

In the present study, we have found that macrophages bound TGF-β with an affinity and with a number of binding sites per cell similar to that observed in other cell types (Massague et al., 1990). We also found that decorin blocks the binding of TGF-β to the macrophages. This could be due to the binding of decorin to TGF-β and by this way to inhibit the interaction with the cell surface receptor. Previously it has been reported that decorin is able to bind *in vitro* a variety of adhesive and nonadhesive proteins including fibronectin, thrombospondin, various types of collagens, C1q as well as TGF-β (Iozzo, 1997; Iozzo and Murdoch, 1996). Therefore, our results indicates that decorin inhibits TGF-β effects in macrophages. Decorin may compete with macrophages for the autocrine TGF-β produced in response to subsaturating amounts of IFN-γ or LPS. This could explain the beneficial effect of decorin on IFN-γ- or LPS-mediated activation. An antibody against TGF-β confirms the effects of decorin.

The data we report here further show that macrophages are under negative regulation through the autocrine production of TGF-β. In fact, studies involving mice models in which TGF-β was inactivated through disruption of the gene, have shown an excessive inflammatory response (Kulkarni et al., 1993), an increased expression of MHC II genes (Geiser et al.,1993) and an increased production of nitric oxide (Vodovotz et al., 1996). In addition, the inflammatory process in TGF-β1 *knockout* mice seems to be closely associated with the development of autoimmunity, as shown by the fact that a massive mononuclear cell infiltration developed in multiple tissues including the lungs, heart, and salivary glands (Dang et al., 1995; Geiser et al.,1993; Kulkarni et al., 1993; Shull et al., 1992; Vodovotz et al., 1996).

All these data suggest that autocrine production of TGF-β plays an important role in the active supression of inflammation in the absence of an adequate proinflammatory stimuli. Therefore, macrophages in the absence of stimuli are in a preactivated stage, which is maintained by inhibitory citokines such as TGF-β. Some genes like MHC class II which expression is induced by IFN-γ showed a basal expression that persist even when important transcription factors as RFX5 and CIITA or molecules involved in signal transcription such as STAT1 that control expression of this genes are disrupted (Chang et al., 1996; Clausen et al., 1998; Meraz et L., 1996). Also we have found that the control of the basal expression seems to be regulated by another mechanism independent of the IFNγ-induced expression (Xaus et al., 2000a).

Our results may have clinical and physiological relevance. Here we presented a mechanism that block the endogenous inhibitor TGF-β. The presence of decorin in the tissues could account for an increased macrophage activation. Macrophages play a critical role during inflammation. In the early steps of the inflammatory process, neutrophils are present at the inflammatory loci but leave after 24 to 48 hours. Macrophages arrive later and remain until the inflammation disappears (Belligan et al., 1996), that is, for as long as stimulated Th1 cells produce IFN-γ. In the late phases of inflammation, macrophages eliminate non-self structures, remove all the debris (including apoptotic bodies), and remodel impaired tissues. However, during chronic inflammations such as rheumatoid arthritis macrophages plays a key role in the pathogenesis (Janossy et al., 1981). In these situations the persistance of macrophages may be related to the presence of molecules that block the process of macrophage deactivation. Several soluble mediators has been described that block macrophage activation such as TGF-β (Bermudez, 1993; Bogdan et al., 1992; Silva et al., 1991; Tsunawaki et al., 1988; Vodovotz et al., 1993), IL10 (Bogdan et al., 1992; O'Farrell et al., 1998), adenosine (Xaus et al., 1999a; 1999b), etc. Recently, it has been shown that macrophages are restrained from tissue-damaging activation by CD200R (a myeloid-specific receptor on the phagocytes) when it engages CD200 a glycoprotein on other cells) (Hoek et al., 2000; Wright et al, 2000). Depending on the balance between activators and inhibitors the macrophages will remain at the inflammatory loci and release enzymes or cytokines that could be deleterious for the articulation (Nathan

and Muller, 2001). In this context decorin or other molecules of the extracellular matrix may contribute to the pathogenesis of chronic inflammations by blocking the inhibitors. In this context, in an animal model of experimental autoimmune encephalomyelitis, systemic administration of antibodies specific for TGF-β identified a role for endogenous TGF-β in supression of the disease (Miller et al., 1992).

El LPS induce apoptosis en los macrófagos principalmente a través de la producción autocrina de TNF-a

El shock endotóxico producido por el LPS se caracteriza por una inflamación sistémica, lesiones en múltiples órganos, colapso circulatorio y muerte. El efecto dañino del LPS durante el shock séptico se ha asociado, principalmente, a la producción de óxido nítrico (NO) y a la secreción de TNF-α. Ambos agentes nocivos son producidos y secretados, principalmente, por los macrófagos tisulares activados por el LPS, por lo que este tipo celular juega un papel clave en el desarrollo del shock endotóxico. En este trabajo hemos estudiado la contribución de ambas vías en las acciones del LPS utilizando como modelo celular los macrófagos derivados de la médula ósea.

La activación de los macrófagos por el LPS induce su muerte celular por procesos de apoptosis. Algunos autores han propuesto que la inducción de apoptosis en los propios macrófagos podría jugar un papel autoregulador, reduciendo así la actividad potencialmente dañina de estas células durante largo tiempo. Ésta se produce de forma rápida, pudiéndose detectar la fragmentación del DNA, un proceso clave en el desarrollo de la apoptosis, ya a las 3 horas de estimulación con LPS, de forma dosis- y tiempo-dependiente. Como hemos mencionado anteriormente, dos mecanismos han sido involucrados en este proceso: la producción de óxido nítrico (NO) y la secreción de TNF-α.

La inducción por el LPS de la expresión de la enzima iNOS y la producción de NO en los macrófagos no se detecta hasta transcurridas 12 horas tras la estimulación con dicho agente bacteriano. Por tanto, la producción de NO es un proceso tardío en la activación de los macrófagos por el LPS. Utilizando un agente químico capaz de producir NO de forma espontánea denominado SNAP hemos demostrado que la producción de NO induce apoptosis en los macrófagos. Sin embargo, nuestros resultados sugieren que la inducción de la iNOS por el LPS no es responsable de los procesos tempranos de la apoptosis inducida por el LPS, puesto que ésta ya es detectable mucho antes (3 horas) que la producción de NO por los macrófagos (12 horas). Además, la inducción temprana de la apoptosis en los macrófagos no se ve modificada significativamente por el tratamiento de los

macrófagos con SMT, un inhibidor de la iNOS, capaz de bloquear totalmente la producción de NO inducida por el LPS. Finalmente, la utilización de ratones *knock-out* (KO) para la iNOS ha demostrado que esta vía no juega ningún papel en la apoptosis temprana inducida por el LPS en los macrófagos derivados de médula ósea.

Por otro lado, el tratamiento con LPS induce la expresión y secreción de TNF-α por los macrófagos. La producción de TNF-α inducida por el LPS es muy rápida (90 minutos). El tratamiento de los macrófagos con TNF-α recombinante induce apoptosis en unos rangos de concentraciones que se corresponden a los niveles secretados durante la estimulación de los macrófagos con LPS. Para estudiar la contribución de la producción autocrina de TNF-α en la inducción de apoptosis por el LPS, se utilizaron macrófagos procedentes de ratones KO del receptor de TNF-α. En estos macrófagos, aunque el LPS sigue siendo capaz de inducir apoptosis, lo hace con una cinética totalmente distinta, no pudiéndose detectar inducción de apoptosis hasta transcurridas 12 horas de estimulación con LPS. Además, la apoptosis tardía inducida en este modelo se bloquea totalmente por el inhibidor SMT, sugiriendo la implicación de la producción de NO por la iNOS.

Así, este trabajo ha permitido demostrar que el LPS induce apoptosis en los macrófagos por dos vías independientes aunque con cinéticas distintas. Los procesos apoptóticos tempranos (3-6 horas) inducidos por el LPS estarían mediados por la producción autocrina de TNF-α. El TNF-α a través de la interacción con los receptores de tipo I (p55) induciría la apoptosis de forma p53-independiente. Por otro lado, en una fase más tardía (12-24 horas) la inducción de la iNOS por el LPS y la producción de NO serían responsables de las lesiones en el DNA que inducirían la expresión de los genes pro-apoptóticos p53 y Bax y la consiguiente inducción de apoptosis.

LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-a

Abstract

The deleterious effects of LPS during endotoxic shock are associated with the secretion of tumor necrosis factor (TNF) -α and the production of nitric oxide (NO), both predominantly released by tissue macrophages. We analyzed the mechanism by which LPS induces apoptosis in bone marrow-derived macrophages (BMDM). LPS-induced apoptosis already reached a plateau at about 6 h of stimulation, whereas the production of NO by the inducible NO-synthase (iNOS) required between 12 to 24 h. Furthermore, LPS-induced early apoptosis was only moderately reduced in the presence of an inhibitor of iNOS or when using macrophages from iNOS -/- mice. In contrast, early apoptosis was paralleled by the rapid secretion of TNF-α and was almost absent in macrophages from mice deficient for one (p55) or both (p55 and p75) TNF-receptors. During the late phase of apoptosis (12-24 h) NO significantly contributed to the death of macrophages even in the absence of TNF-receptor signalling. NO-mediated cell death, but not apoptosis induced by TNF-α, correlated with the induction of p53 and Bax genes. Thus, LPS-induced apoptosis results from two independent mechanisms: first and predominantly, through the autocrine secretion of TNF-α (early apoptotic events), and second, through the production of nitric oxide (late phase of apoptosis).

Introduction

Mononuclear phagocytes represent a large family of cell types that includes tissue macrophages, Kupffer cells in the liver, Langerhans cells in the epidermis, osteoclasts in the bone, microglia in the brain and perhaps some of the interdigitating and follicular dendritic cells found in lymphoid organs (Orgawa, 1993; Valledor et al., 1998). Macrophages exert key functions during the immune response. To perform most of these functions macrophages must be activated (Celada and Nathan, 1994; Schreiber et al., 1985). Thus, macrophages are able to kill bacteria, virus or parasites directly; to secrete several immune regulators (TNF-

α, IL-1β, IL-6, etc); to process antigens and present them to T cells; and finally, to act as scavenger cells and to participate in tissue remodelling.

However, macrophages do not always play a positive role in the homeostasis of the immune system. Under some circumstances, macrophages have deleterious effects. This is the case of the septic shock, which is a severe systemic inflammatory response triggered by the interaction of LPS with macrophages and other host cells (Bone, 1991; Morrison and Ryan, 1987). Although this interaction leads to the progressive release of a variety of proinflammatory cytokines such as IL-8, IL-1β, and IL-6 (Salkowski et al., 1995), experimental evidence points to nitric oxide (NO) and tumor necrosis factor (TNF)- α as the primary mediators of the changes observed during septic shock (Beutler and Cerami, 1988; Parrillo, 1993; Petros et al., 1991). Central to the pathogenesis of the endotoxic shock is the development of circulatory failure, characterized by hypotension, myocardial dysfunction and tissue hypoxia that ultimately leads to multiorgan failure and death (Parker et al., 1987; Parrillo, 1993). Despite major advances in antimicrobial therapy and critical care, septic shock continues to have a mortality rate of 40-70% and remains the leading cause of more than 100.000 deaths per year in the intensive care units of the United States alone (Natanson et al., 1994; Parrillo, 1993).

Although several reports suggest that excessive production of NO by the inducible NO synthase (iNOS) contributes to the circulatory failure during septic shock (Finkel et al., 1992; Hom et al., 1995; Thiemermann, 1994), the role of this enzyme in septic shock remains controversial. The use of iNOS -/- mice revealed the existence of both an iNOS-dependent and -independent pathway for LPS-induced hypotension (Laubach et al., 1995; MacMicking et al., 1995; Wei et al., 1995). Deletion of the iNOS gene or blocking of the activity of iNOS resulted in either no protection (Evans et al., 1994; Laubach et al., 1995), partial (MacMicking et al., 1995; Wei et al., 1995) or total protection (Kilburn et al., 1990; Szabo et al., 1994), or even led to detrimental effects (Billiar et al., 1990; Minnard et al., 1994) during sepsis.

Tumor necrosis factor (TNF) -α affects the growth, differentiation and function of many of cell types and it is a major mediator of inflammatory immune responses (Beutler and Cerami, 1988; 1989; Vasalli, 1992). TNF-α has also been suggested as a key mediator of the septic shock syndrome induced by either LPS or bacterial superantigens (Beutler et al., 1985; Tracey et al., 1986; 1987). The potent regulatory abilities of TNF-α are transduced by two distinct cell surface receptors with 55 kd (Type I) and 75 kd (Type II) relative molecular weight (Goodwin et al., 1991; Lewis et al., 1991).

Most of the known cellular TNF-α responses have been attributed to the activation of p55 type I TNF-αR (Tartaglia et al., 1991; Thoma et al., 1990). In contrast, little is known about the function of p75 type II TNF-αR (Heller et al., 1990; Tartaglia and Goeddel, 1992). Activation of the type I TNF-αR is necessary and sufficient for TNF-α-induced liver failure and hepatocyte apoptosis (Leist et al., 1995) as well as for cytotoxicity and apoptosis in other cell types (Greenblatt et al., 1992; Tartaglia et al., 1993a; 1993b). Although p55 TNF-αR -/- mice seem to be resistant to endotoxic shock, they yet succumb to bacterial infections (Pfeffer et al., 1993).

Thus, LPS-dependent activation of macrophages, exposure to endogenous or exogenous NO, or treatment with TNF-α are enough to induce apoptosis in several cell types (Albina et al., 1993; Murray et al., 1997; Sarih et al., 1993). Apoptosis has been involved in the ultimate multiorgan failure during septic shock. For this reason, we have analyzed the mechanisms involved in the LPS-induced apoptosis of macrophages, which play a crucial role in the pathogenesis of endotoxic shock.

In this report we provide evidence that macrophage apoptosis induced by LPS is mediated by both NO and TNF-α production. However, each of these agents acts separately. TNF-α induces the early apoptotic events (3-6 hours), whereas iNOS-dependent apoptotic events occur later (12-24 hours). NO-induced apoptosis, but not TNF-α-dependent apoptosis, correlates with the induction of p53 and Bax.

Material and methods

Reagents

Lipopolysaccharide (LPS) was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant murine TNF-α was purchased from PrepoTech EC Ltd. (London, UK). Recombinant murine IFN-γ was kindly provided by Genentech Inc. (South San Francisco, CA). 4, 6-diamidino-2-phenylindole (DAPI), (±)-S-Nitroso-N-acetylpenicillamine (SNAP) and S-Methylisothiourea Sulfate (SMT) were all purchased from Calbiochem (La Jolla, CA). All other chemical were of the highest available purity grade and were purchased from Sigma Chemical Co. Deionized water further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Antibodies

For Western blot analysis we used a rabbit antibody against mouse iNOS (M-19; Santa Cruz Biotechnology, Santa Cruz, CA), a sheep anti-mouse p53-PAN antibody (Boehringer Mannheim, Mannheim, Germany) and, as a control, a mouse anti-mouse β-actin antibody (Sigma Chemical Co). Peroxidase-conjugated anti-rabbit IgG (Cappel, Turnhout, Belgium), anti-goat/sheep IgG (Boehringer) or anti-mouse IgG (Cappel), were used as secondary antibodies.

Plasmids and constructions

The plasmid corresponding to the rat iNOS full-length cDNA was kindly provided by Dr. Antonio Felipe (University of Barcelona, Spain). Murine cDNA probes for TNF-α and Bax were kindly provided by Dr. M. Nabholz (ISREC, Epalinges, Switzeland) and Dr. Ramon Merino (University of Cantabria, Spain), respectively. As a control for RNA loading and transfer, we used a 18S rRNA transcript (Torczynski et al., 1983).

Cell culture

Bone marrow-derived macrophages (BMDM) were isolated as previously described (Celada et al., 1984). Six-week-old BALB/C mice (Charles River Laboratories, Inc., Wilmington, MA) were killed by cervical dislocation, and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the marrow tissue flushing by irrigation with media. The marrow plugs were dispersed by passing through a 25-gauge needle, and the cells were suspended by vigorous pipetting and washed by centrifugation. The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 30% L-cell conditioned media as source of M-CSF. Macrophages were obtained as a homogeneous population of adherent cells after 7 days of culture. The cells were incubated at 37º C in a humidified 5% CO_2 atmosphere.

Bone marrow-derived macrophages from iNOS or TNF-αR *knock-out* mice and the corresponding controls were isolated under the same conditions. TNFαRI *knock-out* mice (Zhang et al., 1995) and TNF-αRI/II double *knock-out* mice (Bruce et al., 1996) were kindly donated by Dr. K. Matsushima from Kanazawa University, Japan; and Dr. J. Peschon from University of Kentucky, USA, respectively. The iNOS KO mice were kindly donated by Dr. S. Mudgett (MacMicking et al., 1995) and obtained as previously described (Diefenbach et al., 1998) .

Analysis of DNA content with DAPI

10^6 macrophages previously subjected or not to LPS treatment were resuspended and fixed in ice-cold 70% ethanol. The cells were then washed in PBS, resuspended in 0.2 ml of a solution containing 150 mM NaCl, 80 mM HCl and 0.1% Triton X-100, and incubated at 0-4º C for 10 minutes. Afterwards, 1 ml of a solution containing 180 mM Na_2HPO_4, 90 mM citric acid and 2 μg/ml DAPI, pH 7.4, was added to each sample. After incubating the cells at 4º C for 24 hours, their fluorescence was measured with an Epics Elite flow cytometer (Coulter corporation, Hialeah, FL). For this analysis we used an UV laser with an excitation beam of 25 mW at 333-364 nm and fluorescence was collected with a 525 nm

band-pass filter. Cell doublets were gated out by comparing the pulse area versus the pulse width. 12000 cells were counted for each histogram, and cell cycle distributions were analyzed with the Multicycle program (Phoenix Flow Systems, Inc.; San Diego, CA).

In parallel experiments, cells stained with DAPI were mounted on a slide and visualized in a Zeiss fluorescent microscope. Pictures were taken using a Kodak camera installed to the microscope. Under these conditions, condensed DAPI-stained chromatin was visualized in the nucleus of the apoptotic cells.

Analysis of apoptosis

DNA fragmentation due to internucleosomal cleavage was determined as described previously (Hoggquist et al., 1991). Briefly, $3x10^6$ macrophages were harvested and washed in ice-cold PBS. The cells were lysed in 0.5 ml of lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.0) for 16 hours at 4º C, and the lysates were centrifuged (15000xg) to separate high molecular weight DNA (pellet) from cleaved low molecular weight DNA (supernatant). The DNA supernatants were phenol-extracted twice and precipitated. The pellets were resuspended in Tris-EDTA buffer containing 250 μg/ml RNAse (Boehringer Mannheim). The samples were heated at 65º C for 10 minutes and subjected to electrophoresis in a 2% agarose gel containing ethidium bromide.

Low molecular weight apoptotic DNA, obtained as indicated above, was also measured by an ELISA technique (Cell Death Detection ELISA Plus, Boehringer Mannheim) which is directed against cytoplasmic histone-associated DNA fragments. Each point was performed in triplicate and the results were expressed as the mean ± SD.

Determination of NO production

NO production was estimated by measuring nitrate/nitrite in the cell culture media. Macrophages were cultured in DMEM without phenol-red (GIBCO, Life Technologies, UK) to avoid interference with the Griess absorbance at 550 nm.

Samples were stored at -80º C until assayed. Nitrate was converted to nitrite with *Zea mays* nitrate reductase (Calbiochem). Reduced samples were incubated with an equal volume of Griess reagent, and the absorbance at 550 nm was measured. The total nitrate/nitrite concentration was determined by comparison with a standard curve.

Protein extraction and Western blot analysis

Cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 150 mM NaCl, protease inhibitors). The protein concentration of the samples was determined with the Bio-Rad protein assay. The proteins from the cell lysates (100 μg) were boiled at 95º C in Laemmli SDS loading buffer, separated on 7.5% SDS-PAGE for the detection of iNOS or on 10% SDS-PAGE for p53 immunoblotting. Then, the proteins were electro-transferred to nitrocellulose membranes (Hybond-ECL, Amersham Corp., Arlington Heigths, IL). The membranes were blocked for at least 1 hour at room temperature in Tris buffered saline-0.1% Tween-20 (TBS-T) containing 5% non-fat dry milk and then incubated with TBS-T containing the primary antibody. For iNOS, p53 and β-actin immunoblotting, incubation was performed for 1 hour at room temperature. After three washes of 15 minutes each in TBS-T, the membranes were incubated with peroxidase-conjugated anti-goat/sheep (Boehringer), anti-rabbit or anti-mouse IgG (Cappel) antibodies for 1 hour. After three washes of 15 minutes with TBS-T, ECL detection was performed (Amersham) and the membranes were exposed to X-ray films (Amersham). Quantitation of the blots were carried out by densitometric analysis.

Northern blot analysis

Total cellular RNA (20 μg), extracted with the acidic guanidinium thiocyanate-phenol-chloroform method (Chomcynski and Sacchi, 1987), were separated in 1% agarose with 5 mM MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0/1 M formaldehyde buffer. The RNA was transferred overnight to a GeneScreen nitrocellulose membrane (Life Science Products, Boston, MA) and fixed by UV irradiation (150 mJ). All probes were labeled with ^{32}Pα-dCTP (Amersham) with the

oligolabeling kit method (Pharmacia Biotech, Uppsala, Sweden). To check for differences in RNA loading, the expression of the 18 S rRNA transcript was analyzed. After incubating the membranes for 18 hours at 65º C in hybridization solution (20% formamide, 5X Denhart's, 5X SSC, 10 mM EDTA, 1% SDS, 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 0.2 mg/ml salmon sperm DNA and 10^6 cpm/ml of ^{32}P-labeled probe), they were exposed to Kodak X-AR films (Kodak Company, Rochester, NY). The bands of interest were quantified with a Molecular Analyst system (Bio-Rad Labs., Richmond, CA).

Determination of TNF-α production.

The secretion of TNF-α was measured using a commercial murine TNF-α ELISA kit (Quantikine™ M, R&D Systems, Minneapolis, MN). 10^5 cells were cultured in 24-well plates and stimulated with LPS. Supernatant samples were obtained at the indicated times and subjected to ELISA analysis.

Results

Bone marrow macrophages growing in the presence of M-CSF are unevenly distributed into the different phases of the cell cycle. Upon the activation with LPS, macrophages arrest at the G_0/G_1 phase of the cell cycle and die through the induction of apoptosis.

This conclusion is supported by several observations: i) the staining of the DNA with DAPI revealed that after 6 hours of LPS treatment, 35% of the cells had a sub-diploid DNA content corresponding to that of apoptotic cells, in contrast with 3% of subdiploid cells in non-treated cell cultures (Fig. 1A); ii) macrophages treated with LPS and stained with DAPI showed condensed chromatin in the nucleus (Fig. 1B); iii) electrophoresis on an agarose gel of the DNA obtained from macrophages treated with LPS showed the typical laddering observed after internucleosomal fragmentation of apoptotic DNA (Fig. 1C).

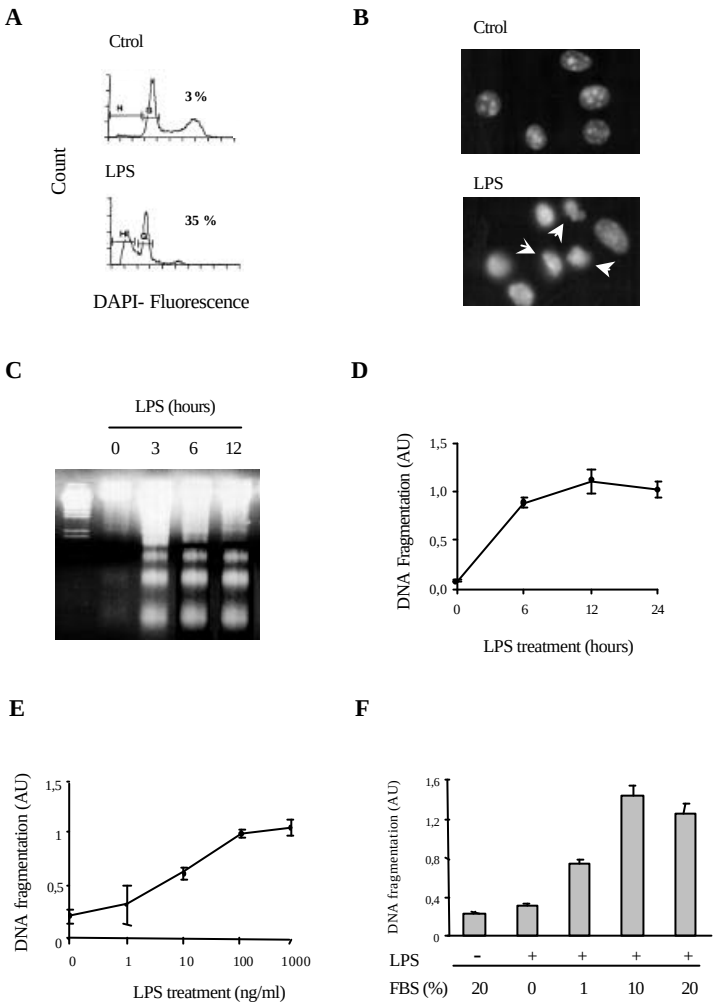


Figure 1: LPS induces apoptosis in bone marrow macrophages. A and B) 10^6 macrophages were stimulated with 100 ng/ml of LPS for 6 hours. DNA was stained with DAPI, and induction of apoptosis was analyzed by cytometric analysis (A) or visualizing the cells in a fluorescence microscope (B). Apoptotic cells are marked with arrows. C and D) LPS induces apoptosis in a time-dependent manner. Apoptotic DNA from macrophages treated with LPS (100 ng/ml) for the indicated times was analyzed by agarose gel electroforesis (C) or by using an ELISA technique directed against histone-associated DNA fragments (D). E) LPS induces apoptosis in a dose-dependent fashion. 10^5 macrophages were treated for 12 hours with the indicated concentrations of LPS. Apoptotic DNA was measured as in D). F) Apoptosis induced by LPS depends on the presence of FBS. The cells were treated with 100 ng/ml LPS for 12 hours in the presence of the indicated concentrations of FBS. Fragmentation of DNA was measured by ELISA. The ELISA experiments were performed in triplicate and represented as the mean value ± SD. These figures are representative of four independent experiments.

Therefore, all these results demonstrate that the treatment of bone marrow macrophages with LPS induces cell death by apoptosis. Moreover, apoptosis was

quantified using an ELISA kit that measures the presence of histone-associated DNA fragments. The induction of macrophage apoptosis by LPS was time- and dose-dependent (Fig. 1D, E). The kinetics of induction of apoptosis was very fast and maximal induction was observed as soon as 3 hours after the start of LPS treatment. The levels of apoptosis did not further increase thereafter and up to 24 hours of stimulation. Maximal induction of apoptosis was obtained at a concentration of 100 ng/ml of LPS (Fig. 1E) and was serum-dependent (Fig. 1F), which can be explained by the fact that the recognition of LPS by its high affinity affinity receptor CD14 requires the previous association of LPS with the serum protein LBP (LPS-binding protein) (Perera et al., 1997; Schumann et al., 1990).

In several cell types, apoptosis induced by LPS has been linked to the cytotoxic effect of iNOS-derived NO (Albina et al., 1993; Heneka et al., 1998; Lakics and Vogel, 1998). Since LPS induces the expression of iNOS in macrophages (Albina et al., 1993; Sarih et al., 1993), we analyzed whether the induction of apoptosis in bone marrow macropahges was also mediated by the generation of NO. The toxic effect of NO was tested by treating bone marrow macrophages with the NO-donor SNAP (Fehsel et al., 1995) that spontaneously produces NO after being added to the culture (Fig. 2A). SNAP induced apoptosis in macrophages in a time- and dose-dependent fashion as determined by measuring DNA fragmentation, DNA laddering (Fig. 2B, C) or by cytometric analysis of DAPI-stained cells (data not shown). Therefore, as has been observed for other types of macrophages (Albina et al., 1993; Sarih et al., 1993), exogenous NO is toxic for bone marrow macrophages.

LPS induces the expression of iNOS in bone marrow macrophages. However, whereas mRNA expression was maximal after 6 hours of LPS treatment (Fig. 3A), the expression of iNOS protein was a late event not observed until 12 hours of LPS treatment, reaching a maximum level after 24 hours (Fig. 3B). The synthesis of iNOS protein correlated with NO production, which was not detected until 12-24 hours of LPS treatment (Fig. 3C).

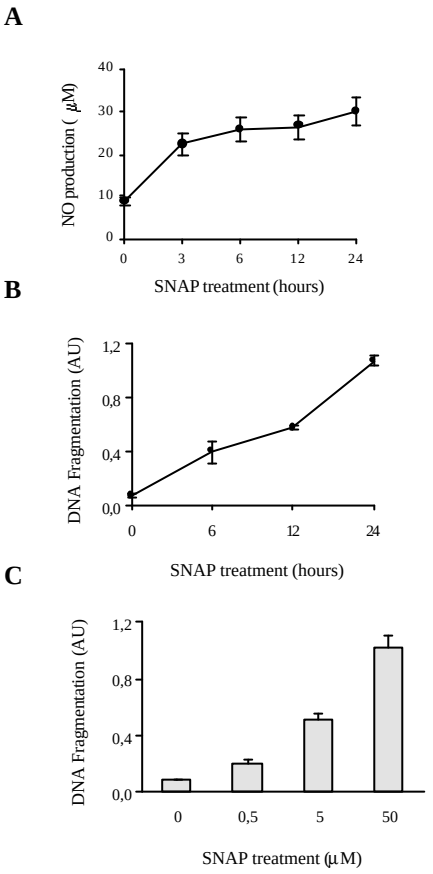


Figure 2: Exogenous NO induces apoptosis in bone marrow macrophages. A) NO production by SNAP. 10^5 macrophages were treated with 50 μM of the NO donor SNAP for the indicated times and the concentration of NO in the supernatant was determined as nitrate/nitrite levels. B) Time-course of SNAP -induced apoptosis. 10^5 macrophages were treated with 50 μM SNAP at the indicated times. Apoptosis was measured by ELISA detection of the histone-associated DNA fragments. C) The cells were treated for 24 hours with the indicated concentrations of SNAP, and apoptosis was detected as indicated above. Each experiment was performed in triplicate and the results of one representative of two independent experiments are represented as the mean value ± SD.

These results suggest that although exogenous NO may induce macrophage apoptosis, the early apoptotic events induced by LPS (3-6 hours) are not related to the production of endogenous NO derived from iNOS. To further determine the role of endogenous NO in LPS-induced apoptosis, we blocked the LPS-induced production of NO by using the iNOS inhibitor SMT (Southam, et al., 1995). SMT did not affect the induction of iNOS mRNA expression by LPS (Fig. 4A). However, SMT totally blocked the NO production induced by LPS (Fig. 4B). The treatment with SMT had a very weak effect on the LPS induction of apoptosis in macrophages (14% inhibition after 6 hours, 23% inhibition after 24 hours of LPS treatment) (Fig. 4C). All this suggests that early macrophage apoptosis induced by LPS is not mediated by the production of endogenous NO.

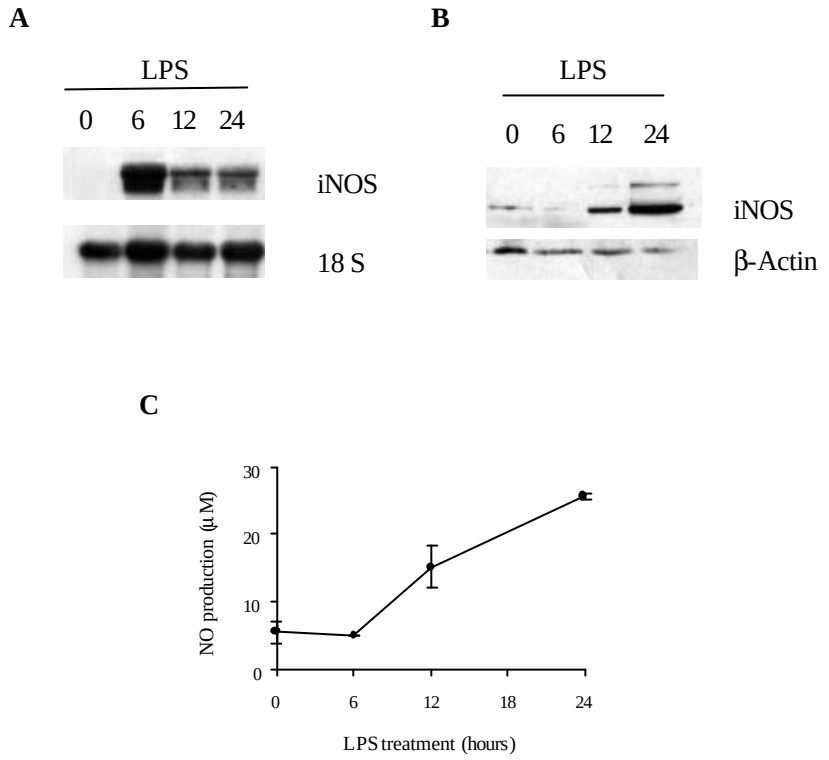


Figure 3: LPS induces the expression of iNOS and the production of NO. The expression of iNOS induced by LPS was measured by Northern blot (A) or Western blot (B). For the northern blot analysis 20μg of total RNA per lane were used. iNOS mRNA expression was analyzed using a ^{32}α-P-labeled rat iNOS cDNA fragment as a probe. The 18S rRNA transcript was used as a control for transfer and RNA loading. Western blots were performed with 100 μg of total protein extracts per lane in a 7.5% SDS-PAGE. A specific 130 kDa band corresponding to murine iNOS protein was detected. The expression of β-actin was used as control. C) LPS induces the production of NO. 10^6 BMDM cultured in media without phenol-red were stimulated with 100 ng/ml LPS and the supernatants harvested at the indicated times. NO production was measured as the nitrite/nitrate levels in the media as described in Material and Methods. Each point was performed in triplicates and represented as the mean ± SD. These figures are representative of three independent experiments.

Since tissue macrophages are major producers of TNF-α (Marsh and Wewers, 1996), we analyzed the role of this cytokine in the LPS-induced apoptosis in macrophages. At 30 minutes after LPS stimulation, bone marrow macrophages already expressed high levels of TNF-α mRNA (Fig. 5A). The protein levels of TNF-α increased very rapidly in the culture supernatants and reached a concentration of 2490 pg/ml after 2 hours. These levels of TNF-α are sufficient to induce apoptosis in BMDM, since doses between 1 and 10 ng/ml of recombinant murine TNF-

α (rmTNF-α) induced significant levels of apoptosis in these cells (Fig. 5C). Moreover, the kinetics of induction of apoptosis by rmTNF-α is very similar to that triggered by LPS (Fig. 5D), with a significant induction of apoptosis within the first 6 hours. Finally, the presence of the iNOS inhibitor SMT did not inhibit the apoptosis induced by TNF-α (data not shown), demonstrating that in bone marrow macrophages the apoptosis induced by TNF-α is not mediated through the production of NO.

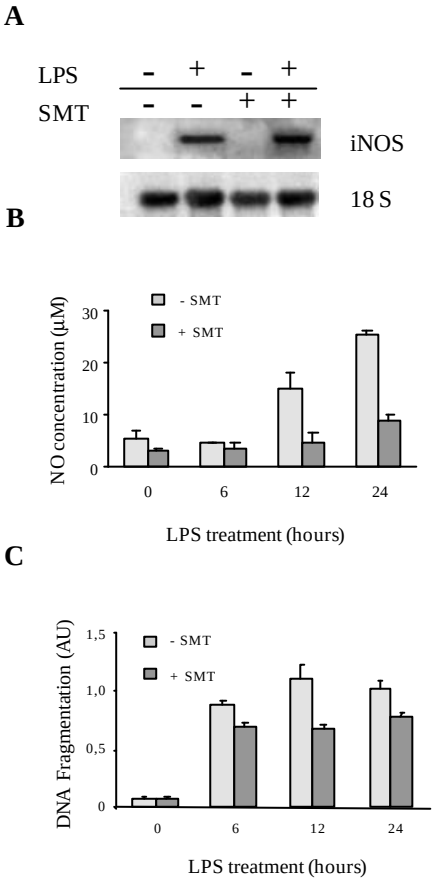


Figure 4: Treatment of macrophages with SMT inhibits LPS-induced NO production but not apoptosis. A) The expression of iNOS was measured by Northern blot in macrophages treated with 100 ng/ml of LPS in the presence or absence of SMT, an iNOS inhibitor (20 μM). B) SMT inhibits LPS-induced NO production. 10^6 macrophages treated with 100 ng/ml of LPS for the indicated times in the presence or absence of the iNOS inhibitor SMT (20 μM). The production of NO was assessed by determination of the nitrate/nitrite levels. Absorbance was measured at 550 nm. C) SMT did not inhibit LPS-induced apoptosis. The cells were treated with 100 ng/ml of LPS for the indicated times in the presence or absence of SMT (20 μM). Each experiment was performed in triplicate and the results of one representative of two independent experiments are represented as the mean value ± SD.

So far we have shown that LPS induces TNF-α production and that TNF-α induces apoptosis in macrophages. LPS-induced secretion of TNF-α and apoptosis occurred almost simultaneously. Therefore, we wanted to determine the role of the autocrine production of TNF-α in the LPS-induced apoptosis of bone marrow-derived macrophages. For these experiments, we used macrophages from mice with both TNF-α receptors disrupted by genetic recombination (TNF-αR KO)

(Bruce et al., 1996; Zhang et al., 1995). The data presented were obtained from TNF-αRI/II double *knock-out* mice. Although not shown, most experiments were repeated with mice with the single type I TNF-α receptor disrupted, from which identical results were obtained.

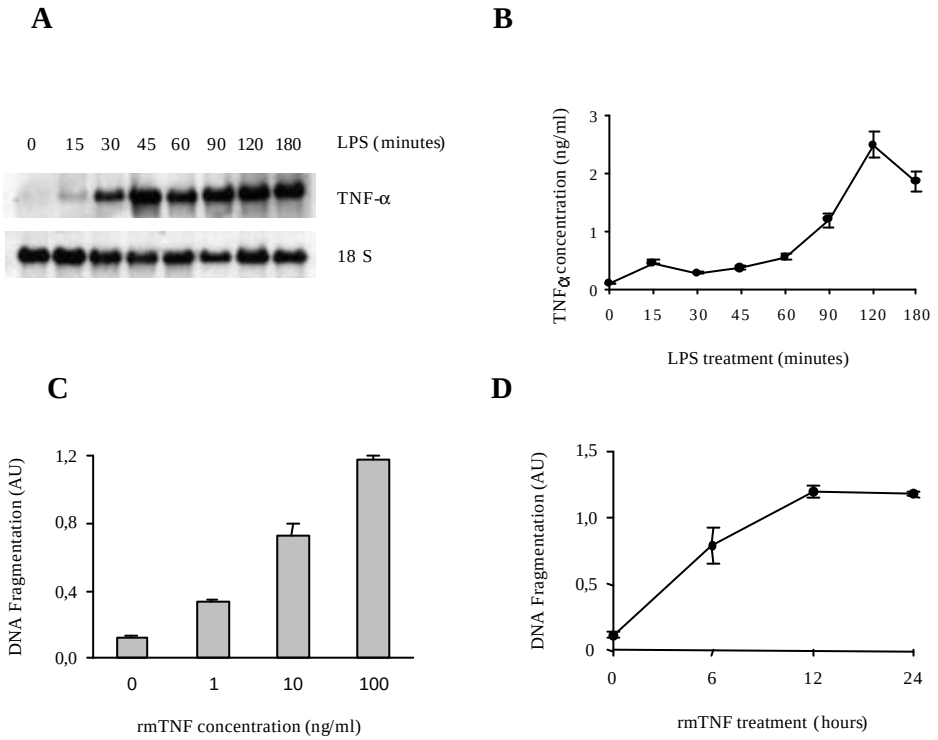


Figure 5: LPS induces the autocrine secretion of TNF-α which produces apoptosis. A) LPS induces the mRNA expression of TNF-α. Total RNA (20 μg per lane) from macrophages treated with 100 ng/ml of LPS for the indicated times was analyzed by Northern blotting with a probe corresponding to a fragment of the murine TNF-α cDNA. 18S rRNA transcript was used as a control of RNA loading and transfer. B) LPS induces the secretion of TNF-α. The concentration of TNF-α in the culture supernatantswas analyzed by ELISA. Each experiment was performed three times and represented as the mean value ± SD. C) TNF-α induces apoptosis in bone marrow macrophages. Macrophages were stimulated for 12 hours with the indicated concentrations of rmTNF-α. Induction of apoptosis was measured by ELISA. D) Time course of rmTNF-α-induced apoptosis. Macrophages were treated with rmTNF-α (100 ng/ml) for the indicated periods of time. Each experiment was performed in triplicate and represented as the mean ± SD, and one of three independent experiments is showed in this figure.

Macrophages from the TNF-αR KO mice did not undergo apoptosis upon exposure to TNF-α (Fig. 6A). Unlike exogenous TNF-α, LPS induced significant apoptosis in these macrophages (Fig. 6A). After 24 hours of LPS treatment,

induction of apoptosis in the TNF-αR KO macrophages was only 36% lower than that observed in the control mice. However, the time-course of apoptosis induction was very different. LPS induced apoptosis within the first 6 hours in macrophages from normal mice, while induction of apoptosis in the TNF-αR KO mice only started at 12 hours and increased up to 24 hours. These results suggest that in wild type macrophages treated with LPS, the autocrine production of TNF-α is the major mediator of early apoptosis. To determine if the apoptosis mediated by the autocrine production of TNF-α is due to the production of NO, we studied the induction of iNOS and the secretion of NO in macrophages from TNF-αR KO mice. In these macrophages, LPS induced a pattern of expression of iNOS mRNA and NO production similar to that in control macrophages (Fig. 6C, D). Furthermore, treatment with SMT also inhibited the production of NO to the same degree in both populations of macrophages (Fig. 6D). These results suggest that in BMDM the autocrine secretion of TNFα is not involved in the control of NO production in response to LPS. Moreover, the treatment with recombinant murine TNF-α did not induce NO secretion in control macrophages (data not shown). The role of NO induction in late apoptosis was also tested by adding the iNOS inhibitor SMT. In the presence of this inhibitor, the LPS-induced apoptosis in macrophages from TNF-αR KO mice decreased 79 % (Fig. 6E). In control macrophages treated with LPS for 24 hours, SMT only resulted in a 23 % reduction of apoptosis. These results were also confirmed by agarose electrophoresis of the apoptotic DNA (Fig. 6E).

Therefore, these results suggest that TNF-α secretion by LPS plays a major role in the induction of early apoptosis, whereas NO production induced by LPS only induces the late apoptotic events. In control macrophages, LPS and TNF-α induced the typical DNA laddering associated with apoptosis, which was not blocked by SMT. In contrast, LPS but not TNF-α induced DNA fragmentation in macrophages from TNF-αR KO mice. In this case, SMT totally blocked the effect of LPS, thus suggesting that the LPS-induced apoptosis of TNFαR KO macrophages was mediated only through NO production.

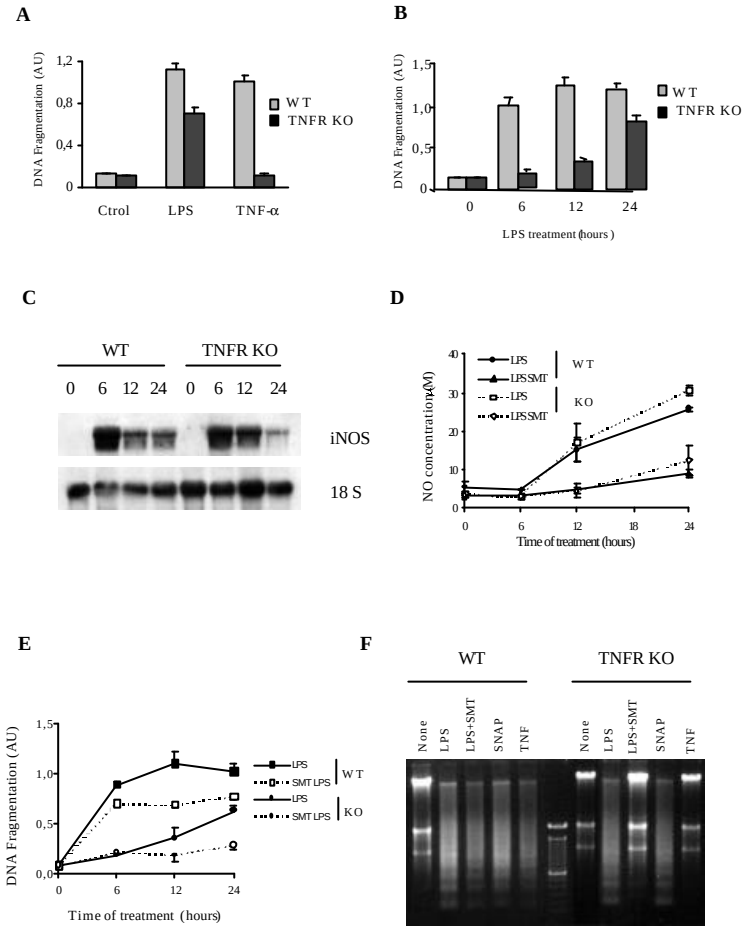
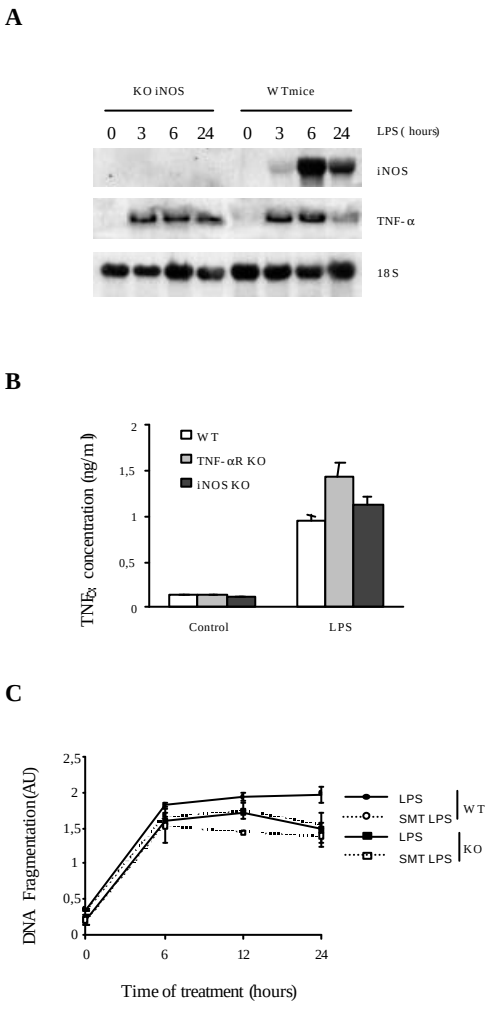


Figure 6: LPS-induced apoptosis in macrophages from TNF-αR *knock-out* mice. A) LPS, but not TNF-α induces apoptosis in macrophages from TNF-αR KO mice. 10^5 macrophages from either wild type or TNF-αR KO mice were treated for 24 hours with LPS (100 ng/ml) or TNF-α (100 ng/ml). DNA fragmentation was evaluated by measuring histone-associated DNA fragments by ELISA. B) Time-course of LPS-induced apoptosis in macrophages from TNF-αR KO mice. Cells from control and KO mice were treated with LPS (100 ng/ml) for the indicated periods of time. Apoptosis was determined as indicated above. C) Macrophages from TNF-αR KO mice express iNOS in response to LPS. Macrophages were treated with 100ng/ml of LPS for the indicated times. 20 μg of total RNA per lane was analyzed by Northern blotting. D) Production of NO in macrophages from TNF-αR KO mice. The production of NO was measured in cultures of macrophages from each group of mice stimulated with 100 ng/ml of LPS for the indicated times in the presence or absence of 20 μM SMT. NO production was determined as the measure of nitrite/nitrate present in the supernatant of the macrophage cultures. E) SMT blocks LPS-induced apoptosis in macrophages from TNF-αR KO mice but not in control macrophages. 10^5 macrophages from control and KO mice were treated with LPS (100 ng/ml) for the indicated periods of time in the presence or absence of SMT (20 μM). Apoptosis was determined by ELISA. Each experiment was performed in triplicate and represented as the mean ± SD. F) LPS-induced apoptosis in macrophages from TNF-αR KO mice is mediated by NO production. DNA fragmentation was analyzed in a 2% agarose gel electrophoresis. Macrophages of each group were treated with 100 ng/ml of LPS in the presence or absence of either 20 μM SMT (iNOS inhibitor), 50 μM SNAP (NO donor) or 100 ng/ml rmTNF-α.

We also analyzed the apoptosis induced in macrophages from iNOS KO mice. After LPS stimulation, these cells expressed similar levels of TNF-α mRNA (Fig. 7A) and secreted similar amounts of this cytokine than macrophages from wild type or TNF-αR KO mice (Fig. 7B). The apoptosis induced by LPS in macrophages from iNOS KO mice was almost identical to that observed in macrophages from control mice and followed similar kinetics (Fig. 7C). Only a slight reduction of apoptosis was observed after 24 hours of LPS treatment (26% reduction), at a time when NO-mediated apoptosis is significant in normal macrophages treated with LPS.



Figutre 7: LPS-induced apoptosis in macrophages from iNOS *knock-out* mice. A) LPS induces TNF-α expression in iNOS KO macrophages. The expression of iNOS and TNF-α mRNA was analyzed by Northern blotting in control and iNOS KO macrophages. B) LPS induces TNF-α expression in iNOS KO macrophages. The secretion of TNF-α was analyzed by ELISA in the supernatant of macrophage cultures from control and iNOS or TNF-αR KO mice. Each experiment was performed three times and represented as the mean value ± SD. C) LPS induced similar rate of apoptosis in macrophages from iNOS KO and in control macrophages. 10^5 macrophages from control and KO mice were treated with LPS (100 ng/ml) for the indicated periods of time in the presence or absence of SMT (20 μM). Apoptosis was determined by ELISA. Each experiment was performed in triplicate and represented as the mean ± SD.

Therefore, the early apoptotic events (those that take place after 6 hours of treatment with LPS) occurred in the absence of p53 and Bax induction. However,

the expression of these pro-apoptotic genes took place at the time at which macrophage apoptosis is dependent on NO-production (24 hours of LPS stimulation). Therefore, we determined whether the expression of these genes could be induced by treatment with a NO donor or TNF-α in macrophages. Treatment with the NO donor SNAP, but not with recombinant TNF-α, induced the expression of p53 and Bax (Fig. 8C, D). These results show that the early (TNF-α-dependent) and late (NO-dependent) mechanisms induced by LPS to produce apoptosis follow two different independent pathways.

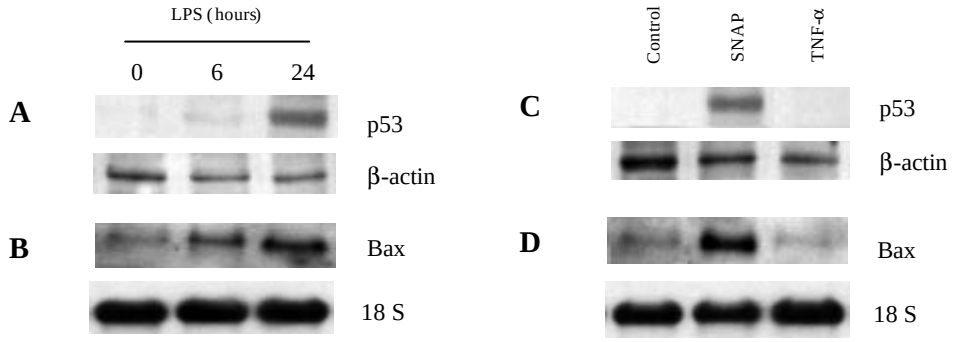


Figure 8: NO-dependent, but not TNF-α-dependent, LPS-induced apoptosis was due to p53 and Bax expression. Macrophages from control mice were treated with LPS (100 ng/ml) for the indicated periods of time. Expression of p53 was analyzed by Western blotting (A), whereas expression of Bax mRNA was analyzed by Northern blotting (B). The expression of p53 (C) and Bax (D) was analyzed in macrophages treated with either 50 μM SNAP or 100 ng/ml rmTNF-α for 24 hours. Northern (20 μg total RNA per lane) and Western (100 μg total protein extracts per lane) blotting were performed as described in Matherial and methods.

Discussion

Endotoxic shock is a potentially lethal complication of systemic infection by gram-negative bacteria (Bone, 1991; Morrison and Ryan, 1987). The toxin responsible for the induction of endotoxic shock is the glycolipid LPS, the major component of the gram-negative bacterial wall. The release of LPS into the circulation activates a series of tissue responses that in their most severe forms lead to septic shock and death. Tissue macrophages play a major role in the generation of the endotoxic response. NO production and TNF-α secretion produced by these cells have been proposed as the primary mediators of this event.

Although an enhanced generation of NO by iNOS has been involved in the pathophysiology of septic shock (Parrillo, 1993; Petros et al., 1991), the inhibition of iNOS in rodent and human models for sepsis and the analysis of iNOS *knock-out* mice has produced conflicting results (Billiar et al., 1990; Evans et al., 1994; Kilburn et al., 1990; Laubach et al., 1995; MacMicking et al., 1995; Minnard et al., 1994; Szabo et al., 1994; Wei et al., 1995). Moreover, the involvement of TNF-α secretion in septic shock came from observations that antibodies or soluble receptors against TNF-α inhibited the deleterious actions of LPS during sepsis (Beutler et al., 1985; Tracey et al., 1986; 1987). However, the use of TNF-αR *knock-out* mice as an experimental model also gave contradictory results. Although mice deficient for the p55 kDa TNF-αR seemed to be resistant to endotoxic shock, they still succumbed to bacterial infection (Pfeffer et al., 1993).

We have analyzed the role of both NO and TNF-α secretion in the LPS-induced apoptosis in bone marrow-derived macrophages. Our results demonstrate that LPS induces apoptosis in macrophages by two independent mechanisms: One is mediated by the autocrine production of TNF-α and the other is triggered by the production of NO. Although both mechanisms are involved in the apoptosis induced by LPS, they act independently, with different kinetics and through separate pathways (Fig. 8).

The cytotoxic effects of TNF-α in most cell types are only evident when RNA or protein synthesis are inhibited, suggesting that *de novo* RNA or protein synthesis protects cells from TNF-α cytotoxicity, probably by the induction of protective genes (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wong et al., 1989). In bone marrow macrophages (as is the case in other inflammatory cell types, including neutrophils and granulocytes, or in endotelial cells and oligodendrocytes), TNF-α alone is sufficient to induce DNA fragmentation and cell death by apoptosis (Akassoglou et al., 1998; Ayala et al., 1997; Denecker et al., 1997; Murray et al., 1997).

The TNF-α signal is transduced by two distinct cell surface receptors, TNF-αR I and II (Tartaglia and Goeddel, 1992; Tartaglia et al., 1991). In this work we have reported the experiments performed with macrophages from the TNF-αRI/II double KO mice (Bruce et al., 1996), but experiments using macrophages from the TNF-αRI KO mice (Zhang et al., 1995) produced identical results. Thus, this confirms that Type I p55 TNF-α receptor mediated the TNF-α-induced apoptosis in macrophages (Leist et al., 1995; Tartaglia et al., 1993; Thoma et al., 1990).

TNFα did not induce the expression of iNOS or NO production in macrophages. Macrophages from TNF-αR KO mice showed levels of LPS-induced expression of iNOS and NO production similar to those measured in control macrophages. In fact, recombinant TNF-α did not induce NO production in macrophages from control mice. Moreover, TNF-α-dependent apoptosis was not blocked by the iNOS inhibitor SMT or in macrophages from iNOS KO mice. Therefore, we conclude that the apoptosis induced by the autocrine production of TNF-α is independent of the production of NO. These results are in disagreement with other previous observations where TNF-α has been associated to the production of NO (Nussler and Billiar, 1993; Nussler et al., 1992).

Recent studies have clarified the mechanism by which the 55 kDa TNF-α receptor signals towards the apoptotic response (Boldin et al., 1996; Chinnaiyan et al., 1995; Hsu et al., 1996). This receptor contains a carboxy-terminal death-domain which appears to be required for the transmission of the apoptotic signal. Binding of TNFα to the receptor triggers the formation of a multiprotein complex where cytoplasmic proteins and the receptor interacts through their respective death domain motifs. Upon TNF-α stimulation, the receptor death domain binds to the death domain of a cytoplasmic protein called TRADD (TNF receptor I-associated death domain), which in turn binds to the death domain of another cytoplasmic protein, termed FADD/MORT-1. This protein also contains a death effector domain (DED) motif, which binds to the DED motif of ICE/Ced-3 protease FLICE/MACH-1 (Caspase 8). It has been suggested that activation of Caspase 8 initiates the activation of a cascade of caspases, which is the effector system for the apoptotic destruction of the cell. This model suggests that ligand binding to the TNF-α

receptor activates the final death effector pathway apparently without any second messengers.

Besides, the LPS-induced apoptosis that is mediated by the production of NO occurs slowly and uses a different signalling pathway. In bone marrow macrophages, NO-dependent apoptosis correlated with the expression of p53 and Bax. Probably the DNA alterations induced by increasing levels of NO induced the expression of p53 (Messmer et al., 1996). p53 regulates the transcription of the Bcl-2-related pro-apoptotic gene Bax (Brune et al., 1997; Kitada et al., 1996). The expression of Bax is sufficient to produce the release of cytochrome C from the mitochondria (Jurgensmeier et al., 1998) and the activation of the mitochondrial apoptotic pathway (Green and Reed, 1998; Reed, 1997), leading to apoptosis.

In summary, we have shown that LPS-induced apoptosis is mediated mostly through the autocrine production of TNF-α. However, when this pathway is inhibited, the apoptosis induced by LPS occurs through the induction of NO. The existence of two independent pathways activated by LPS may explain the inefficiency of several strategies directed against one of these mechanisms so as to prevent the deleterious effects of LPS during the endotoxic shock. This underscores that salvage from ongoing septic shock may require the simultaneous interruption of more than one final pathway, each of them lethal for the host organism.

La PKCε, principalmente a través de la activación de JNK, regula la expresión de TNF-α y, consecuentemente, la apoptosis inducida por el LPS en los macrofagos

El Lipopolisacárido (LPS), componente principal de la pared externa de las bacterias Gram-negativas, es un potente estimulador de los macrófagos. El LPS induce la secreción de citocinas, como el TNF-α, la IL-1β, o la IL-6, que provocan una rápida inducción y amplificación de la respuesta del huésped frente a una infección. Sin embargo, a pesar de su efecto como activador de los macrófagos, el LPS también es un potente inductor de la apoptosis en estas células. Algunos autores sugieren que la inducción de apoptosis en los macrófagos por el LPS podría tratarse de un mecanismo autoregulador de la respuesta inmunitaria. Como hemos demostrado en un trabajo anterior, el LPS ejerce su efecto apoptótico en los macrófagos, principalmente, a través de la secreción autocrina de TNF-α. El objetivo de este estudio era analizar la vía de señalización por la cual el LPS induce la secreción de TNF-α y, en consecuencia, como induce apoptosis en los macrófagos.

El LPS induce de forma rápida la activación de las tres MAP quinasas (ERK, JNK y p38) por lo que cualquiera de ellas podría estar involucrada en la expresión de TNF-α. En este trabajo hemos determinado, mediante la utilización de inhibidores específicos, el papel que desempeña cada una de estas vías en dicha secreción de TNF-α y, por tanto, en la apoptosis inducida por el LPS. Sorprendentemente, las tres MAP quinasas están implicadas en la regulación de la secreción de TNF-α inducida por el LPS, pero a diferentes niveles. Mientras que la actividad de JNK regula la transcripción de TNF-α, puesto que su inhibición afecta tanto a la expresión del mRNA como a la secreción de esta citocina, ERK-1/2 y p38 regulan la producción de TNF-α principalmente a través de mecanismos post-transcripcionales, sin afectar significativamente a los niveles del mensajero inducidos tras la estimulación con LPS.

Tras la estimulación de los macrófagos con LPS, además del papel que ejercen las MAP quinasas, hay otras vías que también son activadas. Así, hemos

observado que la inducción de apoptosis por el LPS y la expresión del mRNA del TNF-α también dependen de la proteína quinasa Cε (PKCε). Además, la inhibición de PKCε bloquea la activación de JNK, pero no la de ERK o p38, por lo que sugiere que la actividad de JNK está mediada por la PKCε en respuesta al LPS y, por tanto, esta vía de señalización juega el principal papel en la regulación de la secreción de TNF-α y en la posterior inducción de apoptosis por el LPS.

Por otro lado, la inhibición de cualquiera de estas vías, ya sea MAPK o PKCε, no afecta a la apoptosis inducida por el tratamiento de los macrófagos con TNF-α recombinante, con lo que se demuestra que estas vías regulan la apoptosis inducida por el LPS en los macrófagos a través de la regulación de la producción de TNF-α y no directamente sobre los mecanismos de señalización hacia la apoptosis desencadenados por el TNF-α, propiamente dicho, en los macrófagos.

La confirmación de nuestro modelo se ha hecho utilizando macrófagos procedentes de ratones *knock-out* de PKCε. La actividad de JNK inducida por el LPS de estos macrófagos se encuentra inhibida, pero no la actividad de ERK o p38. En estos ratones también se observa una disminución de la expresión de TNF-α, y tal y como era de esperar, los macrófagos procedentes de estos ratones son resistentes al tratamiento con LPS.

Por lo tanto, la estimulación de los macrófagos con LPS induce la actividad de PKCε que modula la activación de JNK, por un mecanismo que todavía desconocemos. JNK a su vez es la principal vía que regula los niveles de TNF-α, lo que ocurre a nivel transcripcional, y que son los responsables últimos de la inducción temprana de apoptosis en los macrófagos activados con LPS.

PKCε, mostly through JNK activation, mediates the LPS-induced TNF-α that induces apoptosis in macrophages

Abstract

Lipopolysaccharide (LPS) is a potent stimulator of macrophages and also a powerful apoptosis inducer in these cells. Using primary cultures of bone marrow derived macrophages, we have found that the autocrine production of tumor necrosis factor-α (TNF-α) plays a major role in Lipopolysaccharide (LPS)-induced apoptosis. LPS regulates the different MAKPs, and thus we wanted to determine its involvement either in the secretion of TNF-α or in the induction of apoptosis. Using specific inhibitors we have found that LPS-induced TNF-α dependent apoptosis is mostly mediated by PKCε which is not directly involved in the signaling mechanism of apoptosis but in the process of TNF-α secretion. In our cellular model, all three MAP kinases are involved in the regulation of TNF-α secretion, but at different levels. JNK mainly regulates TNF-α transcription whereas ERK and p38 regulate TNF-α production mostly through post-transcriptional mechanisms. However, only the JNK kinase activity is mediated by PKCε in response to LPS and therefore plays a major role in TNF-α secretion and LPS-induced apoptosis, although any of the 3 kinases can be involved in the mechanism of TNF-α expression and the consequent induction of apoptosis. Finally, using macrophages from PKCε KO mice we demonstrate that PKCε is involved in the regulation of JNK activity, TNF-α expression and the induction of apoptosis by LPS. In conclusion, PKCε and JNK kinase is the major pathway of the TNF-α-dependent apoptosis of macrophages induced by LPS.

Introduction

Lipopolysaccharide (LPS), isolated from the outer membrane of Gram-negative bacteria, is one of the strongest stimulators of macrophages and leads to the secretion of nitrogen intermediates, prostaglandins and cytokines. The secretion of tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and IL-12 leads to rapid induction and amplification of the host response to infection (Bone, 1991; Manthey

and Vogel, 1994; Morrison and Ryan, 1987; Parrillo, 1993). LPS has a clearly inflammatory effect and has also been suggested to be an apoptotic inducer on several cellular types, including macrophages (Akira, 2001; el-Samalouti et al., 2000; Ulevitch, 2000). While the inflammatory response is mediated through several secreted factors, the cytotoxic effects of LPS on macrophages have been ascribed to the production of TNF-α or NO (Albina et al., 1993; Murray et al., 1997; Sarih et al., 1993). Using bone marrow derived macrophages from mice with disrupted genes for the TNF receptors or the inducible NO synthase, we determined that LPS-induced apoptosis results from two independents mechanisms: firstly and primarily through the autocrine secretion of TNF-α (early apoptotic events), and secondly through the production of NO (late apoptosis) (Xaus et al., 2000b).

LPS interacts with LPS-binding protein (LBP), thus allowing binding to CD14 and association with at least one other cell membrane receptor containing an intracellular signaling domain (Diks et al., 2001; Ulevitch and Tobias, 1995). Binding of LPS to these receptors results in the activation of a number of signaling cascades, such as PKC and MAPK kinases. However, the precise mechanism by which LPS triggers apoptosis or the release of cytokines such as TNF-α is unclear (Diks et al., 2001; Ulevitch and Tobias, 1995).

The Protein Kinase C (PKC) family consists of several isoforms that are distributed into three main groups based on their primary structure and activation requirements. In previous works we have described that macrophages derived from bone marrow express only three PKC isoforms: PKCβ_1 (conventional), PKCε (novel) and PKCξ (atypical) (Dekker and Parker, 1994). PKCε is involved in the regulation of important aspects of macrophage biology, in particular proliferation and macrophage activation (Valledor et al., 1999; 2000b). However, LPS also activates the three major MAP Kinase cascades in macrophages, namely the extracellular signal-related kinase (ERK), p38, and c-Jun N-terminal Kinase (JNK) pathways. All three pathways have been linked to activation by LPS and subsequent cytokine gene expression (Geppert et al., 1994; Hambleton et al., 1996; Han et al., 1994; Reimann et al., 1994; Weinstein et al., 1992).

Since TNF-α-dependent apoptosis induced by LPS plays a main role in macrophage biology, we were interested in determining the signaling pathways involved in this process. In this report we provide evidence that TNF-α dependent apoptosis induced by LPS in macrophages is mediated by PKCε. Moreover, although all three MAP kinases are necessary for a correct TNF-α regulation, only the activation of JNK is mediated by PKCε and therefore is mainly responsible for the TNF-α-dependent apoptosis induced by LPS. Both PKCε and MAPK are involved in TNF-α secretion induced by LPS and, thus, in the apoptosis induced by LPS. However, none of them modified the apoptotic events triggered after interaction of TNF-α with its receptor, as we have assessed using recombinant TNF-α.

Material and methods

Reagents

LPS was obtained from Sigma Chemical Co. (St. Louis, MO). In several experiments, the results obtained with commercial LPS was compared to purified LPS kindly donated by Dr. C. Galanos, Max Planck Institute, Freiburg, Germany (Merlin et al., 2001) and no differences were found. Murine recombinant TNF-α was purchased from PrepoTech EC Ltd. (London, UK). Bisindolymaleimyde I (GF109203X), PD98059, SB203580 and Curcumin were obtained from Calbiochem (San Diego, CA). Gö6976 was a kind gift from Dr. A. García de Herreros (Institut Municipal d'Investigació Mèdica, Barcelona, Spain). The phospho-p38 MAP kinase (Thr180/Tyr182) antibody was obtained from Cell Signaling (Beberly, MA). The p38 MAP kinase antibody (sc-535) was purchased from Santa Cruz Biotechnology, INC. (Santa Cruz, CA). All reagents were used following the manufacturer's recommendations.

Cell culture

Bone marrow derived macrophages were isolated as previously described (Celada et al., 1984). Six-week-old BALB/C mice (Charles River Laboratories, Inc., Wilmington, MA) were killed by cervical dislocation and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the marrow tissue

flushed by irrigation with culture media. The marrow plugs were dispersed by passing through a 25-gauge needle and the cells were suspended by vigorous pipetting and washed by centrifugation. The cells were cultured in plastic tissue culture dishes (150 mm) in 40 ml DMEM containing 20% FBS and 30% L-cell conditioned media as source of M-CSF. Once macrophages were 80% confluent (i.e., after 6 days of culture), they were deprived of L-cell conditioned medium for 16-18 hours and treated with LPS (Sigma) in the presence or absence of selective inhibitors. The cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. Macrophages from PKCε KO mice (Castrillo et al., 2001) were obtained in the same way.

Analysis of apoptosis

DNA fragmentation due to internucleosomal cleavage was determined as described elsewhere (Xaus et al., 1999b). Low molecular weight apoptotic DNA was measured by an ELISA technique (Cell Death Detection ELISA Plus, Boehringer Mannheim) which is directed against cytoplasmic histone-associated DNA fragments. Each point was performed in triplicate and the results were expressed as the mean ± SD. In some cases, apoptosis was determined by flow cytometry with DAPI staining or by DNA laddering as previously described (Xaus et al., 1999b; 2000b).

Determination of TNF-α production

TNF-α secretion was measured using a commercial murine TNF-α ELISA kit (Biosource, Camarillo, CA). $5x10^5$ cells were cultured in 24-well plates and stimulated with LPS (10 ng/ml) in the presence or absence of the indicated specific inhibitors. Supernatant samples were obtained 12 hours later and frozen until subjected to ELISA analysis following the manufacturer's protocol.

Protein extraction and Western blot analysis

Western blots were performed as described elsewhere (Valledor et al., 1999). The cells were washed twice in cold PBS and lysed on ice with TGH solution (1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 250 mM NaCl, 1 mM sodium

orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml ioacetamide, 1 mM PMSF). The protein concentration of the samples was determined by Bradford analysis (Bio-Rad Labs, Richmond, CA) and used immediately or stored at –80ºC. For phospho-p38 Western blot analysis, equal amounts of protein (60 μg) were boiled at 95ºC in Laemmli SDS loading buffer and separated on 10% SDS-PAGE. Then, the proteins were electro-transferred to nitrocellulose membranes (Hybond-ECL, Amersham Corp., Arlington Heights, IL). The membranes were blocked for at least 1 hour at room temperature in Tris buffered saline-0.1% Tween-20 (TBS-T) containing 5% (w/v) non-fat dry milk and then incubated with TBS-T containing 5% BSA and the primary antibody (1/1000) overnight at 4ºC. After three washes of 5 minutes each with TBS-T, the membranes were incubated with peroxidase-conjugated anti-rabbit IgG (Cappel, Durham, NC) antibody for 1 hour. After three washes of 5 minutes with TBS-T, ECL detection was performed (Amersham) and the membranes were exposed to X-ray films (Amersham). The bands of interest were quantified by densitometry. The Western blot of p38 MAP kinase was performed as described for the phosphorylated form of p38 MAP kinase with a minor modification: the incubation with primary antibody (1/2000) was performed with TBS-T without 5% BSA.

Northern blot analysis

Total cellular RNA (20 μg), extracted with TRIZOL reagent (Life Technologies, Grand Island, N.Y.) was separated in 1% agarose with 5 mM MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0/1 M formaldehyde buffer. The RNA was transferred overnight to a Hybond-XL nitrocellulose membrane (Amersham) and fixed by UV irradiation (150 mJ) (Valledor et al., 2000). For TNF-α mRNA detection, we used the EcoRI/HindIII fragment of pSP65/TNFa kindly supplied by Dr. M. Nabholz (Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland). To study the expression of IL-1β, we obtained a probe by digesting the construct pGEM/IL-1β provided by Dr. R. Wilson (Glaxo Research and Development Limited, Greenford, U.K.) with EcoRI/PstI. All probes were labeled with ^{32}Pα-dCTP (Amersham) with the oligolabeling kit method (Pharmacia Biotech, Uppsala, Sweden). In order to check for differences in RNA loading, the expression of the 18 S rRNA transcript was analyzed using an 18S probe obtained as previously described (Xaus et al., 2000b). After incubating the membranes for

18 hours at 65ºC in hybridization solution (5x SSC, 5X Denhart's, 1% SDS and 10^6 cpm/ml of ^{32}P-labeled probe), they were exposed to Kodak X-AR films (Kodak Company, Rochester, NY). The bands of interest were quantified with a Molecular Analyst system (Bio-Rad).

Determination of ERK activity by in-gel-kinase assay

ERK activity was analyzed as described (Valledor et al., 1999). Briefly, 50 μg of total protein was separated by 12.5% SDS-PAGE containing 0.1 mg/ml of myelin basic protein (MBP) (Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 hour at room temperature. The gel was then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM β-mercaptoethanol (buffer A) for 1 hour at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hour at room temperature and then renatured by incubating it with five changes of buffer A containing 0.04% Tween-20 for 16 hours at 4ºC. In the phosphorylation assay, the gel was first equilibrated in 40 mM Hepes-NaOH (pH 7.4) containing 2 mM DTT, 0.1 mM EGTA, 15 mM $MgCl_2$, 300 μM sodium orthovanadate for 30 minutes at 25ºC and then incubated for 1 hour in the same solution but also containing 50 μM ATP and 100 μCi γ-^{32}P-ATP (Amersham). The reaction was terminated by washing the gel with 5% TCA containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to X-ray films (Kodak) and quantified with a Bio-Rad Molecular Analyst.

Determination of JNK activity

This assay was performed as described elsewhere (Caelles et al., 1997) with minor modifications. Briefly, the cells were washed with PBS and lysed in cold lysis buffer (1% NP-40, 20 mM Hepes-Na pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodacetamide). 150 μg of total protein were mixed with 75 μl of 20% protein A-sepharose and 1 μl of anti-JNK1 antibody (sc-474, Santa Cruz Biotechnology) in a total volume of 500 μl. The samples were rotated for 2 hours at 4ºC. The immunocomplexes were washed

three times with cold PBS supplemented with 1% NP-40 and 2 mM sodium orthovanadate, once with cold JNK buffer (20 mM Hepes-Na pH 7.5, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 2 mM DTT), and resuspended in JNK reaction buffer (JNK buffer supplemented with 1 μg GST-c-Jun (1-169) (Calbiochem, San Diego, CA) and as a substrate, 20 μM ATP, 1 μCi γ-^{32}P-ATP). The reaction was allowed to proceed for 30 minutes at 30^{o}C and was then stopped by adding 12 μl of 5xLaemli buffer. The samples were incubated for 3 minutes at 100^{o}C and separated by 10% SDS-PAGE. After the electroforesis, the gels were fixed in isopropanol:water:acetic acid (25:65:10), dried and exposed to Kodak X-AR films.

Results

In these studies we used bone marrow derived macrophages, which are a homogeneous population of primary and quiescent cells (Celada et al., 1984). Bone marrow macrophages growing in the presence of M-CSF are unevenly distributed into the different phases of the cell cycle. Upon activation with LPS, macrophages stop at the G_0/G_1 phase of the cell cycle and die through the induction of apoptosis (Xaus et al., 1999b; 2000b). LPS-induced apoptosis was quantified using an ELISA kit that measures the presence of histone-associated DNA fragments (Fig.1). This technique has been used for the quantitative measurement of apoptosis in this cellular model and it shows a good correlation with other methods (Xaus et al., 1999b; 2000b; 2001b). In some experiments, apoptosis was also determined by flow cytometry with DAPI staining or by DNA laddering with qualitatively identical results (data not shown). The induction of macrophage apoptosis by LPS was time- and dose-dependent (Fig. 1). The kinetics of induction of apoptosis was very fast with a maximal induction as soon as 6 hours after the start of LPS treatment. The levels of apoptosis did not increase any further after 6 hours and up to 24 hours of stimulation (Fig.1A).

Maximal induction of apoptosis was obtained at a concentration of 100 ng/ml of LPS (Fig. 1B), a dose that saturates binding of LPS to its high-affinity receptor, CD14/TLR-4 (Ulevitch and Tobias, 1995). It has been reported that other molecules may also transduce signals at very high doses of LPS (1-10 μg/ml) (Perera et al., 1997). In order to avoid signaling through other LPS-receptors

independent of CD14, in this study we used subsaturating amounts of LPS (1-10 ng/ml) that induce apoptosis in macrophages (Fig. 1B). Moreover, since we are interested in the TNF-α-dependent early apoptotic events in response to LPS, we performed all the studies at 6-12 hours after LPS treatment. In order to exclude possible contaminants in the commercial LPS preparation, experiments were repeated using purified LPS (Merlin et al., 2001) and identical results were obtained.

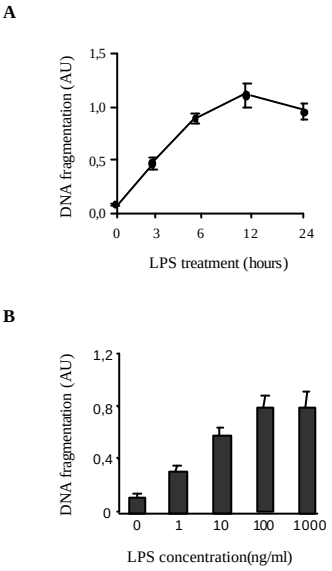


Figure 1: LPS induces apoptosis in bone marrow macrophages. A) LPS induces apoptosis in a time-dependent manner. 10^5 macrophages were stimulated with 100 ng/ml of LPS for the indicated times. Apoptosis was quantified by ELISA detection of histone-associated DNA fragments. B) LPS induces apoptosis in a dose-dependent fashion. 10^5 macrophages were treated for 12 hours with the indicated concentrations of LPS. Apoptosis was measured as in A. The ELISA experiments were performed in triplicate and represented as the mean value ± SD. These figures are representative of four independent experiments.

A high number of LPS-induced processes have been reported to be dependent on PKC activation in macrophages (Fujihara et al., 1994; Liu et al., 1994; Shapira et al., 1994). Hence, we studied the effect of PKC inhibition in macrophages. Our previous studies with specific antibodies had revealed that only PKCβ_1, ε and ξ were present in bone marrow-derived macrophages, whereas the rest of the isozymes were not detected (Valledor et al., 1999). Due to the fact that these cells cannot be transfected efficiently, we needed to use specific chemical inhibitors (Celada et al., 1996). When the cells were preincubated with the PKC inhibitor calphostin C (10-100 nM) before the addition of LPS, we observed a dose-dependent inhibition of the induction of apoptosis in response to LPS (data not shown). The next step was to establish which of the PKC isoforms present in macrophages was involved in LPS-induced apoptosis; in order to do so, we used Bisindolylmaleimyde I (GF109203X) that is a specific inhibitor of conventional PKC (βI) and new PKC (ε). As shown in Fig 2A, GF109203X inhibits LPS-induced apoptosis, thus suggesting that one GF-sensitive PKC isoform is involved in the

regulation of apoptosis by LPS and at the same time discarding the involvement of atypical PKCζ in LPS-induced apoptosis because the doses of GF109203X used in our experiments do not inhibit this isoform (Valledor et al., 1999).

Macrophages were also treated with Gö6976, a selective inhibitor of conventional PKC isoforms, including PKCβ1 (Martiny-Baron et al., 1993). Gö6976 did not modify the levels of apoptosis induced by LPS (Fig. 2B). Taken together, these results suggested that PKC regulates LPS-induced apoptosis and that PKCε is the main PKC isoform involved in this process.

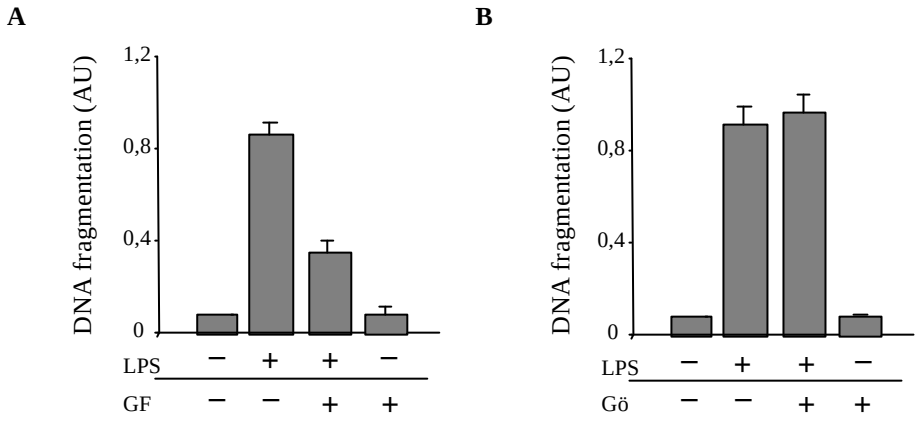


Figure 2: PKCε mediates the apoptosis induced by LPS. A) GF blocks the LPS-induced apopotosis. 10^5 macrophages were preincubated with GF109203X (5μM) for 1 hour and then stimulated with LPS (10 ng/ml) for 12 hours. Apoptosis was quantified by ELISA detection of histone-associated DNA fragments. B) Gö6976 does not protect the apoptosis induced by LPS. 10^5 macrophages were incubated with Gö6976 (2 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for 12 hours; apoptosis was detected as above. Each experiment was performed in triplicate and the results of one representative of two independent experiments are shown as the mean value ± SD.

Because in previous works we had described that TNF-α secretion induced by LPS is mainly responsible for LPS-induced apoptosis (Xaus et al., 2000), we wanted to determine whether the effect of PKCε on LPS-induced apoptosis is mediated through the induction of TNF-α expression or it is a mechanism directly involved with the apoptosis machinery.

Bone marrow macrophages express high levels of TNF-α mRNA after three hours of LPS stimulation (Fig. 3A). LPS also induces the expression of IL-1β mRNA, partly as a consequence of TNF-α secretion. The inhibition of PKCε by

pretreating the cells with GF109203X before LPS stimulation resulted in an almost complete inhibition of the expression of TNF-α and IL1-β mRNA (Fig. 3A).

Stimulation with LPS also resulted in high levels of TNF-α protein secretion in the cell culture supernatant, which arose to concentrations close to 4.5 ng/ml (Fig. 3B). These amounts of TNF-α are clearly sufficient to induce apoptosis in macrophages, because doses between 1 and 10 ng/ml of murine recombinant TNF-α induced significant levels of apoptosis (Xaus et al., 2000b). Again, inhibition of PKCε activity with GF109203X inhibited the secretion of TNF-α (Fig. 3B).

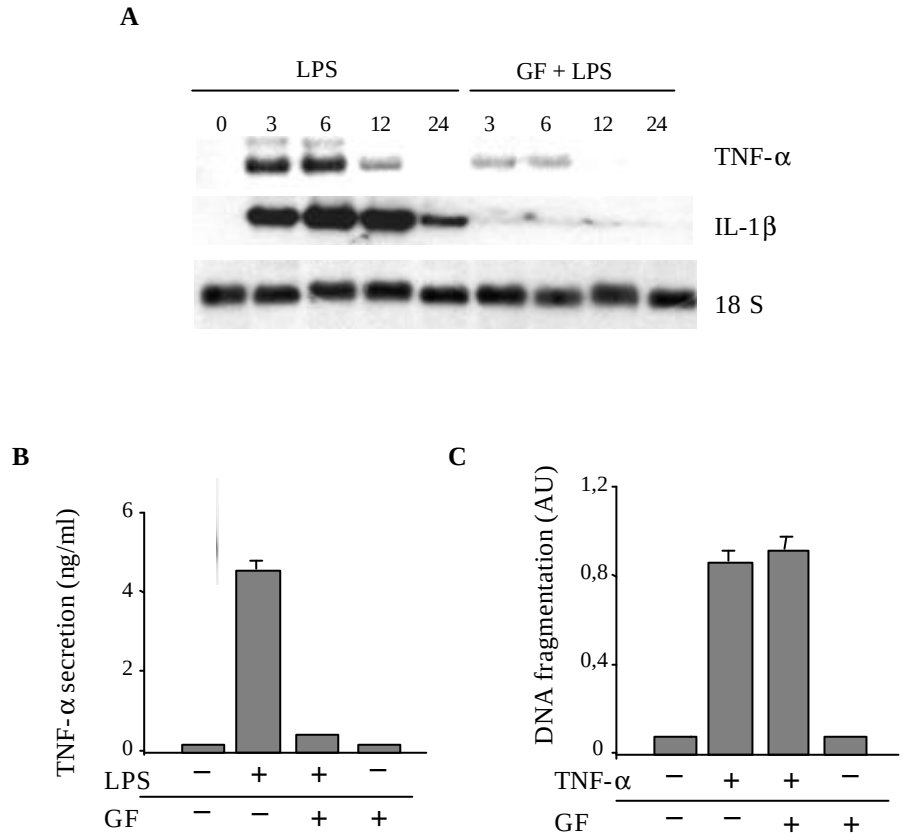


Figure 3: GF inhibits the expression of TNF-a induced by LPS. A) PKCε mediates the expression of cytokines induced by LPS. The cells were incubated with GF109203 (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated times. Expression of TNF-α and IL1-β were analyzed by Northern blotting (20 μg total RNA/lane). The 18S probe was used as a loading and transfer control. This figure is representative of three independent experiments B) Levels of LPS-induced TNF-α protein. Macrophage were incubated with GF109203X (5μM) for 1h and then treated with LPS for 12 hours. The levels of TNF-α protein were determined by ELISA. Each experiment was performed three times and they are represented as the mean value ± SD. C) GF109203X does not inhibits the TNF-α dependent apoptosis. 10^5 macrophages were incubated with GF109203X (5μM) for 1 hour and then stimulated with TNF-α (10 ng/ml) for 12 hours. Apoptosis was quantified by ELISA detection of histone-associated DNA fragments.

Our results suggested that PKCε regulates LPS-induced apoptosis and that there is a correlation with TNF-α production. In order to determine whether LPS induces PKCε-dependent TNF-α secretion or if TNF-α induces the activation of PKCε that is required for apoptosis, we measured the apoptosis induced by recombinant TNF-α in the presence of GF109203X. GF109203X did not affect the apoptosis induced by recombinant TNF-α (Fig. 3C), thus suggesting that PKCε is involved in the apoptosis induced by LPS through the induction of TNF-α production, but not in the TNF-α signaling pathway that induces apoptosis.

LPS also activates the three major MAP kinase cascades in macrophages, namely ERK, p38, and JNK pathways. All three pathways have been linked to activation by LPS and subsequent cytokine gene expression (Guha and Mackman, 2001; Sweet and Hume, 1996). In addition to LPS, the ERK pathway is also activated by growth factors and differentiation signals (Roovers and Assoian, 2000). Activation of the p38 and JNK pathways is often linked to cell stress (Barr and Bogoyevitch, 2001; Hagemann and Black, 2001). Because LPS can activate different signal transduction pathways that result in the activation of MAP kinases, we decided to analyze the involvement of these MAP kinases in apoptosis induced by LPS in macrophages.

Although JNK is activated preferentially by cellular stress signals such as irradiation, heat shock, osmotic stress and protein synthesis inhibitors, the stimulation by growth factors has also been reported (Ichijo, 1999). LPS induced activation of JNK and the preincubation of macrophages with Curcumin (diferulolymethane) inhibited this activation (Fig. 4A). This treatment also inhibited apoptosis induced by LPS (Fig. 4B). Moreover, when we analyzed TNF-α mRNA expression (Fig. 4C) and TNF-α secretion (Fig 4D), we found that the expression of TNF-α decreased when cells were incubated with this JNK inhibitor. This result is similar to what we observed with the PKCε inhibitor and suggests that the JNK pathway can play a role in the expression of TNF-α and the apoptosis induced in response to LPS.

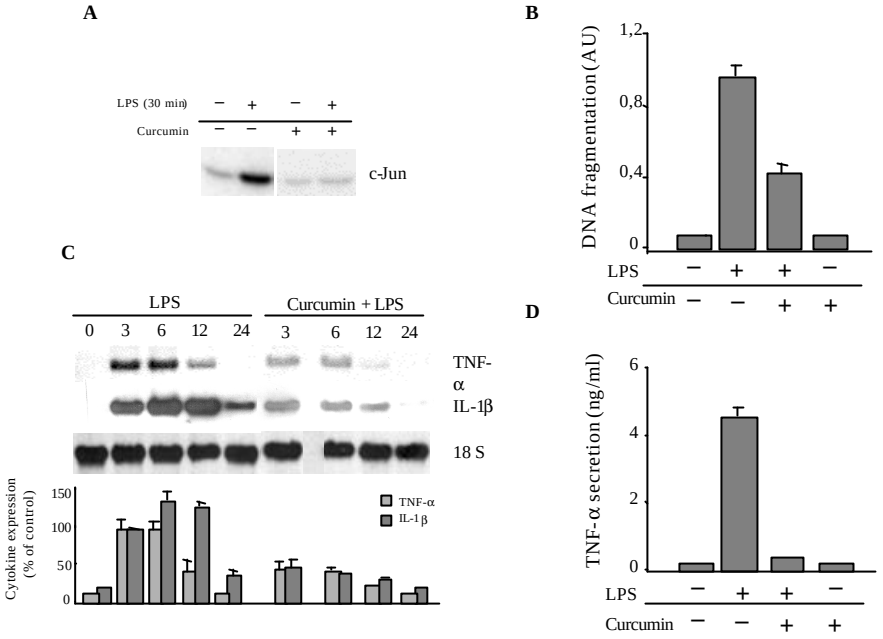


Figure 4: The inhibition of the JNK kinase reduces expression of TNF-α and apoptosis induced by LPS. A) Curcumin inhibits JNK kinase. Quiescent macrophages were incubated with Curcumin (5 μg/ml) for 1 hour and then stimulated with LPS (10 ng/ml) for 30 minutes. JNK activation was measured by the *in vitro* phosphorylation of GST. B) Curcumin inhibits the LPS-induced apoptosis. 10^6 macrophages were incubated with Curcumin (5 μg/ml) for 1 hour and then stimulated with LPS (10 ng/ml) for 12 hours. Each experiment was performed in triplicate and the results of one representative of two independent experiments are shown as the mean value ± SD. C) Curcumin inhibits the expression of TNF-α and IL1-β. The cells were incubated with Curcumin (5 μg/ml) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated periods of time. Cytokine expression was analyzed by Northern blot using 20 μg of total RNA per lane. The quantification of the bands from two representative experiments (mean ± SD) is shown at the bottom of the figure as % of control expression (LPS 3 hours). D) Curcumin inhibits TNF-α secretion. $5x10^5$ macrophages were incubated with Curcumin (5 μg/ml) for 1h and then stimulated with LPS (10 ng/ml) for 12 hours. The concentration of TNF-α in the culture supernatants was analyzed by ELISA. Each experiment was performed three times and represented as the mean value ± SD

In order to determine the role of the other two MAP kinases in TNF-α secretion, specific inhibitors of p38 and ERK-1/2 respectively) were used. At concentrations of 50 μM of PD98059 and 5 μM of SB203580, activation of ERK and p38 , respectively, was blocked in bone marrow macrophages (Fig. 5A). PD98059 slightly decreased the levels of the TNF-α mRNA after LPS treatment, whereas SB203580 did not modify them (Fig. 5B). However, the inhibition of the ERK-1/2 and p38 MAP kinases decreased TNF-α (Fig. 5C). These results showed that ERK-1/2 and p38 MAPK regulate TNF-α expression through post-transcriptional mechanisms, as has been suggested elsewhere (Guha et al., 2001;

Lee and Young, 1996; Rutault et al., 2001). Obviously, the lower TNF-α secretion derived from the response to these inhibitors also decreased LPS-induced apoptosis (Fig. 5D).

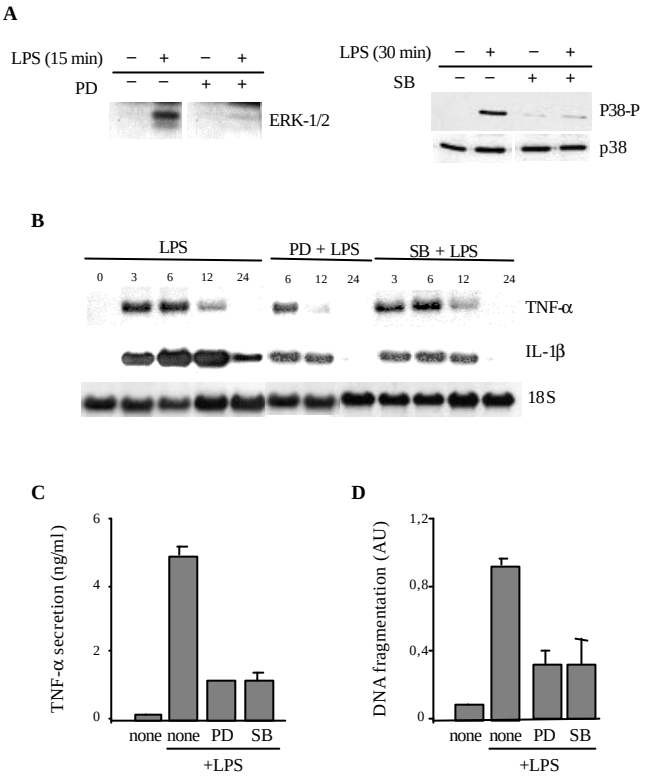


Figure 5: ERK and p38 blockage inhibits the expression of TNF-α and apoptosis induced by LPS. A) PD98059 AND SB203580 inhibits ERK and p38 kinase activity, respectively. Cells were incubated with PD98059 (50 μM) or SB203580 (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for 15 or 30 minutes for ERK kinase assay and for phosphorylated p38 protein detection, respectively. The ERK activity was analyzed by an in-gel-kinase assay. p38 and phosphorylated p38 proteins were detected by Western blot. B) PD98059 and SB203580 reduce LPS-induced apoptosis. 10^5 macrophages were incubated with PD98059 (50 μM) or SB203580 (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for 12 hours. C) PD98059 and SB203580 reduce TNF-α secretion. $5x10^5$ macrophages were cultured and treated as in B. Concentrations of TNF-α in the culture supernatants were analyzed by ELISA. Each experiment was performed three times and represented as the mean value ± SD. D) PD98059 and SB203580 do not modify the expression of cytokines induced by LPS. The cells were cultured and treated as above for the indicated periods of time. Cytokine expression was analyzed by Northern blot using 20 μg of total RNA per lane. One of three independent experiments is shown in this figure.

Taken together, these results suggested an involvement of PKCε and MAP kinases in the apoptosis induced by LPS through the regulation of TNF-α. We also wanted to determine if PKCε is involved in the regulation of MAP kinases, as has already been reported in peritoneal macrophages (Castrillo et al., 2001). Because

of the interest in the differential time-course of ERK kinases in relation to proliferation or differentiation (Valledor et al., 2000b), we performed a selected analysis over time. We observed that JNK activity induced by LPS was clearly inhibited by GF109203X (Fig 6A), but not by Gö6976 (Fig 6B), thus suggesting that PKCε mediates the LPS-induced activation of the JNK pathway. We further extended our analysis to determine the involvement of PKCε in the activation of ERK and p38 MAP kinases pathways by LPS. GF109203X slightly decreased the levels of ERK or p38 activity at short times, but lead to a significant extension of the activity during the response of macrophages to LPS (Figs 6C and D). Again, Gö6976 did not modify the activation of ERK or p38 kinases induced by LPS (data not shown). Thus, PKCε did not inhibit the activation of ERK-1/2 and p38 kinases.

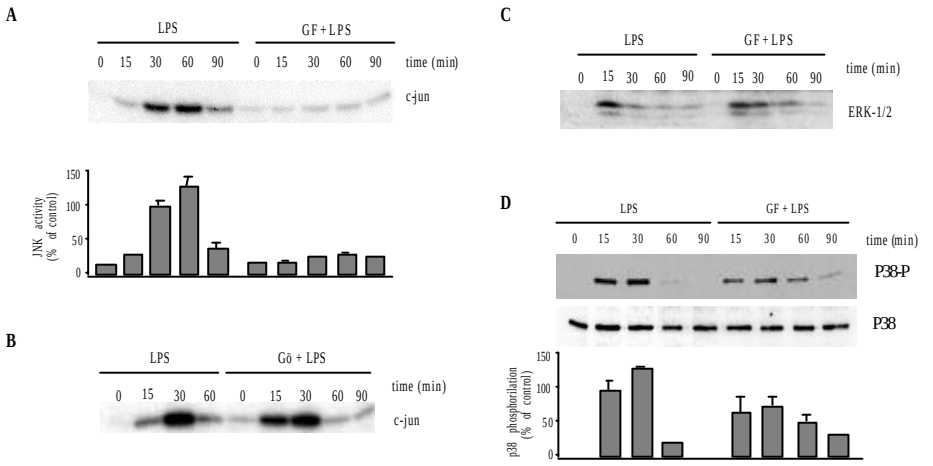


Figure 6: GF109203X inhibits LPS-induced JNK activity and prolongs ERK and p38 Kinase activation. A) GF109203X inhibits LPS-induced JNK activity. Quiescent cells were incubated with GF109203X (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated periods of time. JNK activation was measured by *in vitro* phosphorylation of GST B) Gö6976 do not modify LPS-induced JNK activation. Quiescent cells were incubated with Gö6976 (2 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated periods of time. JNK activation was measured as described above. C) GF109203 induces prolongation of LPS-induced ERK activity. Quiescent cells were incubated with GF109203X (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated periods of time. ERK activity was analyzed by an in-gel-kinase assay. D) GF109203 induces prolongation of LPS-induced p38 Kinase activation. Quiescent cells were incubated with GF109203X (5 μM) for 1 hour and then stimulated with LPS (10 ng/ml) for the indicated periods of time. Expression of p38 kinase and phosphorilated p38-kinase was measured by Western Blot. The bands from two representative experiments (mean ± SD) were quantified by densitometry and the values were represented as % of the control expression (LPS 30 minutes for panel A and LPS 15 minutes for panel B).

Figure 3 showed that inhibition of PKCε by GF109203X was mediated by inhibition of TNF-α expression and not through a direct effect of PKCε on apoptosis signaling. We extended this observation to the three MAP kinases. None of the three inhibitors (i.e., Curcumin, PD or SB) blocked the apoptosis induced by recombinant TNF-α (Fig. 7A), thus suggesting that the role of the three MAP kinases in apoptosis induced by LPS is not mediated through modulation of the mechanisms involved in TNF-α-induced apoptosis signaling but through the direct regulation of TNF-α expression induced by LPS.

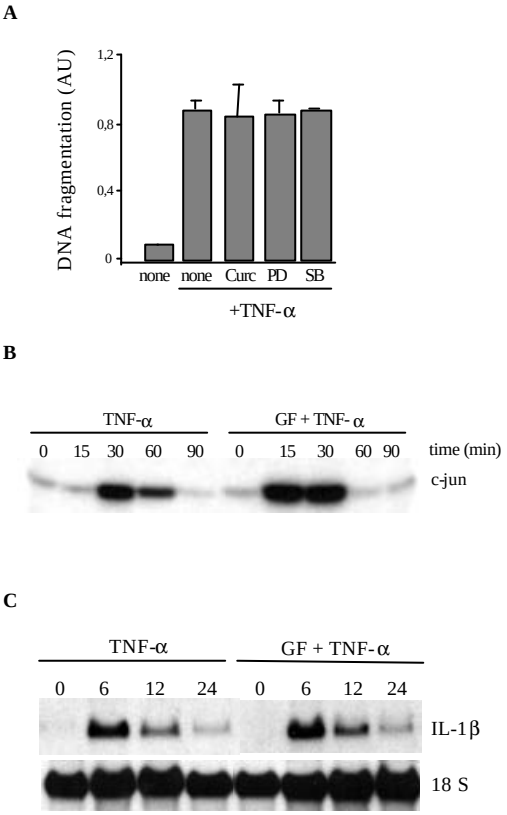


Figure 7: TNF-α-induced apoptosis is not mediated by PKCε. A) Inhibition of MAP Kinases does no modify the TNF-α-induced apoptosis. 10^5 macrophages were incubated with Curcumin (5 μg/ml), PD98059 (50 μM) or SB109203 (5 μM) for 1 hour and then stimulated with TNF-α (10 ng/ml) for 12 hours; apoptosis was detected as indicated under Fig. 1. Each experiment was performed in triplicate and the results of one representative of two independent experiments are shown as the mean value ± SD. B) GF109203 does not inhibits the TNF-induced JNK activity. Quiescent cells were incubated with GF109203X (5 μM) for 1 hour and then stimulated with TNF-α (10 ng/ml) for the indicated periods of time. JNK activation was measured as described in Material and methods. C) GF109203 does not inhibit the TNF-α-induced cytokine expression. The quiescent cells were incubated with GF109203 (5 μM) for 1 hour and then stimulated with TNF-α (10 ng/ml) for the indicated times. Total RNA (20 μg) was analyzed by Northern blotting. These figures are representative of three independent experiments

GF109203X did not inhibit JNK activation (Fig. 7B) or IL1-β expression (Fig. 7C) induced by TNF-α. TNF-α did not induce the activation of ERK or p38 either (data not shown). Moreover, the fact that curcumin did not affect apoptosis induced by recombinant TNF-α indicated that JNK activation in macrophages in response to TNF-α did not modulate the signaling cascade leading to apoptosis in response to

TNF-α. These results confirmed that LPS activates PKCε, which in turn activates the c-jun kinase and induces the production of TNF-α production with the subsequent triggering of apoptosis.

Finally, to further confirm our results we analyzed the apoptosis induced by LPS in bone marrow-derived macrophages obtained from mice where the PKCε gene had been disrupted by homologous recombination (PKCε KO). The macrophages from these mice were resistant to induction of apoptosis by LPS (Fig 8A). Moreover, we also observed an important reduction of JNK activity in macrophages from KO PKCε mice (Fig 8B), while ERK-1/2 and p38 were not inhibited after LPS treatment (Fig 8B). PKCε KO macrophages also showed lower levels of induction of TNF-α mRNA by LPS (Fig 8C).

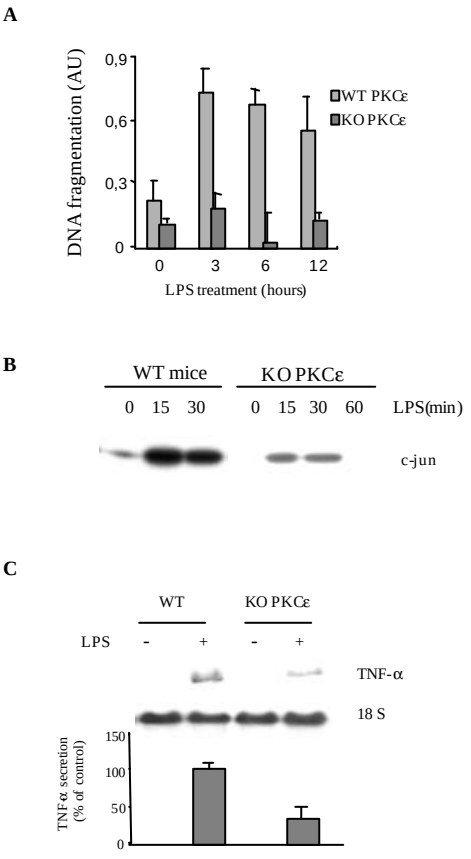


Figure 8: LPS does not induce apoptosis in macrophages from PKCε *knock-out* mice. A) LPS does not induce apoptosis in macrophages from PKCε *knock-out* mice. 10^5 macrophages from WT or PKCε KO were stimulated with 100 ng/ml of LPS for the indicated periods of time. Apoptotic DNA was analyzed by using an ELISA technique directed against histone-associated DNA fragments. The ELISA experiments were performed in triplicate and are shown as the mean value ± SD. B) LPS-induced JNK activity is reduced in macrophages from PKCε KO mice. Quiescent cells were stimulated with LPS (100 ng/ml) for the indicated periods of time. . JNK activation was measured by *in vitro* phosphorylation of GST. C) LPS-induced expression of mRNA of TNF-α is reduced in macrophages from PKCε KO mice. Quiescent cells were stimulated with LPS (100 ng/ml) for 1 hour. TNF-α expression was analyzed by Northern blot using 20 μg of total RNA per lane. The bands from two representative experiments were quantified by densitometry and represented as the mean ± SD.

Discussion

This report describes the role of PKCε and JNK in the cascade of events that start after interaction of LPS with the cell membrane and finish with autocrine

secretion of TNF-α, which induces apoptosis in macrophages. In a previous work we established that bone marrow-derived macrophages die through apoptosis in response to LPS predominantly through autocrine secretion of TNF-α (Xaus et al., 2000b). We have studied the signal transduction pathway that leads to the secretion of TNF-α and subsequent apoptosis.

Several processes induced by LPS in macrophages have been described to be dependent on PKC activation (Fujihara et al., 1994; Liu et al., 1994; Shapira et al., 1994), such as the induction of MAPK phosphatase (MKP)-1 (Valledor et al., 2000a), NO production (Fujihara et al., 1994; Diaz-Guerra et al., 1996) or the expression of TNF-α (Shapira et al., 1994). As cell model for macrophages we used bone marrow-derived macrophages, which are primary cell cultures that can proliferate, activate, differentiate or suffer apoptosis when induced with different stimuli. However, transfection efficiency is very low in this cell model (as is the case in other primary cell cultures) (Celada et al., 1996). Therefore, we had to use specific chemical inhibitors to study the signaling pathways involved in macrophage biology. Our results suggest that LPS induces TNF-α secretion and apoptosis through a pathway that involves the activation of PKC. The activation of this kinase is sufficient to induce apoptosis in several cell types (Garcia-Bermejo et al., 2002; Park et al., 2001; Siegmund et al., 2001), and by using two unrelated PKC inhibitors we have shown that PKC is also involved in the induction of apoptosis in macrophages by LPS. Although PKC participates in the control of several LPS-induced events (Fujihara et al., 1994; Liu et al., 1994; Shapira et al., 1994), it is still unclear which isoform(s) are involved in each of these effects. In fact, the comparison of different macrophage cell lines, or even different primary monocytic/macrophagic populations (Chang and Beezhold, 1993; Meldrum et al., 1998; Monick et al., 1998), shows significant variations in the expression of PKC isozymes (Balsinde et al., 1997; Büscher et al., 1995; Lin and Chen, 1998), perhaps as a consequence of their specific state of differentation/maturation. We had previously found that bone marrow macrophages express PKC isoforms βI, ε and ζ (Valledor et al., 1999). Although LPS shows a high structural similarity with ceramide (Wright and Kolesnick, 1995), a second messenger that activates PKCζ (Lozano et al., 1994), it is unlikely that this isoform mediates the induction of apoptosis by LPS for several reasons. Firstly, the PKC inhibitor GF109203X that blocks apoptosis induction was used at doses that inhibit conventional and novel

PKCs, but not atypical isoforms such as including PKCζ (Martiny-Baron et al., 1993). Secondly, we detected no PKCζ translocation in response to LPS (Valledor et al., 2000a). Thirdly, although PI-3K is activated by LPS and mediates the synthesis of PIP3, a second messenger that activates PKCζ (Nakanishi et al., 1993), wortmannin (a specific inhibitor of PI-3K) does not protect macrophages from apoptosis induced by LPS (data not shown). Finally, it has been shown that LPS and ceramide use divergent signaling pathways in macrophages to induce cell death (Lakics and Vogel, 1998). These observations suggest that PKCζ is not involved in the LPS signaling pathway that leads to apoptosis induction.

Several observations support the involvement of PKCε rather than PKCβI in the induction of TNF-α and apoptosis by LPS. Firstly, GF109203X inhibits conventional PKC isoforms better than novel ones (Martiny-Baron et al., 1993). Concentrations of GF109203X up to 1 μM completely inhibit the activation of conventional PKCs, including βI, whereas concentrations up to 5 μM are needed to completely block novel isoforms, including ε. In our experiments, maximal inhibition of apoptosis or TNF-α production were reached at concentrations of 3-5 μM of GF109203X. Secondly, Gö6976, a selective inhibitor of conventional PKCs, does not block induction of TNF-α or apoptosis by LPS. And finally, LPS does not induce apoptosis or TNF-α gene expression in bone marrow derived macrophages from mice with an inactivated PKCε gene. All these results demonstrate that PKCε is specifically involved in the LPS-induction of TNF-α and apoptosis in bone marrow macrophages.

The signaling events that occur after the interaction of LPS with CD14/TLR4 are not completely known, but several studies have suggested the involvement of MAP kinase activation (Geppert et al., 1994; Hambleton et al., 1996; Han et al., 1994; Reimann et al., 1994; Weinstein et al., 1992). A partial block of LPS-induced apoptosis was found when macrophages were incubated with the MAP kinases inhibitors for ERK and p38. However, the inhibition of the JNK kinase activity inhibited almost completely the apoptosis induced by LPS. The three MAP kinases regulate the biosynthesis of TNF-α but they act at different transcriptional and post-transcriptional levels. The inhibition of JNK kinase had a most drastic effect on TNF-α gene expression, whereas TNF-α mRNA levels slightly decreased or did not

change after blocking the ERK-1/2 or p38 pathways, respectively. However, the inhibitors of ERK-1/2 and p38 clearly decreased the levels of secreted TNF-α protein. Thus, our results suggest that ERK-1/2 and p38 MAP kinases regulate TNF-α production mostly at a post/transcriptional level whereas JNK does so at a transcriptional level. JNK has been involved in the transcriptional regulation of TNF-α through the regulation of the formation of the AP-1 transcription factor complex and the binding to the TNF-α promotor (Spriggs et al., 1992). At the post-transcriptional level, several reports support the involvement of the three MAP kinase pathways in TNF-α mRNA stability and translation (Rutault et al., 2001; Swantek et al., 1997; Williams et al., 1999).

The ultimate fate of a cell exposed to TNF-α is determined by signal integration between its different effectors, including IκB kinase (IKK), c-Jun N-terminal protein kinase (JNK) and caspases (Baud and Karin, 2001). Activation of caspases is required for apoptosis (Thornberry and Lazebnik, 1998), whereas activation of IKK inhibits apoptosis through the transcription factor NF-κB, whose target genes include inhibitors of caspases (Wang et al., 1998). JNK activates the transcription factor c-Jun/AP-1 as well as other targets (Chang and Karin, 2001). However, the role of JNK activation in apoptosis induced by TNF-α is less clear (Liu et al., 1996; Natoli et al., 1997).

In our experiments, the fact that PKC and MAPK inhibitors blocked apoptosis induced by LPS and TNF-α expression but were unable to inhibit apoptosis induced by recombinant TNF-α treatment indicates that PKCε and the three MAP kinases can modulate LPS-induced apoptosis through the production of TNF-α but not through a direct effect on the apoptotic mechanism. This is not surprising, because no involvement of MAP kinases or PKCε has been described in the pathway induced by TNF-α that leads to the induction of apoptosis (Boldin et al., 1996; Chinnaiyan et al., 1995; Hsu et al., 1996). Binding of TNF-α to the p55 receptor triggers the formation of a multiple protein complex in which cytoplasmic proteins and the receptor interact through their respective death-domains. After TNF-α stimulation, the receptor death-domain binds to the death-domain of a cytoplasmic protein called TRADD (TNF receptor I-associated death domain), which in turn binds to the death-domain of another cytoplasmic protein,

FADD/MORT-1. This protein also contains a death-domain (DED) motif, which binds to the DED motif of ICE/Ced-3 protease FLICE/MACH-1 (Caspase 8). It has also been suggested that activation of Caspase 8 initiates the activation of a cascade of caspases, which is the effector system for the apoptotic destruction of the cell. This model suggests that ligand binding to the TNF-α receptor activates the final death effector pathway apparently without any second messengers (Baud and Karin, 2001).

Recently, research conducted on fibroblasts has found that the NF-κB pathway negatively modulates JNK activation mediated by TNF-α and contributes to the inhibition of apoptosis (De Smaele et al., 2001; Tang et al., 2001). However, our results with curcumin do not confirm these observations. These differences could be due to different responses to TNF-α between macrophages and fibroblasts (Locksley et al., 2001; Xaus et al., 2000), since TNF-α induces apoptosis in macrophages without inhibiting protein synthesis or the NFκB pathway.

Although all three MAPK could modulate TNF-α expression and thus the apoptosis induced by LPS, our results suggest that only JNK is down-stream of the PKCε activity. This supports the existence of a new pathway in macrophages where LPS induces PKCε which activates JNK, which in turn modulates TNF-α expression at transcriptional level and thus mediates apoptosis induced by LPS. The kinase activity experiments support our hypothesis, since the JNK kinase activity induced by LPS is inhibited when macrophages are pre/treated with the PKC inhibitor GF109203X. Our results were confirmed with the use of mice with a disrupted PKCε gene (Castrillo et al., 2001). These mice showed a lower resistance to infection by Gram-positive or –negative bacteria, and the peritoneal macrophages showed a dramatic reduction of LPS-induced TNF-α production with a partial reduction of LPS-induced ERK or p38 MAP kinase activities (Castrillo et al., 2001). Our results confirm and extend these observations. In our experiments, the bone marrow-derived macrophages from these mice were resistant to LPS-induced apoptosis, and both TNF-α secretion and JNK activity were lower. This suggests a preferential induction of JNK activity over the other two MAP kinases.

Discusión

Los macrófagos, pertenecientes a la inmunidad natural o inmediata, son células especializadas en reconocer múltiples antígenos de forma no específica y, según su naturaleza, responderán de una manera u otra, condicionando así la posterior respuesta inmunitaria. Es por ello que desde el punto de vista de defensa del organismo es conveniente que exista un número elevado de macrófagos dispuestos a actuar como la primera línea de defensa. En este sentido, y a diferencia de lo que ocurre con los linfocitos, los macrófagos se encuentran proliferando en condiciones normales para poder mantener un tamaño de población constante y elevado, y tras un estímulo activador los macrófagos dejan de proliferar y pasan a desarrollar sus funciones.

Tanto los macrófagos como el resto de las celulas del sistema inmunitario son producidos en exceso y sólo aquellos que sean necesarios o capaces de realizar su función lograrán sobrevivir. Este es el caso por ejemplo de los linfocitos B cuya activación a través de los receptores de su superficie induce una prolongación de la vida media de estas células (Lam y col., 1997). De manera similar la supervivencia de los linfocitos T depende del reconocimiento por el TCR de las moléculas del MHC en las células presentadoras de antígeno (Tanchot y col., 1997). De acuerdo con la cantidad de sangre circulante, y sabiendo que la capacidad de regeneración de estas células es de unas 18 horas, la médula ósea produce, aproximadamente, 5 $x10^9$ macrófagos por día. La mayoría de los macrófagos serán eliminados mediante procesos de apoptosis y sólo sobrevivirán aquellos que tengan una funcionalidad (Xaus y col., 2001a). Al igual que ocurre con los linfocitos, la adquisición de esta capacidad funcional permite aumentar la supervivencia de los macrófagos en los tejidos. En el caso de los macrófagos diferenciados en los distintos tejidos, la vida media suele ser muy larga (varios años) (Fig. 16).

Así, a nivel tisular, los macrófagos pueden tener diversas opciones en respuesta al estímulo que reciban (Fig. 16). Según el tejido y las necesidades del mismo, el macrófago puede diferenciarse en células dendríticas, células de Kuppfer, células de la microglía, etc. (Abbas y col., 1991). Los macrófagos no diferenciados que están en los tejidos son capaces de proliferar gracias a la presencia o a la producción autocrina de M-CSF, así como a la presencia de otros

factores de crecimiento y citocinas no específicos como el GM-CSF y la IL-3 (Celada y Maki, 1992). En ausencia de citocinas o factores de crecimiento, los macrófagos entran en un estado de quiescencia y, si la falta de factores de crecimiento persiste, acaban por morir a través de procesos apoptóticos (Xaus y col., 1999b). Sin embargo, los macrófagos también pueden ser eliminados por procesos de apoptosis inducida no por la falta de factores de supervivencia, sino a través de la señalización por factores extracelulares producidos durante una infección o un proceso inflamatorio (Xaus y col., 2000b). Los estudios previos de nuestro grupo, y algunos de los presentados en esta Tesis, demuestran que tanto las citocinas como el IFN-γ (Xaus y col., 1999b) o los componentes de la matriz extracelular como la decorina (Xaus y col., 2001b) condicionan la supervivencia de estas células.

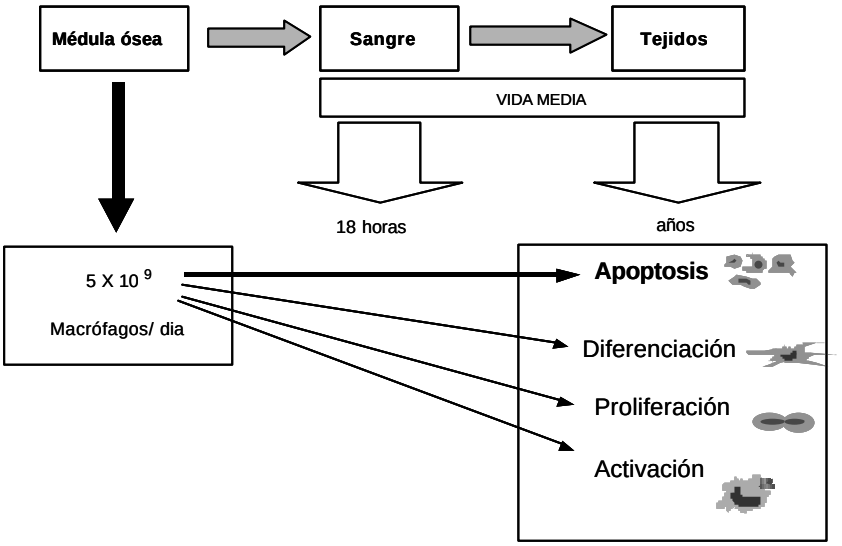


Figura 16. Proceso de producción y eliminación de los macrófagos. La médula ósea produce aproximadamente 5 x 10^9 monocitos por día. Los monocitos abandonan la médula ósea y, a través del torrente circulatorio, acceden a los distintos tejidos del organismo. La mayoría de los macrófagos serán eliminados mediante procesos de apoptosis y sólo sobrevivirán aquellos que tengan una funcionalidad. En este sentido, la vida de los macrófagos tisulares puede ser muy larga (años). En función de los estímulos a los que se vean expuestos, los macrófagos desarrollarán diferentes actividades: proliferación, diferenciación o activación.

Originalmente, los procesos de proliferación, activación y apoptosis eran considerados como sucesos aislados y regulados de forma independiente. Sin embargo, actualmente se ha observado que existe una intercomunicación entre las vías de señalización intracelulares que condiciona las decisiones de los

macrófagos hacia una de estas respuestas en función del balance de los estímulos que reciben del exterior, ya sean señales mitogénicas, activadoras, de la matriz extracelular o señales apoptóticas. Aunque los mecanismos reguladores de cada una de estas actividades pueden llegar a ser muy complejos, existe un número limitado de vías esenciales que regulan tanto de forma independiente como de manera integrada dichas decisiones. Intentar clarificar el papel de estas vías en la regulación de las actividades de los macrófagos ha sido el objetivo principal de este trabajo.

La integración de todos los resultados obtenidos en función de los procesos de proliferación, activación y apoptosis, no ha sido una tarea fácil. Por este motivo, para agilizar la lectura, hemos decidido dividir esta discusión en tres apartados: proliferación y activación, supervivencia, y apoptosis. Sin embargo todos estos procesos están íntimamente ligados y es difícil, en algunos casos, separarlos en apartados concretos. Por ejemplo, la respuesta de supervivencia es antagonista de la de apoptosis. Podríamos haber dicho que la falta de activación de una vía de señalización es la responsable de la apoptosis y no que esta vía de señalización es inducida en respuesta a la supervivencia. Otro caso se da cuando analizamos la vía que induce la expresión de citocinas, como el TNF-α que es responsable de la apoptosis en los macrófagos. Por otra parte, también las citocinas son responsables de la activación y la respuesta inflamatoria. En este caso, hemos preferido integrarla en el apartado de apoptosis. Por último, hemos creído conveniente discutir el modelo celular utilizado.

Como modelo celular hemos utilizado los macrófagos derivados de la médula ósea, ya que son cultivos primarios, no transformados, que presentan la maquinaria del ciclo celular intacta y responden a la presencia de factores que inducen la proliferación, la diferenciación, la activación o la apoptosis. Sin embargo, este modelo celular como todos los cultivos primarios tiene un gran inconveniente. Estas células son difíciles de transfectar por lo que no es factible realizar estudios con dominantes negativos o estudios de sobrexpresión (Celada y col., 1996). Esto implica que para el estudio de las vías de señalización tengamos que utilizar inhibidores químicos.

La utilización de drogas o compuestos químicos para inhibir la actividad de determinadas proteínas se asocia con la posibilidad, y por lo tanto con la crítica, de que estos inhibidores no sean del todo específicos y puedan activar o inhibir otras proteínas según las condiciones experimentales. Por este motivo, muchos de los resultados obtenidos han sido contrastados mediante la utilización de macrófagos procedentes de ratones en los que se ha eliminado, por recombinación homóloga, el gen objeto de estudio. Además, siempre que ha sido posible, se han utilizado varios inhibidores que ejercen el mismo efecto sobre la proteína, permitiéndonos corroborar nuestros resultados. Los inhibidores utilizados y las dianas de cada uno de ellos están resumidos en una tabla al inicio de esta Tesis.

Cabe mencionar que no siempre se pueden extrapolar los resultados obtenidos en los macrófagos derivados de la médula ósea a otras poblaciones de fagocitos mononucleares ni, incluso, con tipos celulares similares aunque en distinto estadío de diferenciación/maduración, puesto que existen diferencias considerables entre ellos. Tanto es así que, por ejemplo, mientras que los macrófagos medulares o la microglía son capaces de proliferar, los monocitos circulantes o los macrófagos peritoneales tienen esta capacidad muy limitada (Celada y Maki, 1992). Estas diferencias pueden deberse a la expresión diferencial de diversos genes en estas subpoblaciones. Así, se ha descrito que según las líneas celulares de macrófagos existen diferencias en el patrón de expresión de las isoformas de PKC (Balsinde y col., 1997; Büscher y col., 1995; Lin y Chen, 1997; 1998). Dichas diferencias pueden deberse también a las distintas condiciones ambientales o extracelulares en los que se encuentra cada población. Por ejemplo, el proceso de adhesión de los monocitos es suficiente para estimular la activación de las quinasas ERK (Hoog y Means, 2001). Además, las poblaciones de macrófagos circulantes o tisulares a diferencia de los macrófagos de la médula ósea han podido recibir señales estimuladoras o inhibidoras durante su tránsito dentro del organismo o en el momento de su aislamiento *in vitro*, mientras que los macrófagos de la médula ósea son obtenidos como células inmaduras no estimuladas que son diferenciadas *in vitro* bajo condiciones controladas.

Proliferación y activación

Los macrófagos, o bien proliferan o bien se activan. Estos son dos procesos totalmente diferenciados en este tipo celular. Los agentes que inducen proliferación no inducen activación y los que inducen activación además bloquean la proliferación (Xaus y col., 1999b). Los inductores de estas acciones utilizan distintos receptores y cabe esperar que la transducción de señales hacia el interior celular sea distinto.

Al estudiar las vías de transducción de señales que regulan la proliferación y la activación encontramos con sorpresa la participación, en ambas, de la vía de señalización MEK/ERK-1/2 (Valledor y col.,1999; 2000a). Además, en ambos casos, de forma independiente a la activación de MEK/ERK se produce la expresión de MKP-1 que es la fosfatasa que defosforila y, por lo tanto, inactiva a las quinasas ERK-1/2. Los macrófagos expresan tres isoformas de PKC (βI, ε y ζ), pero sólo la ε está implicada en la expresión de MKP-1 en ambos procesos (Valledor y col., 1999; 2000a). ¿Cómo pueden utilizar una misma vía procesos de señalización tan dispares? Nuestros resultados han mostrado que el punto crítico está basado en las distintas cinéticas de activación de ERK-1/2, las cuales determinan que el macrófago prolifere o se active.

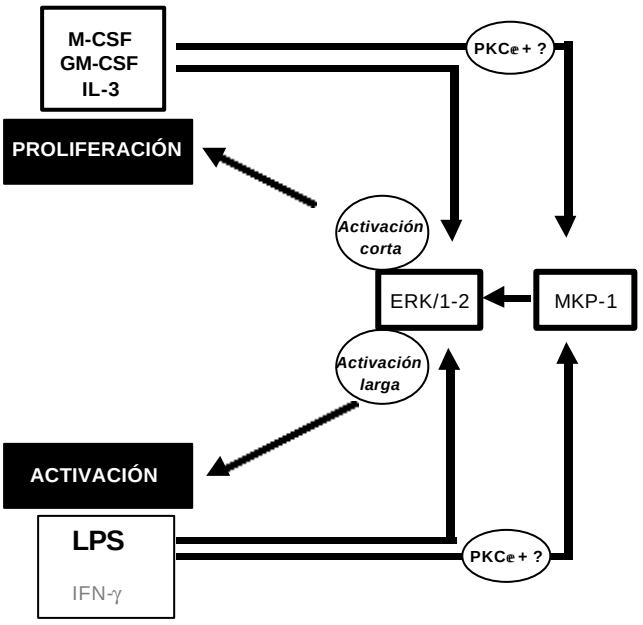


Figura 17. El patrón de activación de ERK-1/2 determina las respuestas en los macrófagos. La activación de ERK-1/2 a los cinco minutos (*activación corta*) de estimulación induce proliferación, mientras que un pico de activación de ERK-1/2 a los 15 minutos (*activación larga*) conduce a los macrófagos a secretar citocinas y participar en la respuesta inmunitaria. Tanto los estímulos proliferativos como los activadores a través de la activación de PKCε inducen la expresión de MKP-1. Esta fosfatasa es la responsable de la defosforilación y por tanto, inactivación de las quinasas ERK-1/2 inducidas por estos estímulos.

La activación de la vía MEK/ERK es necesaria para la proliferación en respuesta al M-CSF induciendo la activación de las quinasas con un pico a los cinco minutos y decayendo su actividad a partir del minuto 20-30 de tratamiento (*activación corta*). En otros tipos celulares, en respuesta a sus factores de crecimiento, la activación de estas quinasas también es necesaria para la proliferación (Foey y col., 1998; Scherle y col., 1998). El bloqueo de la activación de esta vía causa una parada a nivel de la fase G_1 del ciclo celular, sin inducir la muerte por apoptosis (Fig. 17).

En cuanto a la activación, las quinasas ERK aparecen fosforiladas y activas a partir de los diez minutos y alcanzan un pico de activación a los 15 minutos después del inicio de la estimulación, y la actividad decrece a partir de los 30-45 minutos (*activación larga*). Nuestros resultados coinciden con las observaciones de Jaworoski y col. (1996) en las que se reflejan las mismas diferencias en la cinética de activación de ERK en respuesta al LPS y al M-CSF.

El mecanismo por el cual los agentes proliferantes inducen un pico temprano de activación de ERK en comparación con el inducido por los agentes activadores debe ser consecuencia de la activación de diferentes vías de señalización que culminan con la activación de las quinasas ERK. En la línea macrofágica BAC1.2F5 se ha postulado que la activación de ERK-1/2 por el M-CSF puede ser mediada por mecanismos dependientes e independientes de Ras, mientras que la activación por el LPS implica parcialmente a la acción de las isoformas de fosfolipasa C específicas de fosfatidilcolina (Busher y col., 1995). La existencia de vías distintas para la activación de ERK-1/2 por el M-CSF y el LPS está apoyada también, por nuestros resultados con la ciclosporina A (CsA) y el FK506 (Comalada y col., 2002a). Estas drogas químicamente diferentes son inmunosupresoras y ejercen su efecto biológico principalmente mediante la inhibición específica de la Calcineurina en los linfocitos (Fathman y Myers, 1992; Kahan, 1993; Thomson, 1990).

Sin embargo, en nuestro modelo hemos descartado la implicación de la Calcineurina en la regulación de la proliferación de los macrófagos. Nuestros resultados, junto con los descritos en otros sistemas (de Hoog y col., 2000), nos indican que la vía Ca^{2+}/CaM/CaN no esta implicada en la proliferación de los

macrófagos. A pesar de ello, tanto la CsA como el FK506 a concentraciones elevadas inhiben la proliferación de los macrófagos mediante el bloqueo de la activación de las quinasas ERK. Mientras que la CsA inhibe la activación de ERK-1/2 y la expresión de MKP-1, el FK506 induce altos niveles de expresión de esta fosfatasa. Estos resultados nos sugieren que estos inmunosupresores ejercen su papel inhibidor en diferentes puntos de la vía de señalización.

También hemos visto que el efecto de la CsA y el FK506 sobre los macrófagos activados por el LPS se produce a diferente nivel. Mientras que la CsA potencia la expresión de las citocinas proinflamatorias, el FK506 ejerce el efecto contrario. Estas diferencias se correlacionan con las diferencias que vemos a nivel de activación de ERK-1/2. La CsA inhibe la expresión de la fosfatasa MKP-1 e induce un alargamiento del patrón de activación de ERK-1/2 a diferencia de lo que se observaba con el M-CSF. El FK506, tal y como ocurría con el M-CSF, incrementa la expresión de MKP-1 y por tanto inhibe la activación de ERK-1/2. Winter y colaboradores (1998) también han detectado un aumento de la inducción de MKP-1 tras el tratamiento con FK506.

Lo curioso ha sido observar que la CsA ejercía un efecto distinto en la activación del patrón de ERK-1/2 inducido por el LPS o por el M-CSF. Mientras que la vía de señalización de Raf-1/MEK/ERK inducida por el M-CSF puede ser inhibida a altas concentraciones de CsA, no ocurre lo mismo con la activación de ERK-1/2 por el LPS. Esta diferencia nos indica la existencia de vías independientes para la activación de las ERK por el M-CSF y el LPS tal y como hemos comentado anteriormente. Raf-1 podría interaccionar con una inmunofilina descrita recientemente (Stancato y col.,1994; Coss y col., 1998), lo que explicaría cómo, a altas dosis, la CsA pueda inhibir esta vía. Además, ha sido descrita una nueva familia de proteína quinasas llamadas Cot o Tpl-2 que pueden mediar la activación de ERK en respuesta al LPS independientemente de Raf-1 (Salmeron y col., 1996). Esta quinasa pertenece al primer módulo de la familia de las MAPKKK pudiendo activar tanto a ERK como a JNK (Fanger y col., 1997; Hunter, 1997; Robinson y Cobb, 1997). La transfección de esta quinasa en una línea celular de linfocitos T induce la expresión de TNF-α. Además, la activación de Cot/Tpl-2 no se inhibe por la CsA (Ballester y col., 1998) lo que nos sugiere que podría ser la responsable de la activación de ERK-1/2 por el LPS.

Por otro lado, estos inhibidores podrían ejercer su efecto sobre la peptidil-prolyl cis-trans isomerasa (PPIase). Esta proteína es similar a la ciclofilina, el receptor específico de la CsA (Takahashi y col., 1989). La CsA podría interaccionar con esta proteína inhibiendo su actividad y, por lo tanto, inhibiendo la actividad de otras proteínas que podrían participar en la señalización en respuesta al M-CSF. Esto abre la posibilidad a nuevas moléculas que independientemente de la Calcineurina puedan ser substratos de la CsA y el FK506. De hecho, aunque la ciclofilina y el FKBP12 son las inmunofilinas mejor caracterizadas, se sabe que existen otros miembros de esta familia que se expresan en menor abundancia (revisado en Schreiber, 1991). La CsA puede inhibir la activación de otros miembros de la familia de las MAP quinasas como JNK y p38 (Matsuda y col., 1998) posiblemente a través de otra inmunofilina independiente de la que inhibe a Raf-1, ya que la activación de estas quinasas se puede producir independientemente de Raf-1 (Fanger y col., 2000; Hunter, 1997).

Los resultados con la CsA sobre la activación de los macrófagos por el LPS podrían también sugerir, en un principio, que un alargamiento de la actividad de las quinasas ERK-1/2 inducida por el LPS, podría ser indicativo de una mayor expresión de citocinas. Sin embargo, la fibronectina aunque alarga el patrón de activación de las ERK no induce un aumento de la expresión de las citocinas sino, que las inhibe parcialmente (Comalada y col., 2002b). Algo similar ocurre cuando se tratan los macrófagos con GF109203X (Valledor y col., 2000a). Por lo tanto, teniendo en cuenta todos los resultados, podemos relacionar un alargamiento del patrón de activación de las quinasas ERK-1/2 con una disminución de la proliferación y con la expresión de citocinas en respuesta a ciertos estímulos. Sin embargo, aunque este alargamiento de la actividad quinasa ERK-1/2 sea una condición necesaria no es suficiente para la producción de citocinas.

Así, hemos visto que las diferencias observadas en la cinética de activación de ERK-1/2 entre los estímulos activadores y mitogénicos dependen por un lado de las diferentes vías de señalización que conducen a la activación de ERK-1/2 pero también, por otro lado, de los mecanismos de desactivación de ERK-1/2. Ampliando nuestros resultados hemos podido demostrar también que el efecto antriproliferativo del IFN-γ se debe a la inhibición de la expresión de la fosfatasa

MKP-1, y el consiguiente alargamiento del patrón de activación de las ERK-1/2 (Comalada y col., 2002c). Esta inhibición está mediada por STAT1 y podría deberse a la competición entre los factores de transcripción STAT1 y AP-1 por el activador CBP u otros activadores necesarios para la transcripción de MKP-1 (Genot y col., 1995) (Fig. 18).

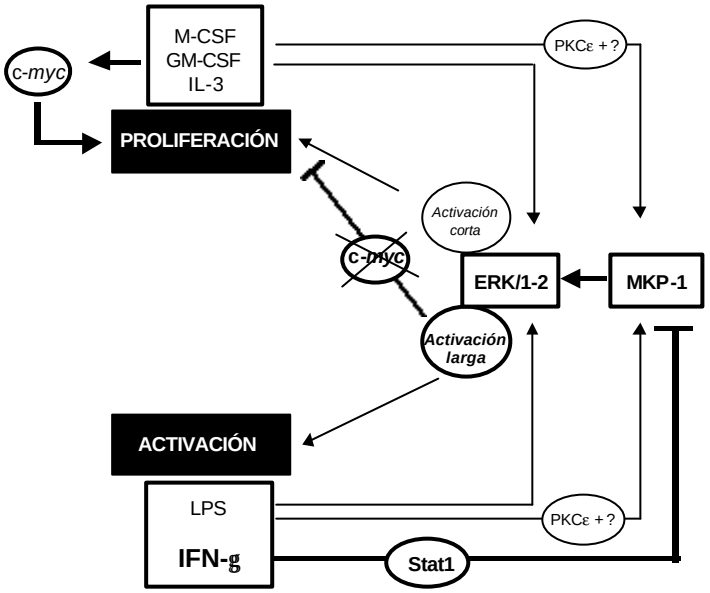


Figura 18. El patrón prolongado de activación de ERK-1/2 es el responsable de la inhibición de la proliferación. El IFN-γ a través de la activación de STAT1 inhibe la expresión de MKP-1 y en consecuencia induce un alargamiento de la cinética de activación de las quinasas ERK. La activación prolongada de estas quinasas es la responsable de la inhibición de la expresión de c-*myc* y por tanto de la inhibición de la proliferación.

En algunos modelos celulares la inhibición de la proliferación observada tras la diferenciación terminal y la senescencia está relacionada con una activación prolongada de ERK la cual conduce a un aumento de la expresión del inhibidor de las cdk $p21^{Waf1}$, siendo éste el responsable de dicha inhibición de la proliferación (Fanton y col., 2001; Lin y col., 1998). Aunque el IFN-γ en los macrófagos induce $p21^{Waf1}$ a través de STAT1, éste inhibidor del ciclo celular no juega un papel importante en la regulación de la proliferación en nuestro modelo ya que el IFN-γ continúa inhibiendo la proliferación de los macrófagos procedentes de ratones *knock-out* de $p21^{Waf1}$. En otros sistemas celulares se ha llegado a conclusiones similares (Sharma y col., 1998; Vivo y col., 2001). Además, la inhibición de las ERK-1/2 no bloquea la expresión de $p21^{Waf1}$ por el M-CSF sugiriendo que la expresión de $p21^{Waf1}$ no está mediada por la activación de las ERK-1/2. Esto sugiere que el alargamiento de la cinética de activación de ERK-1/2 podría ser la

responsable de la inhibición de la proliferación en los macrófagos pero por algún mecanismo independiente de $p21^{Waf1}$ (Fig. 18).

En conclusión, hemos establecido que la activación de las quinasa ERK-1/2 es crítica para la determinación de algunas respuestas celulares como la proliferación. Sin embargo, desconocemos el mecanismo exacto por el cual, ERK-1/2 participa en la proliferación de los macrófagos. Una vez activadas, ERK-1/2 son capaces de fosforilar y regular, ya sea positivamente o negativamente, numerosas proteínas citoplasmáticas, o pueden traslocarse al núcleo donde regulan la expresión de genes de acción inmediata (Treisman, 1996). Por ejemplo, ERK-1/2 pueden fosforilar el factor de transcripción Elk-1 participando en la expresión del gen *c-fos*, implicado en procesos proliferativos (Janknecht y col., 1993; Price y col., 1995). También se ha descrito que estas quinasas podrían mediar la fosforilación en la Ser-62 de la proteína c-Myc (Gupta y col., 1993; Seth y col., 1992). La inducción y activación de este factor de transcripción es indispensable para la proliferación inducida por el M-CSF (Roussel, 1997). De forma similar, la actividad de ERK-1/2 es necesaria para la expresión de la ciclina D_1 (Lavoie y col., 1996), la cual se requiere para la progresión a través de la fase G_1 del ciclo celular en respuesta al M-CSF. Además, la activación de las quinasas ERK-1/2 está implicada en la regulación postranscripcional de esta ciclina, mediante la activación de Mnk1 por las ERK-1/2 (Fukunga y Hunter, 1997). La Mnk1 es una serina/treonina quinasa que ejerce su efecto sobre el factor regulador de la traducción, elF-4E, que contribuiría en el transporte núcleo-citoplasmático del mRNA de la ciclina D_1 (Pyronnet y col., 1999; Rousseau y col., 1996).

En nuestro modelo, los efectos del IFN-γ no se deben a la inhibición de la expresión de la ciclina D_1 como se ha descrito en ciertos tipos celulares. Aunque ERK-1/2 participa como un regulador positivo de la expresión de la ciclina D_1, el IFN-γ no ejerce ningún efecto sobre la expresión de este gen (resultados no presentados), aunque no descartamos la implicación de una regulación postranscripcional de este gen mediada por el IFN-γ. Sin embargo, nuestros resultados apuntan en otra dirección ya que tras el tratamiento con el IFN-γ detectamos una clara inhibición de la expresión de *c-myc* (Xaus y col., 1999b) que confirma otras observaciones (Dey y col., 1999; Vairo y col., 1996). La expresión inmediata de *c-myc* es una característica de la estimulación mitogénica en las

células quiescentes y es inducida por una amplia variedad de mitógenos, citocinas y factores de crecimiento (Roussel y col., 1991). También existen evidencias de que *c-myc* es requerido para el inicio del ciclo celular y su sobrexpresión induce la proliferación a diferentes niveles (Karn y col., 1989; Sorrentino y col., 1986; Stern y col., 1986). Por otro lado, los procesos de diferenciación terminal y pérdida de la capacidad proliferativa de diversos tipos celulares se correlacionan con una disminución de la expresión de *c-myc*, y la expresión ectópica de esta proteína puede inhibir la diferenciación (Henriksson y Lusher, 1996).

Sin embargo, el mecanismo por el cual IFN-γ podría inhibir la expresión de *c-myc* no se ha definido con exactitud hasta el momento. Se ha sugerido que la regulación de *c-myc* por el IFN-γ es a nivel postranscripcional (Meurs y col., 1990; Raveh y col., 1996). El IFN-γ activa a la serina /treonina quinasa PKR (*double-stranded RNA* (dsRNA)-*protein kinase*) que fosforila a la subunidad α del factor elF-2, bloqueando así el inicio de la traducción (Hershey, 1991). En contraposición, se ha descrito también que el IFN-γ, a través de la regulación de la expresión de proteínas como MAD1 o la p202 puede regular la interacción de c-Myc con el promotor de diferentes genes (Chin y col., 1996; Wang y col., 2000).

Nuestros resultados indican que el IFN-γ puede inhibir la expresión de *c-myc* a nivel transcripcional. La expresión de *c-myc* inducida por el M-CSF no necesita la activación de las quinasas ERK-1/2. En contraposición, el alargamiento de la actividad ERK provocado por la inhibición de la expresión de MKP-1 inducida por el IFN-γ es el responsable de la inhibición de los niveles de mRNA de *c-myc*. Por tanto, nuestros resultados sugieren un nuevo y provocativo modelo para explicar la vía de señalización utilizada por el IFN-γ para inhibir la expresión de *c-myc*. En este sistema, la activación de ERK-1/2 juega un papel inhibitorio y no estimulador de la expresión de *c-myc* y explica la necesidad de un pico rápido y corto de la activación de ERK-1/2 en respuesta a los factores de crecimiento (Fig. 18). Nuestros resultados concuerdan con los procesos de diferenciación terminal que relacionan un patrón prolongado de activación de ERK-1/2 con una reducción de los niveles de la expresión de *c-myc* (Henrikson y Lusher, 1996). De todas formas, hacen falta más estudios para determinar el mecanismo exacto por el cual la activación prolongada de ERK-1/2 puede regular negativamente la transcripción de *c-myc*.

A continuación, nos planteamos si este mismo patrón de activación diferencial se extendía a la vía de JNK, otro miembro de la súperfamilia de las MAP quinasas. Aunque la activación de JNK se ha implicado en procesos relacionados con apoptosis o supervivencia, también es cierto que estas quinasas pueden ser activadas a través de receptores con actividad tirosina quinasa, como es el caso del M-CSFR. Numerosos estudios han implicado a las proteínas GTPasas de la familia *Rho*, como iniciadoras de la activación del primer módulo MAPKKK, culminando la señalización en la activación de JNK (Fanger y col., 2000; Schleissinger, 2000). En nuestro modelo celular, JNK se activa, en general, tanto por procesos mitogénicos como activadores. No detectamos diferencias en el patrón de activación de JNK inducido por estímulos activadores o por estímulos proliferativos. Además, la inhibición de la proteína JNK no desempeña ningún papel en la proliferación de los macrófagos aunque juega un papel crítico en la activación de estas células. Al igual que ERK, la activación de JNK es necesaria para la correcta expresión y secreción de TNF-α. Esta citocina proinflamatoria es secretada en respuesta a la activación de los macrófagos por el LPS, así como el IFN-γ y es la responsable, en la mayor parte, de la apoptosis de los macrófagos (discutido mas adelante).

La regulación de la proliferación, supervivencia y del crecimiento celular, así como de los fenómenos de activación celular no sólo se producen tras la interacción con factores solubles, sino también a través de la interacción con las macromoléculas del entorno que forman parte de lo que se denomina matriz extracelular (Howe y col., 1998; Ruoslahti, 1989; Ruoslahti y Reed, 1994). Estos estímulos pueden afectar a la adhesión, migración, diferenciación, proliferación, activación y apoptosis de los macrófagos. Por lo tanto, la matriz extracelular circundante a las células es de vital importancia para las funciones celulares.

La decorina es un proteoglicano presente en la matriz extracelular de la mayoría de tejidos de los mamíferos (Iozzo, 1997). La importancia biológica de esta molécula no está bien definida, aunque algunos estudios indican que interviene en la fibrilogénesis del colágeno y que podría jugar un papel como inhibidor del crecimiento de las células tumorales (Iozzo, 1997; Santra y col., 1995; 2000). Sin embargo, hasta la fecha poco se conoce del efecto de la decorina sobre las células normales.

En nuestros trabajos, hemos observado que la decorina por un lado inhibe la proliferación de los macrófagos aunque aumenta la supervivencia en ausencia de factores de crecimiento, y por otro lado aumenta la capacidad de activación de los macrófagos. Debido a que la decorina aumenta la adhesión a la matriz extracelular, y puesto que estos fenómenos juegan un papel clave en la diferenciación de los monocitos (Berton y Lowell, 1999; Jun y col., 1995), quisimos averiguar cuales de los efectos de la decorina eran debidos a la adhesión y cuales de ellos eran debidos a mecanismos independientes. Para ello hemos comparado los efectos de la decorina frente a otros componentes de la matriz extracelular como la fibronectina que también induce un aumento de la adhesión.

El primer efecto que nos sorprendió fué observar que existía una correlación inversa entre los procesos de adhesión y la capacidad de proliferación. Por lo tanto, parecía que el efecto de la decorina sobre la proliferación era solo un efecto indirecto mediado a través de los mecanismos de adhesión.

La adhesión de las células como los fibroblastos o los macrófagos a la matriz extracelular se produce principalmente a través de las integrinas, las cuales pueden modular el paso de las células a través de la fase G_1 a S del ciclo celular en respuesta a los factores de crecimiento (Howe y col., 1998; Meredith y Schwartz, 1997; Ruoslahti y Reed, 1994; Zhu y col., 1996). Utilizando anticuerpos anti-$\beta1$ y péptidos RGD hemos confirmado esta hipótesis y nos ha proporcionado información adicional sobre el receptor de la decorina. El efecto de la decorina sobre la adhesión de los macrófagos se encuentra mediado a través de un receptor no relacionado con las integrinas $\beta1$ a diferencia de la fibronectina que utiliza la integrina $\beta1$ para adherirse. De hecho, se sabe que la fibronectina puede interaccionar con los macrófagos a través de los receptores de integrinas $\alpha5\beta1$ y $\alpha4\beta1$ induciendo la migración de estas células (Fougerolles y col., 2000; Hershkoviz y col., 1992; Wesley y col., 1998). Además, debido a que la decorina presenta un efecto sinérgico sobre la adhesión de los macrófagos con la fibronectina nos confirma que la decorina utiliza un receptor independiente de ésta. Sin embargo, son necesarios más estudios para poder determinar el receptor específico de la decorina.

Al igual que ocurre con el IFN-γ, la decorina inhibe la expresión de MKP-1 e induce un alargamiento de la actividad ERK. Además, la decorina también induce la expresión de $p21^{Waf1}$. Sin embargo, a diferencia del IFN-γ, la decorina también induce la expresión de $p27^{Kip1}$ que es el responsable del bloqueo sobre la proliferación (Fig. 19). El hecho de que la adhesión de los macrófagos a la fibronectina ejerce los mismos efectos antiproliferativos que la decorina como el alargamiento de las ERK por inhibición de MKP-1 y la expresión de $p27^{Kip1}$, nos sugiere que, estas actividades de la decorina son debidas al aumento de la adhesión propiamente dicha y no a un sistema de señalización específico (Fig. 19).

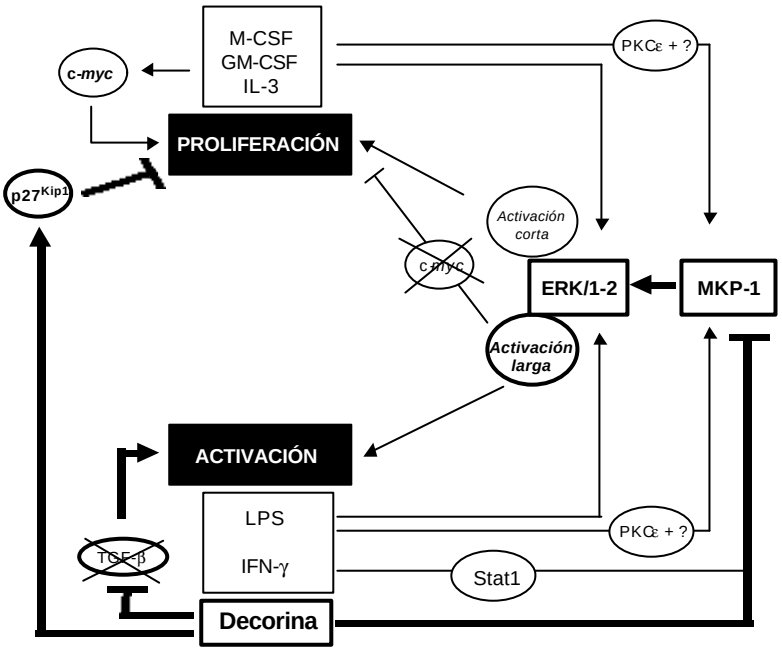


Figura 19. Efecto de la decorina sobre los mecanismos de proliferación y activacion de los macrófagos. La decorina y la fibronectina inhiben la proliferación de los macrófagos mediante un aumento de adhesión que se correlaciona con un aumento de los niveles de expresión de $p27^{Kip1}$ y un alargamiento del patrón de activación de ERK-1/2 mediante la inhibición de MKP-1. También, la decorina potencia la activación de los macrófagos inducida por el IFN-γ y el LPS mediante el secuestro del TGF-β producido endógenamente.

Lamentablemente no hemos podido analizar la expresión de *c-myc* tras el tratamiento con decorina y determinar su relación con la elongación del patrón de las ERK. Sin embargo, los resultados obtenidos con el IFN-γ nos hacen pensar que ambos agentes podrían tener un mecanismo de actuación similar. Otro punto que queda por resolver es la relación existente entre la expresión de $p27^{Kip1}$ y los

procesos que acabamos de mencionar. Aunque en algunos casos, como puede ser la diferenciación terminal o la senescencia celular, se ha asociado un alargamiento de la actividad de las ERK-1/2 con la expresión de los inhibidores del ciclo celular , estos suelen ser $p21^{Waf1}$ o $p16^{Ink4a}$, pero no $p27^{Kip1}$ (Liu y col., 1996; Moscatello y col., 1998; Santra y col., 2000).

Una vez dilucidado el papel de la decorina sobre la proliferación de los macrófagos quisimos analizar cual era su papel en la activación de estas células. La decorina, a diferencia de otros inhibidores de la proliferación no es capaz de inducir la activación. Sin embargo, la decorina potencia la activación mediada por el IFN-γ o por el LPS. Como estos activadores utilizan diferentes vías de señalización, nos hemos centrado en encontrar un mecanismo común.

La decorina es capaz de unirse al TGF-β y modular la actividad de esta citocina que es producida por los macrófagos de manera autocrina y es la responsable de la regulación negativa de su activación (Assoian y col., 1987; Grotendorst y col., 1989). El TGF-β antagoniza muchas de las respuestas inducidas por el IFN-γ como la producción de H_2O_2, de NO, la inhibición de la iNOS, la secreción de TNF-α o incluso la capacidad que posee el IFN-γ de matar a microorganismos intracelulares (Bermudez, 1993; Bogdan y col., 1992; Ding y col., 1990; Silva y col., 1991; Tsunawaki y col., 1988; Vodovotz y col., 1993). Además, el TGF-β inhibe la expresión de las moléculas de clase II a través de elementos conservados situados en el promotor proximal (Czarniecki y col., 1988; Reimold y col., 1993). Se ha descrito que el efecto represor del TGF-β sobre el IFN-γ se debe a una interacción entre moléculas involucradas en sus respectivas señalizaciones. La vía del TGF-β/SMAD se inhibe por la activación de la vía IFN-γ/STAT y viceversa (Pitts y col., 2001; Ulloa y col., 1999). Además, el TGF-β también tiene un efecto inhibidor sobre los macrófagos activados por el LPS, inhibiendo la expresión de iNOS (Werner y col., 2000) y reduciendo las citocinas pro-inflamatorias durante el shock séptico (Imai y col., 2000). Por lo tanto, la producción autocrina del TGF-β desempeña una importante función en la supresión de la inflamación en ausencia de un estímulo pro-inflamatorio adecuado (Fig. 19). En los animales en los que el gen del TGF-β ha sido inactivado, se ha visto que presentan una excesiva respuesta inflamatoria (Kulkarni y col., 1993) y un aumento de la expresión del MHC II (Geiser y col., 1993). Además, el aumento

de la capacidad de la respuesta inflamatoria en estos ratones esta asociado con el desarrollo de autoinmunidad, detectándose una infiltración masiva de células mononucleares en numerosos tejidos (Dang y col., 1995; Geiser y col., 1993; Kulkarni y col., 1993; Shull y col., 1992; Vodovotz y col., 1996)

Se ha demostrado *in vitro* que la decorina es capaz de interaccionar con el TGF-β, y con otras proteínas como la fibronectina, C1q, trombospondina y varios tipos de colágeno (Iozzo, 1997; Iozzo y Murdoch, 1996). Esto nos sugirió que la decorina podrían impedir la interacción del TGF-β con su receptor específico y la posterior inhibición de la activación de los macrófagos. En este sentido, hemos demostrado que la decorina bloquea la interacción del TGF-β con los macrófagos. Por lo tanto, la decorina actúa secuestrando el TGF-β e impidiendo que éste pueda interaccionar con los macrófagos para regular negativamente la respuesta inflamatoria.

Nuestros resultados han mostrado que hay un balance entre la activación y la supresión que esta mediado por la producción autocrina del TGF-β. En condiciones basales los macrófagos secretan TGF-β que mantiene a estas células en un estado de inactivación. La células no responden a bajas concentraciones de estímulos activadores ya que el TGF-β actúa bloqueando la activación. Si el estímulo supera un basal de pre-activación, el TGF-β secretado por los macrófagos no será suficiente para inhibir la activación de estas células y en consecuencia tendrá lugar la respuesta inmunitaria. Además, los macrófagos también secretan decorina frente a diferentes estímulos y en condiciones de estrés tisular modulando así la capacidad de respuesta de estas células (Bidanset y col., 1992; Krumdieck y col., 1992; Shimidt y col., 1987).

Como conclusión de este primer bloque, nuestros resultados demuestran que la actividad de ERK-1/2 es imprescindible tanto para los procesos proliferativos como para la activación de los macrófagos. En los procesos mitogénicos es necesario que la activación de las ERK-1/2 se produzca de forma muy rápida y transitoria, probablemente porque estas quinasas son necesarias para desencadenar los procesos más tempranos para la progresión del ciclo celular. La activación de ERK-1/2 debe ser rápidamente inhibida ya que un

alargamiento de la actividad de ERK-1/2 supone la inhibición de la proliferación por un bloqueo en la expresión de *c-myc*.

Supervivencia

Para mantener la viabilidad de muchos tipos celulares es necesario la presencia de factores de crecimiento (Roussel, 1997). Los macrófagos no son una excepción a esta regla y requieren la estimulación constante con factores de crecimiento, tanto para mantener su viabilidad como para proliferar o diferenciarse (Celada, 1994). La falta de factores de crecimiento induce la activación del programa de apoptosis a causa de un defecto en una o en varias vías de señalización. En este sentido la primera duda que se nos planteó fue intentar averiguar si los procesos de proliferación y supervivencia están controlados por la misma vía o si existen vías independientes que regulan ambos procesos.

En algunos sistemas celulares, la activación de la vía de señalización de Raf-1/MEK/ ERK es la responsable de inhibir la apoptosis inducida por la falta de factores de crecimiento (Borasio y col.,1993; Xue y col., 1999). Puesto que ya habíamos observado el papel clave que jugaban las proteínas ERK en la regulación de los procesos de proliferación y activación en los macrófagos, esto nos llevó a analizar el papel de estas quinasas en el mecanismo de supervivencia inducido por los factores de crecimiento. Así, la inhibición de ERK-1/2, inhibe la proliferación de los macrófagos, pero no protege de la apoptosis.

La interacción del M-CSF con su receptor, además de las ERK-1/2 también induce la activación de otras vías de señalización, entre ellas los otros miembros de la familia de las MAPK quinasas, JNK y p38. La inhibición de estas quinasas no bloquea ni la proliferación ni disminuye la viabilidad de los macrófagos inducida por los factores de crecimiento.

En respuesta al M-CSF también es activada la vía de señalización de PI-3K (Bourette y col.,1997) y en otros tipos celulares es crítica para la supervivencia (revisado en Datta y col., 1999). La inhibición de PI-3K en nuestro modelo experimental, demuestra que esta fosfatidilquinasa juega un papel clave en la

regulación de la supervivencia. Además, la inhibición de PI-3K no afecta a la proliferación y nos confirma, de nuevo, la independencia entre ambos procesos.

La PI-3K puede ejercer su efecto sobre distintos substratos, como son las PKC atípicas (PKCζ en nuestro modelo), la $p70^{S6}$ quinasa y Akt los cuales se han implicado en los procesos de supervivencia en algunos casos (Coffer y col.,1998; Dekker y Parker, 1994; Rameh y Cantley, 1999). Hemos podido demostrar que mientras que la PKCζ y la $p70^{S6K}$ no tienen un papel relevante en la supervivencia, la activación de la Akt por parte de PI-3K es la principal responsable de su supervivencia. Esta quinasa puede inhibir la apoptosis inducida por la ausencia de factores de crecimiento o por la irradiación de las células neuronales, los fibroblastos y los linfocitos (Coffer y col., 1998; Hemmings, 1997).

Hasta este punto, nuestros resultados sugieren que la activación de la vía de señalización de Raf-1/MEK/ERK desempeña un papel primordial en la proliferación mientras que la vía de PI-3K/Akt regula los procesos de supervivencia. Como era de esperar, una célula animal no es un sistema que se pueda simplificar tan fácilmente, por lo que no es de extrañar que, además de estas dos vías principales, existan otras vías como es el caso de PKCε que puedan participar en procesos paralelos (datos no mostrados).

Actualmente desconocemos el mecanismo por el cual la vía PI-3K/Akt regula la supervivencia de los macrófagos. Recientemente, han sido identificados un conjunto de sustratos que pueden ser fosforilados por Akt y que podrían tener un efecto antiapoptótico. Entre ellos se incluyen dos componentes de la maquinaria de la muerte celular, BAD (Datta y col., 1997) y la caspasa 9 (Cardone y col., 1998); factores de transcripción como los *Forkhead* (Brunet y col., 1999) y el E2F (Brenna y col., 1997) que están implicados en la regulación del ciclo celular; y quinasas como la IKK que regulan al factor de transcripción NF-κB (Madrid y col., 2000). Aunque no descartamos que estos substratos de Akt puedan desempeñar un papel importante en la supervivencia, nuestros resultados han aportado un nuevo mecanismo por el cual la vía PI-3K/Akt puede regular la supervivencia de los macrófagos que es la expresión del inhibidor del ciclo celular $p21^{Waf1}$ (Fig. 20).

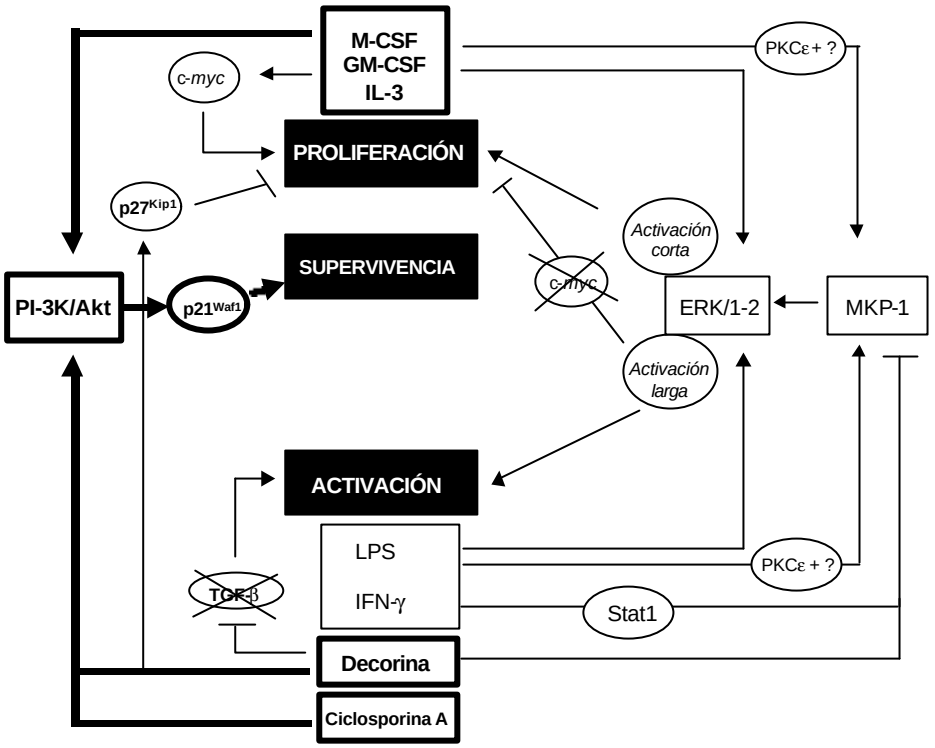


Figura 20. La vía de señalización PI-3K/Akt/p21^{Waf1} es la responsable de la supervivencia de los macrófagos. Los estímulos proliferativos, como el M-CSF, GM-CSF e IL-3 mediante la activación de la vía de PI-3K/Akt/p21^{Waf1} regulan la supervivencia. Además, los estímulos como la decorina y la ciclosporina A, también activan esta vía de señalización protegiendo a los macrófagos de la apoptosis inducida por la falta de factores de crecimiento.

La proteína p21^{Waf1} fue identificada inicialmente como un inhibidor del ciclo celular en numerosos sistemas jugando un papel importante en la progresión del ciclo celular. Posteriormente, numerosos estudios han descrito funciones adicionales de p21^{Waf1}, que se deben a su elevada capacidad de unión a otras proteínas y de regulación de sus actividades. Por ejemplo, se ha visto que p21^{Waf1} puede participar en la activación de los complejos ciclina D_1/cdk4, en la activación de la transcripción, en el transporte nuclear, en la replicación del DNA, etc (Delavaine, 1999; Kitaura y col, 2000; Perkins, 1997; Zhang y col., 1994). Sin embargo, en los últimos años se ha implicado a p21^{Waf1} en la regulación de la apoptosis (Ver apartado 2.2.3.2.1 de la introducción). Los estudios previos de nuestro grupo demostraron que p21^{Waf1} juega un papel clave como regulador de procesos apoptóticos (Xaus y col., 1999b).

Los inhibidores de la proliferación de los macrófagos no afectan a la expresión de $p21^{Waf1}$ en respuesta a los factores de crecimiento. Sin embargo, todos aquellos compuestos que inhiben la supervivencia son capaces de inhibir también la expresión de $p21^{Waf1}$; sugiriendo que $p21^{Waf1}$ podría estar mediando los efectos antiapoptóticos inducidos a través de la vía PI-3K/Akt.

Las señales mediadas por la matriz extracelular también son importantes para la supervivencia celular (Ruoslahti y Redd, 1994). Por ejemplo se sabe que una matriz extracelular rica en laminina induce la supervivencia tanto *in vivo* como *in vitro* de las células epiteliales (Finlay y col., 2000). Se ha sugerido que la laminina ejerce su efecto a través de los receptores de las integrinas $\alpha_6\beta_1$, y actúan estabilizando la vía de PI-3K inducida por los factores de crecimiento como la insulina o el IGF-1 (Boudreau y col., 1995; Farrelly y col., 1999). Nuestros resultados indican que la decorina tiene un efecto protector de la apoptosis inducida por la falta de factores de crecimiento. Hemos demostrado que este efecto no se debe a un aumento de la adhesión. En ratones deficientes en el gen de $p21^{Waf1}$, la decorina es incapaz de proteger de la apoptosis lo que muestra que $p21^{Waf1}$ es responsable del efecto antiapoptotico producido por la falta de factores de crecimiento. Al igual que la decorina, la CsA a través de la activación de la vía de PI-3K/Akt, también induce la expresión de $p21^{Waf1}$ y protege de la inducción de la apoptosis (Fig. 20).

La inducción de la expresión de $p21^{Waf1}$ por el IFN-γ no está mediada a través de la vía de PI-3K/Akt. La mayoría de las respuestas inducidas por el IFN-γ se produce mediante la activación de la vía de JAK/STAT (Bach y col., 1997). El complejo Stat1 fosforilado activa la transcripción de los genes que contienen secuencias GAS, entre los que se ha descrito a $p21^{Waf1}$ (Chin y col., 1996).

Por tanto, parece ser que la expresión de $p21^{Waf1}$ podría ser un mecanismo general de regulación de la supervivencia. El mecanismo por el cual Akt activa la transcripción de $p21^{Waf1}$ hoy en día lo desconocemos. Sin embargo, se ha implicado la fosforilación de STAT1 por parte de Akt como el responsable de la protección de la apoptosis (Nguyen y col., 2001). Se sabe que el M-CSF activa a STAT1 aunque no se ha podido demostrar la interacción directa de este factor con el receptor fosforilado del M-CSF (Bourette y col., 1997). Actualmente nos

disponemos a estudiar si el efecto de la decorina y la CsA está relacionado con STAT1 utilizando macrófagos procedentes de ratones KO de STAT1, pudiendo así establecer una relación entre la protección de la apoptosis y la inducción de $p21^{Waf1}$ inducida por el IFN-γ y otros estímulos PI-3K/Akt-dependientes en los macrófagos.

Como resumen de este bloque, hemos demostrado que en los macrófagos derivados de la médula ósea existen dos vías independientes: la vía de MEK/ERK, que regula la proliferación y la vía PI-3K/Akt que regula no solo la supervivencia de los macrófagos en presencia de factores de crecimiento, sino que es un mecanismo más general implicado también en la supervivencia inducida por otro tipos de estímulos como la decorina o la ciclosporina A (Comalada y col, 2002d).

Apoptosis

Se ha sugerido una relación entre la activación celular y los procesos de muerte celular programada o apoptosis, especialmente en las células del sistema inmunitario. Esta relación ha sido interpretada como un mecanismo de autorregulación de la actividad de estas células, la cual si se produce de forma descontrolada y persistente podría llegar a ser perjudicial para el organismo. Este efecto inductor de apoptosis tras la activación de las células del sistema inmunitario está potencializado si la activación no se produce en condiciones óptimas como puede ser el caso de la activación de los linfocitos por el entrecruzamiento de los receptores de membrana en ausencia de los coestímulos adecuados (Freitas y Rocha, 2000).

Los macrófagos no son una excepción a esta regla y también se ha asociado su activación mediada por el LPS con la muerte por apoptosis (Albina y col., 1993; Sarih y col., 1993; Xaus y col., 2000b). Los trabajos de nuestro laboratorio sobre el papel protector que el IFN-γ ejerce sobre la apoptosis inducida por el LPS en los macrófagos han aportado nuevas ideas sobre este tema (Xaus y col., 1999b). Aunque no está tan claramente estudiada como en los linfocitos, la activación de los macrófagos de forma no adecuada también induce su muerte por apoptosis. En este caso el reconocimiento previo del IFN-γ, el activador endógeno

de los macrófagos protege de la apoptosis. En condiciones fisiológicas es difícil que un macrófago pueda interactuar con el LPS en ausencia del IFN-γ y, si esto llega a producirse, suele desencadenar una respuesta patológica como la que ocurre durante el shock endotóxico (Sicher y col., 1995) (Fig. 21).

En un primer paso durante una infección limitada por bacterias gram-negativas, el LPS y los productos bacterianos son contenidos en los puntos de inflamación principalmente por los neutrófilos y algunos macrófagos residentes. Como resultado de la incipiente respuesta inmunitaria se producirán agentes quimiotácticos (quimiocinas y productos del sistema del complemento) y secreción de citocinas. Así el IFN-γ producido por las células NK y los linfocitos T activados difunde hacia los tejidos adyacentes (Marchant y col., 1994; Pfeifer y col., 1992). A medida que se aproximan al punto de inflamación los macrófagos reclutados encontrarán primero al IFN-γ, de manera que su contacto con el LPS se producirá una vez éstos han estado ya activados. Esto permitirá entre otras cosas un aumento de la expresión de las moléculas del MHC de clase II y, por tanto, de la presentación antigénica. Por otro lado, el LPS actúa de forma sinérgica con el IFN-γ en aquellas actividades antibacterianas de los macrófagos, como puede ser la producción de óxido nítrico por la iNOS o la secreción de citocinas como el TNF-α. Por último, el IFN-γ confiere viabilidad a los macrófagos activados con LPS, de manera que los macrófagos, a diferencia de los neutrófilos, permanecerán en los puntos de inflamación durante un largo periodo de tiempo participando así en la resolución de la respuesta inmunitaria y en la reparación de los tejidos dañados (Bellingan y col., 1996).

Sin embargo, este panorama se ve alterado cuando se produce una infección generalizada de bacterias gram-negativas desencadenante del shock séptico. En estas condiciones, los neutrófilos y los macrófagos residentes no son capaces de delimitar las bacterias en los puntos de entrada produciéndose una infección generalizada. En estas situaciones el LPS difunde también a los tejidos adyacentes, de manera que los macrófagos reclutados podrán entrar en contacto con el LPS antes de haber sido activados por el IFN-γ. Esto provocará, por un lado, una respuesta inmunitaria ineficaz debido a la incapacidad de activación como es la expresión de las moléculas del MHC de clase II (Xaus y col., 2000a), y,

por otro lado, la disminución de la viabilidad celular de los macrófagos, los cuales sucumbirán por apoptosis (Xaus y col., 1999b) (Fig. 21).

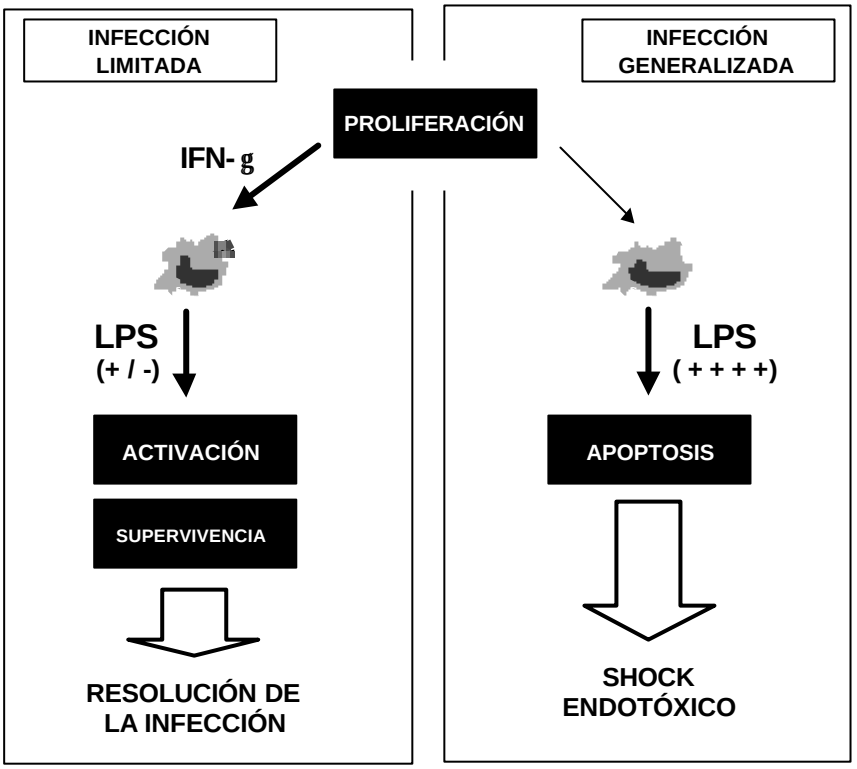


Figura 21. Respuesta de los macrófagos frente a la infección bacteriana. En una infección limitada, el IFN-γ producido por las células NK y los linfocitos T activados difunde a los tejidos adyacentes. Los macrófagos reclutados encontraran primero al IFN-γ, de manera que su contacto con el LPS se producirá una vez estos han estado ya activados permitiendo la resolución de la infección. Además, el IFN-γ proporcionará supervivencia a los macrófagos. En una infección generalizada, el LPS difunde a los tejidos adyacentes, de manera que los macrófagos reclutados podrán entrar en contacto con el LPS antes de haber sido activados por el IFN-γ. Los macrófagos sucumbirán a la apoptosis conduciendo al individuo al shock endotóxico.

Con este modelo no intentamos restar importancia a la apoptosis inducida tras la activación como mecanismo regulador de la actividad de los macrófagos en el desarrollo de la respuesta inmunitaria, pero existe un gran número de mecanismos reguladores más específicos que aseguran la correcta activación o inhibición tras la eliminación del estímulo activador. Entre estos mecanismos hay que destacar la existencia de sistemas represores endógenos como la secreción autocrina de TGF-β o de moléculas coestimuladoras como CD200R (Hoek y col., 2000; Wright y col., 2000) que aseguran que la respuesta inflamatoria no se

produzca de forma inadecuada. Además, en fases avanzadas de la respuesta inmunitaria se produce la expresión de otras moléculas inhibidoras como la PGE_2 o la adenosina (Xaus y col., 1999a; 1999c). Por último, tras la eliminación del antígeno se produce la reducción de la producción de IFN-γ por los linfocitos T activados. Todo ello hace que finalice la respuesta inflamatoria.

Hasta el momento, la inducción de apoptosis en los macrófagos tras la activación con el LPS se había asociado con la activación de la iNOS y la consiguiente producción de óxido nítrico (NO) (Albina y col., 1993; Sarih y col., 1993). Algunos autores habían propuesto que las dosis moderadas de óxido nítrico pueden tener un efecto beneficioso y protector de la inducción posterior de apoptosis (Brune y col., 1996). Nuestros resultados sobre la actividad del LPS en los macrófagos planteaba algunas contradicciones con las hipótesis previas. La inducción de apoptosis en los macrófagos era un fenómeno temprano que se observaba a las 3-6 horas de la estimulación con LPS mientas que la producción de óxido nítrico es un fenómeno tardío que empieza a detectarse a partir de las 12 horas. Consecuentemente, la inducción temprana de apoptosis por el LPS no podía estar mediada por la producción de óxido nítrico; y, por lo tanto, otros mecanismos debían estar involucrados.

El LPS induce en los macrófagos de forma muy rápida la secreción de TNF-α pudiéndose detectar a los 90 minutos. Esta citocina es capaz de inducir apoptosis tanto en células tumorales (Tartaglia y col., 1993) como en diversos tipos celulares, entre los que se incluyen hepatocitos, células endoteliales, oligodendrocitos y muchas otras (Akassoglou y col., 1998; Leist y col., 1995; Stehlik y col., 1998). Por este motivo, nos planteamos si el TNF-α secretado de forma autocrina podía ser responsable de la inducción temprana de apoptosis en los macrófagos estimulados con LPS.

En los macrófagos el TNF-α induce la muerte por apoptosis. Para este efecto no es necesaria la inhibición de la síntesis macromolecular, a diferencia de lo descrito en ciertos tipos celulares, donde se postula que la inhibición de la expresión de NF-κB sería necesaria para la inducción de apoptosis por el TNF-α (Beg y Baltimore, 1996; Van Antwerp y col., 1996). La utilización de ratones deficientes de los receptores de TNF-α o de la iNOS, ha permitido determinar la

implicación relativa de la síntesis autocrina de TNF-α y de la producción de óxido nítrico en la inducción de apoptosis por el LPS. En este sentido, nuestros resultados muestran que el LPS induce apoptosis en los macrófagos a través de ambos mecanismos, aunque con cinéticas de actuación distintas. En las fases tempranas de acción del LPS, el principal sistema de inducción de la apoptosis depende de la producción autocrina de TNF-α, mientras que en fases más tardías, la producción de óxido nítrico también jugaría un papel como inductor de apoptosis.

La utilización de ratones KO para el receptor I del TNF-α o bien para los receptores I y II ha dado lugar a resultados idénticos, sugiriendo que el receptor I (p55) del TNF-α es el principal mediador de la inducción de apoptosis con LPS. Estos datos concuerdan con lo que se había descrito en otros tipos celulares (Leist y col., 1995; Tartaglia y col., 1993a; 1993b). Por su parte, los receptores de tipo II parecen tener un papel importante en la inducción de apoptosis en aquellos modelos donde la interacción célula-célula es necesaria (Greeblatt y Elias, 1992; Heller y col., 1990), de manera que el receptor de TNF-α de tipo II podría mediar señales apoptóticas mediante el reconocimiento de moléculas de TNF-α asociadas a la membrana celular.

Nuestros resultados permiten sugerir que el TNF-α y el óxido nítrico inducen apoptosis en los macrófagos por dos vías independientes. Sin embargo, debido a la cinética de actuación de ambos agentes, en situaciones normales será la expresión de TNF-α el principal mediador de la apoptosis y la vía del óxido nítrico pasara a jugar un papel importante sólo en aquellas situaciones donde la expresión de TNF-α se vea inhibida o bloqueada o en aquellos tipos celulares que hayan demostrado resistencia a la inducción de apoptosis por esta citocina. Por este motivo, dedicamos especial interés en averiguar la vía de señalización por la cual el LPS induce la expresión del TNF-α y en consecuencia la inducción de la apoptosis en los macrófagos.

El papel de la PKC en la regulación de diversas actividades en los macrófagos ha sido ampliamente descrita en numerosos trabajos tanto por la estimulación con LPS como por factores de crecimiento (Fujihara y col., 1994; Liu y col., 1994; Shapira y col., 1994). En muchos de estos casos, la especificidad de

la isoforma de PKC determina el tipo de respuesta de los macrófagos. En un reciente estudio *in vivo* con ratones KO de PKCε se ha demostrando que éstos no pueden defenderse de las infecciones bacterianas debido a una insuficiente activación de los macrófagos (Castrillo y col., 2001). En los trabajos presentados en esta Tesis hemos demostrado que PKCε es una molécula clave en la biología de los macrófagos puesto que es la isoforma de PKC responsable de modular la proliferación, supervivencia y activación de los macrófagos.

Así, hemos visto que la inhibición de PKCε bloquea la inducción de apoptosis por el LPS. Sin embargo, el LPS también activa otras vías de señalización además de PKCε en los macrófagos, entre ellas JNK, p38 y ERK-1/2, las tres familias de MAPK quinasas. Hemos demostrado que todas ellas pueden regular la expresión del TNF-α en respuesta al LPS y así la apoptosis de los macrófagos (Fig. 22). En nuestro modelo, JNK tendría un efecto principalmente a nivel transcripcional, mientras que ERK y p38 actuarían a nivel post-transcripcional. De hecho, recientemente se han relacionado estas tres MAPK quinasas en la regulación de TNF-α a diferentes niveles (Dumitru y col., 2000; Kontoyiannis y col., 1999; Kotlyarov y col., 1999). Estudios anteriores al nuestro, han sugerido que ERK-1/2 juega un papel regulador de la transcripción del TNF-α en los monocitos humanos (Van der Bruggen y col., 1999) y en la línea celular RAW 264.7 (Geppert y col., 1994). Sin embargo, otros autores han descrito que en los macrófagos peritoneales ERK-1/2 no ejerce ningún efecto en la transcripción del TNF-α pero si contribuirían en el transporte citoplasmático al núcleo del mRNA (Dumitru y col., 2000). Además, en contraposición a nuestros resultados, otros autores han atribuido a las quinasas JNK y p38 un papel regulador de la traducción o quizás de la estabilidad del RNA mensajero (Kontoyiannis y col., 1999). Todas estas diferencias pueden ser debidas a las diferencias existentes entre los diferentes tipos celulares, como ya habíamos descrito para las acciones de PKC.

A pesar de que las tres MAPK pueden tener un efecto regulando la expresión del TNF-α, nuestros resultados han demostrado que sólo la actividad de JNK está bajo la influencia de PKCε. Hasta el momento desconocemos el mecanismo por el cual la PKCε puede señalizar la activación de JNK aunque se ha

descrito una posible interacción entre PKC y MEKK, un miembro del módulo MAPKKK que conduce a la activación de JNK (Karin, 1995).

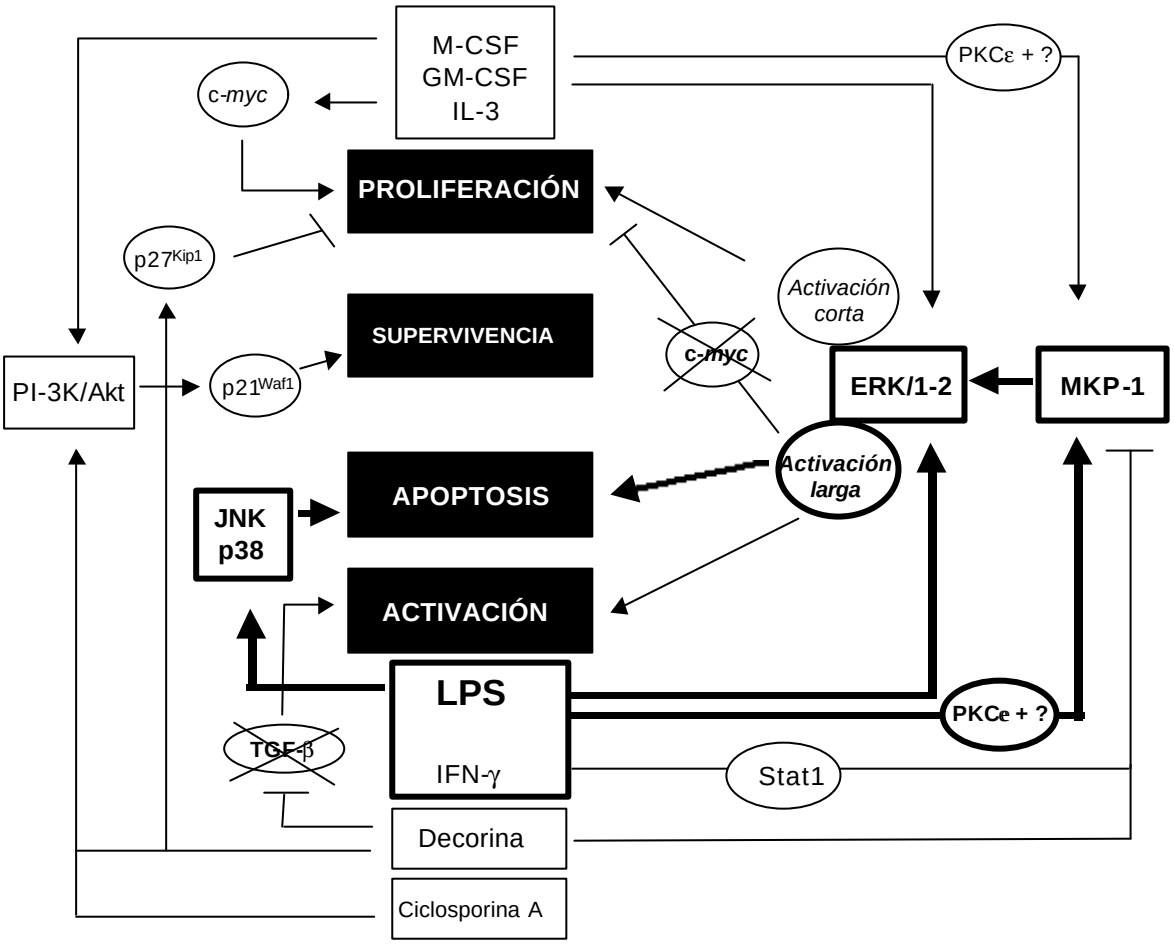


Figura 22. El LPS induce apoptosis a través de la producción autocrina de TNF-a que está condicionada por la activación de PKCe y de las MAPKs.

La activación de JNK por PKCε es la responsable de la inducción de la expresión de TNF-α, a través de las secuencias TRE presentes en su promotor que pueden ser reconocidas por el factor de transcripción AP-1. Así se ha implicado a PKCε en la activación del factor de transcripción AP-1 en diversos sistemas (Reifel-Miller y col., 1996; Tseng y col., 1994). Precisamente, durante la activación de los linfocitos T, PKCε es la principal isoforma involucrada en la activación de AP-1 (Genot y col., 1995). Aunque estamos seguros que la activación de JNK no se encuentra relacionada con la expresión de MKP-1 inducida en respuesta al M-CSF, no podemos asegurar que la activación de JNK por el LPS no juegue un papel en la expresión de MKP-1 y por lo tanto del

alargamiento del patrón de activación de p38 y ERK-1/2. Existen estudios pendientes encaminados a esclarecer esta cuestión.

Como resumen global, debemos señalar que en los macrófagos existe un conjunto de vías de transmisión de señales que funcionan como un eje principal o esqueleto que permite a estas células tomar las decisiones correctas en respuesta al balance entre los estímulos mitogénicos, activadores o de supervivencia que reciben del exterior. En este sentido el patrón de activación de las ERK es clave para las decisiones hacia la proliferación y la activación, mientras que el eje PI-3K/Akt/$p21^{Waf1}$ es un mecanismo general para controlar la supervivencia de estas células. Finalmente, la activación de PKCε podría jugar un papel central en los macrófagos regulando o modulando de forma conjunta las diversas decisiones de los macrófagos, tanto a nivel de proliferación, activación como supervivencia.

Conclusiones

Las principales conclusiones que se pueden derivar de los resultados experimentales presentados en este trabajo son las siguientes:

1. La vía de las MEK/ERK es esencial en la regulación de los procesos de proliferación y activación de los macrófagos aunque con una cinética de activación diferente.

2. La vía de PI-3K/Akt regula la supervivencia de los macrófagos a través de la expresión de $p21^{Waf1}$.

3. El IFN-γ inhibe la proliferación de los macrófagos a través de la inhibición de la expresión de la fosfatasa MKP-1 y una prolongación de la activación de ERK que es capaz de regular la expresión de c-*myc*.

4. La ciclosporina inhibe la activación de las ERK inducida por el M-CSF lo que produce un bloqueo de la proliferación. Este efecto es independiente de la fosfatasa calcineurina.

5. El aumento de adhesión inducido por la decorina inhibe la proliferación de los macrófagos a través de la expresión de $p27^{Kip1}$ y la prolongación de la actividad de las ERK. La decorina aumenta la activación de los macrófagos a través del secuestro del TGF-β producido endógenamente.

6. El LPS induce apoptosis en los macrófagos principalmente mediante la secreción autocrina de TNF-α que está regulada mayoritariamente por PKCε a través de la modulación de la actividad de JNK.

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Anexo

Los trabajos o publicaciones científicas que se han derivado del trabajo realizado durante la realización de esta Tesis, estén o no recogidos en este trabajo, son los siguientes:

1. Valledor, A.F., Xaus, J., **Comalada, M**., Soler, C., y Celada, A. 2000. Protein kinase Cε is required for the induction of mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *J. Immunol*. 164: 29-37.
2. Valledor, A.F., **Comalada, M**., Xaus, J., y Celada, A. 2000. The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. *J. Biol. Chem*. 275: 7403-7409.
3. Xaus, J., **Comalada, M**., Barrachina, M., Herrero, C., Gonalons, E., Soler, C., Lloberas, J. y Celada, A. 2000. The expression of MHCII class II genes in macrophages is cell cycle dependent. *J. Immunol*. 165: 6364-6371.
4. Xaus*, J., **Comalada*, M**., Valledor, A.F., Lloberas, J., Lopez-Soriano, F., Argiles, J.M., Bogdan, C., y Celada, A. 2000. LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-α. *Blood*. 95: 3823-3831. (* coautores)
5. Xaus, J., **Comalada, M.**, Valledor, A.F., Cardó, M., Herrero, C., Soler, C., Lloberas, J., y Celada, A. 2001. Molecular mechanism involved in macrophage survival, proliferation, activation or apoptosis. *Immunobiology*. 204: 543-550.
6. Xaus*, J., **Comalada*, M.**, Cardó*, M., Valledor, A.F., y Celada, A. 2001. Decorin inhibits M-CSF-proliferation of macrophages and enhances cell survival trough induction of $p27^{kip1}$ and $p21^{waf1}$. *Blood*. 98: 2124-2133. (*coautores)
7. Soler, C., García-Manteiga, J., Valdés, R., Xaus, J., **Comalada, M.**, Casado, F.J., Pastor-Anglada, M., Celada, A., y Felipe, A. 2001. Macrophages require different nucleoside transport systems for proliferation and activation. *FASEB J.* 15: 1979-1988.
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macrophages determines the up-regulation of concentrative nucleoside transporters Cnt1 and Cnt2 through tumor necrosis factor-α-dependent and –independent mechanisms. *J. Biol. Chem*. 276: 30043-30049.

9. Herrero, C., Sebastián, C., Marqués, L., **Comalada, M**., Xaus, J., Valledor, A.F., Lloberas, J., y Celada, A. 2002. Immunosenescence of macrophages: reduced MHC class II gene expression. *Exp. Gerontol*. 37: 389-394.
10. **Comalada, M**., Valledor, A.F., Umbert, I., Xaus, J., y Celada, A. 2002. Cyclosporin A and FK506 block macrophage proliferation and inhibit the activation of extracellular signal-regulated kinases through a calcineurin-independent mechanism. (*Sometido a publicación.*)
11. **Comalada, M**., Cardó, M., Xaus, J., Valledor, A.F., Lloberas, J., Ventura, F., y Celada, A. 2002. Decorin reverses the repressive effect of the autocrine produced TGF-β on macrophage activation. (*Sometido a publicación*)
12. **Comalada, M.**, Valledor, A.F., Xaus, J., Gil, M.P., Schreiber, R.D., y Celada, A. 2002. IFN-γ inhibits macrophage proliferation via the prolongation of the activation pattern of ERK-1/2, which regulates c-*myc* expression. (*Sometido a publicación*)
13. **Comalada, M**., Xaus, J., Valledor, A.F., Sanchez, E., y Celada, A. 2002. M-CSF-dependent survival of macrophages, but not proliferation, involves de $p21^{Waf1}$ induction through PI-3kinase/AKT pathway. (*Sometido a publicación).*
14. **Comalada, M**., Xaus, J., Valledor, A.F., Lopez-Lopez, C., Bosca, L., Parker, P.J., y Celada, A. 2002. PKCε, mostly through JNK activation, mediates the LPS-induced TNF-α that induces apoptosis in macrophages. (*Sometido a publicación*).