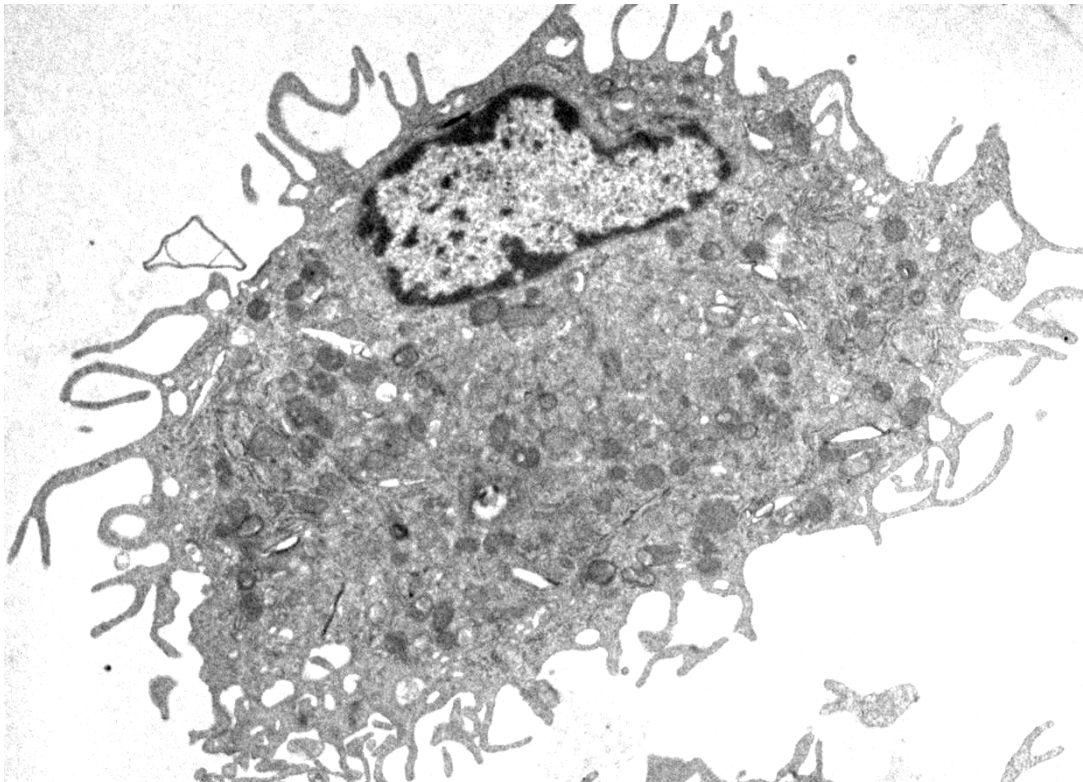# Early steps regulating proliferation and activation in macrophages

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DISCUSSION

DISCUSSION

Macrophages play a key role in natural and acquired specific immune responses. As the first line of defense, these cells are able to recognize foreign pathogens and strange particles and phagocytes them. Consequently, they are able to release cytokines to resolve the infection locally and chemokines to recruit a specific cellular response. The presence of certain cytokines promotes the antigenic presentation leading to a specific response. Resolving of inflammation also involves macrophage participation.

In consequence, the macrophage repertoire must be maintained for a correct immune response. In adults, monocytes are generated in bone marrow and transvasate to different tissues differentiating to macrophages. Locally, in absence of factors, macrophages remain quiescent or proliferate in presence of local or autocrine mitogens such as M-CSF, GM-CSF or IL-3, maintaining a high and constant number of cells.

However, when activating signals appear such as LPS or IFN-γ in a Th1 environment, macrophages stop their proliferation program and acquire effectors functions. LPS activates macrophages to increase the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6, IL-8 and to up-regulate NOS2 expression, which play important roles in the immune response (Marchant, Bruyns et al. 1994). This response contributes to an efficient control of growth and dissemination of invading pathogens. Conversely, products induced by LPS can act as intracellular or extracellular messengers.

On the other hand, IFN-γ promotes inhibition of proliferation increasing macrophage survival together with up-regulation of class II MHC molecules to trigger T cell responses. In addition, synergistically both LPS and IFN-γ increase microbicidal activity enhancing cytokine and NO production. The macrophage response thus differs from that of T lymphocytes which are in a quiescent state in normal conditions and their stimulation is responsible for increasing their clonal expansion.

To avoid possible problems of cell line models, our studies have been carried on using non-transformed primary cultures of macrophages obtained from bone marrow resembling physiological conditions in animal models. Physiologically, monocytes differentiate to several types of macrophages depending on the tissue they reach, including microglia in CNS, Kuppfer cells in liver, Langerhans cells, alveolar and peritoneal macrophages, etc. This specificity is reflected by different cellular responses and the activation of different signal transduction pathways with a common protein subset. As an example of these differences, medullar and microglia macrophages are highly proliferating populations whereas peritoneal or blood monocytes are not (Celada

and Maki 1992). We obtained our cultures *in vitro* in well established conditions. These cells thus had not been previously activated and respond to growth factors with intact cell cycle machinery, to activation, to differentiation or can be induced to die by apoptosis.

Based on our cellular model, we studied proliferation and activation signal transduction mechanisms trying to elucidate cross talking processes that balance differential responses to extracellular stimuli. Because of the high complexity of these pathways, we focused on the main pathways involved in macrophage proliferation and activation and the effects of a new cyclophilin A binding drug on macrophage biology.

Most of the studies on differentiated macrophages were based on pharmacological inhibitors due to the difficulty of transfecting primary cultures of macrophages with plasmids (Marais, Light et al. 1995; Mason, Springer et al. 1999; Chong, Vikis et al. 2003). However, macrophages are able to incorporate small sequences such as interference RNA. Because of the limitations of inhibitor use such as cell permeability, specificity, mechanism of action, reversibility and half life, these studies are open to criticism. Consequently, some studies were corroborated using alternative inhibitors, extensive unspecific controls, interference RNA or macrophages from knock-out mice. Studies of IC50 and toxicity were also performed to use minimal non toxic and effective doses.

As previously explained in another thesis, proliferation and activation are opposite responses in bone marrow derived macrophages (Xaus, Comalada et al. 2001). Studies using different mitogenic and activating stimuli revealed that they use different receptors. However, both responses share similar signal transduction pathways. Both stimuli require the activation of the classical Raf, MEK, ERK-1/2 pathway which has been clearly implicated in proliferation and proinflammatory cytokine production (Reimann, Buscher et al. 1994; Zheng and Guan 1994; Valledor, Xaus et al. 1999; Valledor, Comalada et al. 2000).

The differences observed in proliferation and activation are mainly based on different kinetics of activation of ERK kinase module (Valledor, Comalada et al. 2000). M-CSF induces an early activation of the classical module giving peak of ERK activation at 5 min. Other mitogenic stimuli such as GM-CSF, IL-3 and phorbol esters induce a similar pattern of activation, as well. Our results thus suggest that ERK is not alone in displaying an early kinetics of activation, upstream kinases such as Raf-1 and MEK-1 do so as well.

For its part, LPS is able to block macrophage proliferation and induce the production of proinflammatory cytokines and NOS2. In comparison to proliferating stimuli, LPS causes a delayed activation of ERK-1/2 with a peak at 15 min (Buscher, Hipskind et al. 1995), which correlates with delayed activation of Raf-1 and MEK-1 components as well. A delayed ERK activation has also been observed with exogenous PC-

PLC. The correlation of distinct kinetics of ERK-1/2 activation and deactivation with a range of macrophagic functions has also been observed (Buscher, Hipskind et al. 1995).

This finding indicates that specific events that occur upstream of Raf-1 signaling may be responsible for these differences. Upstream molecules implicated could be Ras which has been involved in M-CSF, but not in LPS, signaling towards Raf-1 activation, whereas phosphatidylcholine phospholipase C (PL-PLC) appears to mediate Raf-1 activation in LPS-stimulated cells (Hamilton 1997). Also, differences in receptor assembly and activation could account for the differential time-courses; *c-fms* receptor requires non-covalent dimerization and autophosphorylation of tyrosine kinase domains (Shimazu, Akashi et al. 1999; Akashi, Shimazu et al. 2000), whereas the recognition of LPS requires its binding to LBP, which transfers LPS monomer to the membrane bound CD14 molecule which presents it to the MD-2/TLR4 complex causing its dimerization (Palsson-McDermott and O'Neill 2004). Moreover, the functional integrity of the LPS receptor also depends on glycosilations of MD-2/TLR4 (Valledor, Comalada et al. 2000). At high doses, a TLR2 recognition of LPS has also been described (Ulevitch and Tobias 1995; Chow, Young et al. 1999; Baumgarten, Knuefermann et al. 2001). All these data suggest that early intracellular event confers specificity on macrophage response.

Furthermore, we have observed that while ERK phosphorylation in response to M-CSF is a Raf-1-dependent process, in response to LPS alternative pathways may direct the activation of these kinases. This was also reflected at functional level, since ERK-1/2 is required for both cell cycle progression and for expression of proinflammatory cytokines (TNF-α, IL-1β and IL-6). The inhibition of Raf-1 activity compromised the progression of macrophages through the cell cycle whereas no effect was observed in proinflammatory response upon LPS stimulation. The involvement of Raf-1 in mediating proliferation has also been described in fibroblasts (Jamal and Ziff 1990; Qureshi, Rim et al. 1991; Bruder, Heidecker et al. 1992). In this context, the Raf-1 knockout has confirmed that Raf-1 is required for the viability of mice embryos and for ERK activation by mitogens in fibroblasts (Jamal and Ziff 1990).

From several cell cycle regulators studied, G1 blockage caused by Raf-1 inhibition seems to be mediated by increased expression of cyclin-dependent kinase inhibitors, p21^{Waf-1} and p27^{Kip-1}. In agreement with our data, oncogenic activation of Raf-1 suppresses the expression of p27^{Kip-1} in NIH3T3 cells (Valledor, Xaus et al. 2000). The data were corroborated by the use of other disruptors of Raf-1 activity, specific inhibitor and small interference RNA to Raf-1.

Independently of the time-course of activation of MAPKs, deactivation kinetics is also crucial for the macrophage decision to proliferate or activate. As an example, IFN-γ

activation of macrophages prolongs ERK activation in presence of M-CSF blocking macrophage proliferation (Xaus, Cardo et al. 1999). Through STAT-1 dependent mechanisms, IFN-γ causes this effect through the inhibition of phosphatases such as MKP-1 and other MKPs which are responsible for dephosphorylating MAPK activation. All these data suggest that MKPs are a potential target to regulate ERK dependent macrophage responses (Camps, Nichols et al. 2000; Keyse 2000).

MKP-1 expression is also subjected to a kinetically differential induction by proliferating and activating signals, M-CSF induces a 30 min MKP-1 peak of expression whereas LPS at 45 min. Interestingly, deactivation of ERK-1/2 is correlated temporally with MKP-1 induction in both stimuli (Valledor, Xaus et al. 2000; Comalada, Valledor et al. 2003).

Regulation of MKP-1 expression has been widely studied in several cell types. Previous results in our group demonstrated that from all PKC isoforms expressed in bone marrow macrophages, PKCβI, PKCϵ and PKCζ, only PKCϵ isoform, constitutively located in the membrane, is a key regulator in ERK-independent MKP-1 induction (Valledor, Xaus et al. 1999). All these data centered our interest in MKP-1 regulation by other kinases, including Raf-1 and ERK, JNK and p38 MAPKs.

From the data presented with pharmacological inhibitors and siRNA, it can be concluded that Raf-1 activity is required for both M-CSF and LPS induction of MKP-1 phosphatase (Fig. 25). Previous reports showed that ectopic expression of v-raf in macrophages also induces MKP-1 (Sun, Charles et al. 1993; Wu and Bennett 2005). In accordance with its role as MAPK phosphatase, a prolongation of both ERK and p38 patterns of activation was detected in MKP-1 absence.

The mechanism used by Raf-1 to induce MKP-1 expression in macrophages probably involves the activation of PKCϵ. Our data demonstrate that Raf-1 interacts and mediates the activation of PKCϵ in response to M-CSF and to LPS. However, our observations are in agreement with previous studies that demonstrated that Ras recruits Raf-1 to the plasma membrane where it can interact with PKCϵ and act as a co-regulator and cooperator of PKC-derived signals (Valledor, Xaus et al. 1999).

The study in parallel of Raf-1 influence on cytokine production by LPS revealed that Raf-1 may limit the levels of cytokine production. Several hypotheses could explain this. First, an extended MAPK activation in absence of MKP-1 in absence of Raf-1 activity may enhance the proinflammatory response. On the other hand, it has been previously described that MKP-1 may limit cytokine production through inactivation of MAPKs involved in their expression and stabilization (Guha and Mackman 2002; Shelton, Steelman et al. 2003). Corroborating this, the use of MKP-1 knock-out mice revealed an

increase in cytokine production by LPS. Moreover, Raf-1 and PI3K interactions have also been described, and some authors suggested that PI3K acts also as attenuator of LPS proinflammatory response (Guthridge, Eidlen et al. 1997; Monick, Carter et al. 2000). Further experiments will be required to elucidate this.
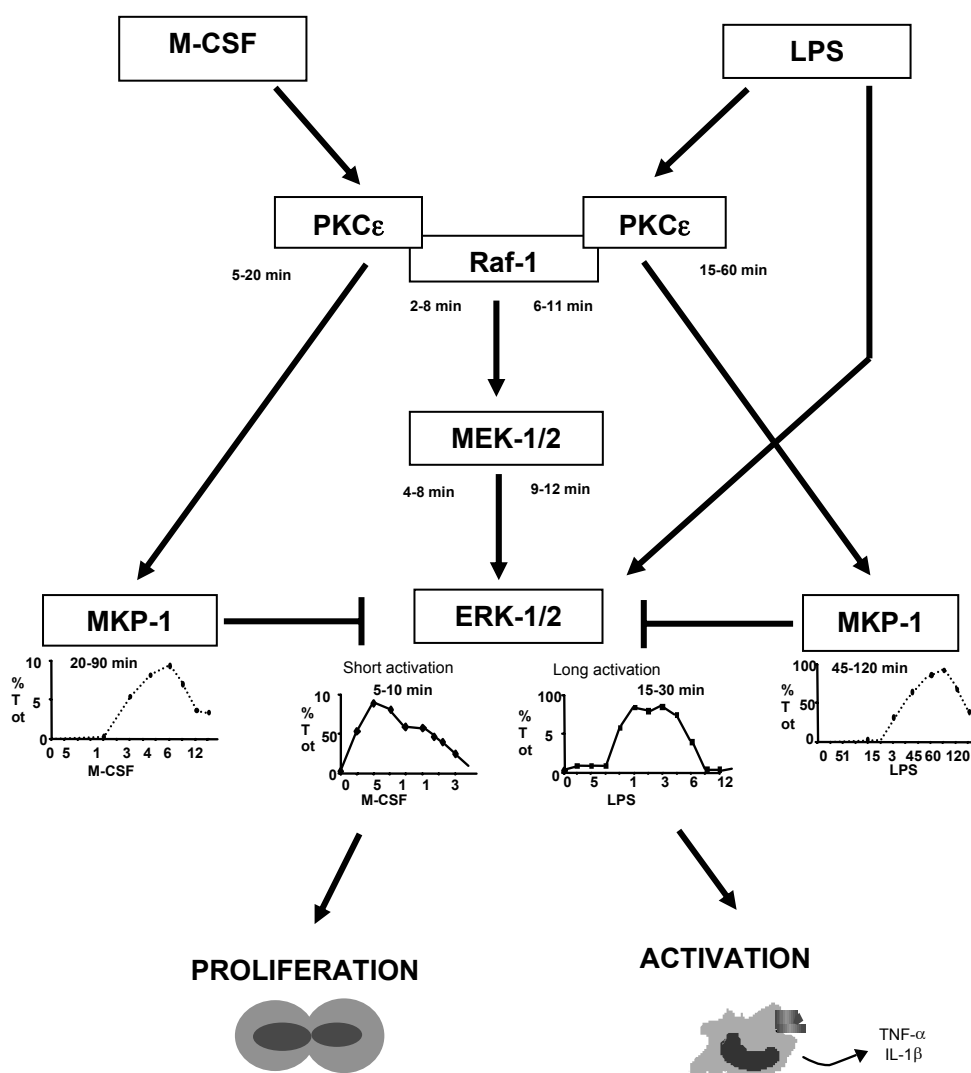

Figure 25. Different kinetics of Raf-1, MEK and ERK-1/2 activation determine macrophage response. Short and early activation of Raf, MEK, ERK lead macrophage response to proliferation whereas a long and delayed activation corresponds to macrophage activation. Both stimuli induce Raf-1 and PKCε activation for expressing MKP-1 phosphatase responsible of deactivating ERK-1/2 activity. Raf-1 activity is indispensable for M-CSF induced activation of ERK-1/2 whereas LPS can use alternative pathways.

As a summary, during macrophage signal transduction to M-CSF, Raf-1 plays two crucial roles that result in tight regulation of the pattern of ERK activation. Firstly, Raf-1

activity accounts for the positive regulation of the MEK-ERK module, which is later required for macrophage proliferation. Secondly, Raf-1 is involved in expression of the phosphatase MKP-1, a key negative regulator of MAPK activity. On the other hand, LPS activates signaling events that are capable of bypassing Raf-1 activation and still inducing ERK-1/2 activity and pro-inflammatory cytokine production. Other macrophagic cells, including the RAW 264.7 cell line and primary alveolar macrophages, phosphorylate ERK during LPS stimulation without activation of Raf-1 (Salmeron, Ahmad et al. 1996; Waterfield, Zhang et al. 2003). Although MEK-1/2 are the major substrates of Raf proteins, the presence of an alternative upstream MAPKKK has been proposed and includes the serine/threonine kinase Cot/Tpl2 (Nebreda, Hill et al. 1993), the proto-oncogene Mos kinase (Lange-Carter and Johnson 1994) and a 73-kDa MEKK in EGF-treated PC-12 cells (Krautwald, Buscher et al. 1995). In fact, Cot/Tpl2 kinases have been able to activate both ERK and JNK (Fanger, Johnson et al. 1997; Robinson and Cobb 1997) and transfection of this kinase in T lymphocytes induces the TNF-α expression (Salmeron, Ahmad et al. 1996).

The role of MAPKs in MKP-1 phosphatase expression and MKP-1 specificity is highly controversial, as reflected in this manuscript. The origin of cell type, differentiation stage, different gene expression and experimental conditions seem to be involved in these discrepancies and highly controlled culture conditions should be used. In our model, studies of MAPK involvement in MKP-1 induction by both proliferative and activating stimuli revealed that in contrast to the findings in other cell types such as fibroblasts (Brondello, Brunet et al. 1997; Valledor, Xaus et al. 1999; Valledor, Xaus et al. 2000), MKP-1 induction by M-CSF and LPS in macrophages is independent of ERK activation (Xaus, Comalada et al. 2001; Comalada, Valledor et al. 2003). Interestingly, a number of conditions that inhibit MKP-1 expression in macrophages, for example extracellular matrix proteins such as decorin or fibrinogen, or treatment with cyclosporine A or FK506, prolongs ERK activity and reduce proliferation (Valledor, Xaus et al. 1999; Valledor, Comalada et al. 2000; Valledor, Xaus et al. 2000) also suggesting ERK independent pathways of MKP-1 induction in macrophages.

As assessed by using specific inhibitors, our results revealed that from all MAPKs, both M-CSF and LPS require only JNK activation for MKP-1 induction and that neither ERK-1/2 nor p38 were involved. Recently, it has also been shown that in fibroblasts the expression of MKP-1 is induced by activation of the stress-response pathways MEKK-SEK-SAPK rather than the Raf-MEK-MAP-signaling pathway stimulation (Bokemeyer, Sorokin et al. 1996). However, we cannot rule out ERK-1/2 or p38 implication in MKP-1 expression or in increasing stability by phosphorylation, as previously described (Brondello, Pouyssegur et al. 1999).

Two JNK genes, *jnk1* and *jnk2,* are constitutively expressed in macrophages. Using JNK1 and JNK2 single knock-out mice, a kind gift from Dra. Carme Caelles, we have determined that JNK1 but not JNK2 isoforms are involved in MKP-1 induction by both stimuli. Studies with JNK3 knock-out mice were also performed and no alteration of macrophage biology tested was observed (data not shown).

Targeted disruption of the JNK-kinase and stress and extracellular signal-activated kinase 1 (SEK-1), causes defects in the transcriptional activity of AP-1 implicated in the regulation of cytokine gene expression (Yang, Tournier et al. 1997; Foletta, Segal et al. 1998). Accordingly, we demonstrated a role for JNK1 but not for JNK2 in proinflammatory cytokine biosynthesis (TNF-α, IL-1β and IL-6) and NO production induced by LPS and TNF-α activation, suggesting that JNK activation mediates macrophage activation by LPS. The data give evidence of major JNK1 involvement in MKP-1 transcriptional induction by both M-CSF and LPS probably through c-jun transcriptional regulation as assessed in JNK1 knock-out macrophages. Indeed, gain-of function JNK1 studies in fibroblast showed MKP-1 increased induction (Han, Kim et al. 2002). Furthermore, JNK2 knock-out macrophages showed a slightly increased proinflammatory response to LPS that could be related to the described role of JNK2 in interfering JNK1 activation (Liu, Minemoto et al. 2004).

As expected, blockage of JNK activation causes a slight prolongation of ERK and p38 activation as observed inhibiting PKCϵ, suggesting that stimuli that lead to MAPK activation also lead to induction of attenuators providing a negative feedback mechanism to control the activation of other MAPKs. Other studies have also reported a JNK role in attenuating ERK activation (Shen, Godlewski et al. 2003). Although the activation of JNK by PKCϵ has not been explored, there are reports of an interaction between PKC and MEKK which is responsible for JNK activation (Karin 1995).

These results demonstrate conclusively that JNK1 is involved in two mechanisms critical for macrophage functions (Fig. 26). It acts as a regulator of MKP-1 phosphatase which controls the extent of other MAPKs, presenting a scenario where the same kinases that need to be dephosphorylated regulate the expression of their phosphatases. In addition, JNK1 is involved in the proinflammatory response induced by LPS. These data offer new insights to target abnormal inflammatory response by activated macrophages. Little is known about the role of JNK in human cancer, but it has been observed that activated JNK may promote a transformed phenotype (Raitano, Halpern et al. 1995; Rodrigues, Park et al. 1997) and constitutively active JNK activity has been described in human tumor lines and inflammatory diseases (Bost, McKay et al. 1997; Teng, Perry et al. 1997). In addition, MKP-1 has been found over expressed in

many malignant human tumors (Bang, Kwon et al. 1998; Liao, Guo et al. 2003), suggesting a correlation between JNK and MKP-1 expression. Therefore, we speculate that JNK could be a novel therapeutic target to regulate MKP-1 expression in tumors or in exaggerated inflammatory responses seen in some lung diseases such as asthma and acute respiratory distress syndrome.
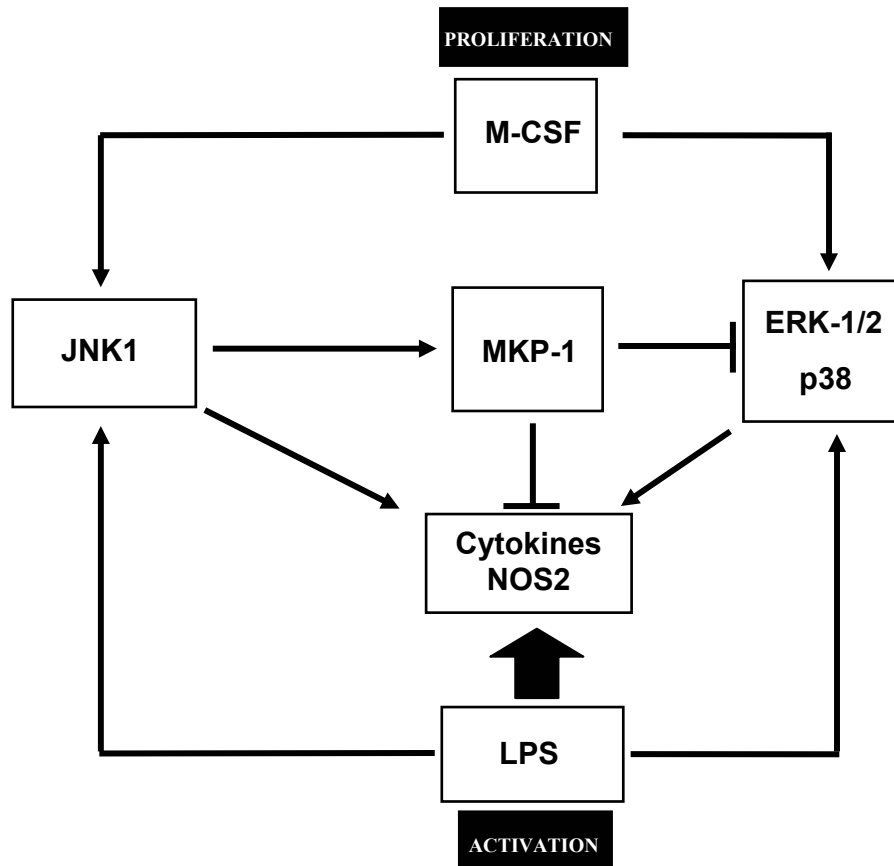

Figure 26. JNK1 implication in macrophage biology. JNK1 activity is required for M-CSF and LPS induction of MKP-1 phosphatase responsible of dephosphorylating MAPKs. Proinflammatory response induced by LPS requires JNK1, ERK-1/2 and p38 activity and it is attenuated by MKP-1.

Comparing the requirement of JNK isoforms in macrophages and other cell types, JNK1 seem to downregulate T cell proliferation and increase apoptotic mechanisms promoting a Th2 response (Yang, Tournier et al. 1997). In this regard, JNK1 does not alter macrophage proliferation and is involved in LPS induced cytokine production, typical traits of a Th1 response (Sabapathy, Hu et al. 1999). Although JNK2 has been described to be required for IFN-γ production by Th1 cells (Kuan, Yang et al. 1999), our results

demonstrate that it has no significant role in macrophages in any of the experiments tested.

Importantly, we have determined a role for JNK1 in the activation of macrophages with IFN-γ (data not shown). IFN-γ has been described to block macrophage proliferation and S phase progression causing a G1 blockage (Xaus, Cardo et al. 1999). Curiously, in JNK1 deficient mice the blockage of cell proliferation and cell cycle progression by IFN-γ was altered, suggesting a role for JNK in macrophage activation induced by IFN-γ. Furthermore, studies with JNK inhibitor showed that macrophages were not able to induce class II MHC proteins or CIITA expression correctly due to early defects in phosphorylation of STAT-1 signal transducer of IFN-γ signaling. Other studies suggest that several members of the AP-1 family and ATF-2 proteins are recruited to the class II MHC promoter (Neve Ombra, Autiero et al. 1993; Zhu, Linhoff et al. 2000). In support of an emerging role for JNK in the innate immune response, studies of JNK1 gain-of-function fibroblasts showed increased expression of interferon-responsive genes (Han, Kim et al. 2002). Further studies are required but regulation of the expression of MHC II molecules by JNK may become a critical point in the control and maintenance of the immune response.

Our results thus support different and non-redundant physiological roles of JNK1 and JNK2 isoforms on macrophage biology in response to M-CSF and to LPS; though we cannot rule out the possibility that unaffected biological processes could be due to redundant or at least overlapping functions of both coexpressed isoforms of JNK. Although we have observed no defects in proliferation of macrophages from JNK single knock-outs (data not shown), JNK1/JNK2 double knock-out fibroblasts die embryonically and display complete absence of JNK activity proliferating more slowly (Fehr, Kallen et al. 1999; Sabapathy, Jochum et al. 1999; Sanglier, Quesniaux et al. 1999; Tournier, Hess et al. 2000). The role of the JNK2 in apoptosis and cell cycle progression remains to be clarified as well. However, JNK may also play a protective role supporting cell survival through Akt interaction (Logan, Falasca et al. 1997; Lopez-Ilasaca, Li et al. 1997; Aikin, Maysinger et al. 2004; Sunayama, Tsuruta et al. 2005).

Both M-CSF and LPS activate JNK with the same kinetics (Kyriakis and Avruch 1996). This excludes the possibility of being responsible for the different kinetics displayed by MKP-1 induction with M-CSF and LPS. We hypothesize that Raf-1 and PKCε, which can interact and display different kinetics in response to these stimuli, may be involved in differences in MKP-1 induction.

The involvement of Raf-1 and JNK1 in MKP-1 transcriptional induction and promoter requirements is currently being studied. However, the literature suggests a role of Raf-1 in regulating nuclear events such as c-fos and c-jun which regulate Ets-1 and AP-1 binding sites (Cook, Aziz et al. 1999). Not presented data of inhibition of c-jun and c-fos transcriptional expression in Raf-1 inhibited cells also demonstrate this hypothesis. A PKCε and JNK role in AP-1 activation has been described as well (Derijard, Hibi et al. 1994; Genot, Parker et al. 1995; Reifel-Miller, Conarty et al. 1996; Davis 2000). Accordingly, the promoter of the MKP-1 gene contains AP-1 sites at -450 bp position which can be dependent on all these kinases (Charles, Sun et al. 1993; Kwak, Hakes et al. 1994; Marais, Light et al. 1995). Regulation of AP-1 activity occurs by induced transcription of c-jun and c-fos as well as by posttranslational phosphorylation of their products (Foletta, Segal et al. 1998; Morton, Davis et al. 2003). In addition to an AP-1 element present in the MKP-1 promoter, two cyclic AMP responsive elements (CRE), AP-2 and E-box elements are present (Kwak, Hakes et al. 1994; Ryser, Massiha et al. 2004). The activity of CRE elements may also respond to c-jun, CREM and ATF-2 transcription factors (Cook, Beltman et al. 1997).

MKPs involved in MAPK inactivation are induced by a great variety of stimuli such as serum and growth factor stimulation, phorbol esters, oxidative stress and heat shock (Keyse 1995; Keyse 2000; Theodosiou and Ashworth 2002). The wide family of MKP members and different specificity supports a spatially and temporally ordered manner to control the extent, location and duration of MAPK activation. In fact, although some prolongation in phospho ERK MAPKs is observed in LPS and M-CSF stimulated macrophages and in fibroblasts derived from MKP-1 knock-out mice (Dorfman, Carrasco et al. 1996) and data not shown), ERK-1/2 was briefly dephosphorylated suggesting that other MKPs or phosphatases could be induced to compensate the loss of MKP-1 activity.

MKP-1 deficient mice and RNA interference technology demonstrated that other phosphatases such as MKP-4 could be subserving the loss of MKP-1 activity (Dorfman, Carrasco et al. 1996). Unpublished results in our group seem to indicate that IFN-γ elongates ERK activity by inhibiting both nuclear MKP-1 and cytoplasmic MKP-4 expression (manuscript in preparation). The study of expression of several MKPs in JNK1 knock-out mice stimulated with both M-CSF and LPS was also performed (data not shown). Since MKP-1 but not MKP-4 inhibition in JNK1 macrophages was observed, these data could explain the correct ERK dephosphorylation during LPS stimulation.

Furthermore, several data indicate that from all MKPs induced by M-CSF (MKP-1, MKP-4, PAC-1, MKP-2, MKP-X and CPG21), only MKP-1 phosphatase is dependent on JNK1 activity, and that IFN-γ downregulation of MKP-4 is mediated by a JNK1 independent

mechanism. Low or no induction of MKP-3, and MKP-5 were observed in presence of M-CSF.

Curiously, kinetics and JNK1 dependence of MKP expression by LPS differs from that of M-CSF. As described previously in RAW 264.7 macrophages (Camps, Nichols et al. 2000; Keyse 2000; Matsuguchi, Musikacharoen et al. 2001), our studies show that apart from all MKPs induced by M-CSF, LPS also induces the expression of MKP-5 and MKP-M. However, MKP-3, which has been suggested to have a role in cytoplasmic retention of inactive ERK, was down-regulated during LPS stimulation as described previously (Muda, Boschert et al. 1996; Karlsson, Mathers et al. 2004). However, apart from MKP-1, only MKP-5 phosphatase seems to be critically down-regulated in JNK1 knock-out macrophages during LPS stimulation. Since MKP-5 has been described to be localized in both the nucleus and cytoplasm with specific inhibitory activity against p38 and JNK (Tanoue, Moriguchi et al. 1999; Theodosiou, Smith et al. 1999; Tamura, Hanada et al. 2002), results suggest that JNK1 could be activating self-regulatory mechanisms that require further studies. The time course induction of several MKPs by M-CSF and LPS and the involvement of MAPKs in their induction remain to be determined.