Role of NFAT5 in macrophage polarisation

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ABSTRACT

Macrophages are exquisite sensors of tissue homeostasis that can rapidly switch between pro- and anti-inflammatory or regulatory modes to respond to perturbations in the microenvironment. This functional plasticity involves a precise orchestration of gene expression patterns whose transcriptional regulators have not been fully characterised. We have previously identified the transcription factor NFAT5 as an activator of Toll-like receptor-induced responses, and here we explore its contribution to macrophage functions in different polarisation settings. We found that both in classically and in alternatively-polarised macrophages NFAT5 enhanced functions associated with a pro-inflammatory profile such as bactericidal capacity and the ability to promote Th1 polarisation over Th2 responses. In this regard, NFAT5 upregulated the Th1-stimulatory cytokine IL-12 in classically-activated macrophages, whereas in alternatively polarised ones it enhanced the expression of the pro-Th1 mediators *Relma* and *Arg1*, indicating that it could promote pro-inflammatory readiness by regulating independent genes in differently polarised macrophages. Finally, *in vivo* assays revealed a reduced anti-tumour capacity in NFAT5-deficient macrophages against syngeneic Lewis lung carcinoma and ID8 ovarian carcinoma cells, a defect that in the ID8 model was associated with a reduced accumulation of effector CD8 T cells at the tumour site. Altogether, analysis of the effect of NFAT5 in the pro- and anti-inflammatory macrophages uncovered its ability to regulate distinct genes under both polarisation modes and revealed its predominant role in promoting pro-inflammatory macrophage functions ranging from facilitating Th1 responses to restraining tumour progression.

RESUM DE LA TESI

Els macròfags són cèl·lules del sistema immunitari innat que es troben en tots els òrgans. Als teixits, els macròfags actuen per mantenir la homeòstasi canviant ràpidament entre estats inflamatoris i antiinflamatoris per respondre eficientment a les pertorbacions que succeeixen en el seu entorn. Aquesta plasticitat funcional requereix l'expressió coordinada de gens que estan regulats per factors que encara no han estat completament caracteritzats. El nostre grup havia descrit prèviament que el factor de transcripció NFAT5 actua com a activador de les respostes induïdes pels receptors de tipus Toll (TLRs), i en aquest treball explorem la seva contribució com a regulador de les funcions dels macròfags en diferents condicions de polarització. Hem vist que tant en els macròfags clàssicament activats (M1) com en els activats de manera alternativa (M2), NFAT5 participa en la inducció de les seves funcions inflamatòries, com ara la capacitat per a eliminar bacteris intracel·lulars o la inducció de respostes Th1 en limfòcits T CD4. En aquest sentit, NFAT5 és necessari per a l'expressió de la citocina IL-12 en macròfags clàssicament activats, important inductora de respostes de tipus 1, i també participa en l'expressió de gens com *Relma* i *Arg1* en macròfags M2, mediadors que limiten les respostes Th2. Aquestes observacions indiquen que NFAT5 facilita l'adquisició d'un estat proinflamatori en macròfags. A més a més, els assajos *in vivo* amb tumors singènics de pulmó (Lewis lung carcinoma) i el carcinoma d'ovari (ID8) indiquen que els macròfags deficients en NFAT5 tenen una menor capacitat antitumoral. En el cas del model de la ID8, la incapacitat per a limitar el creixement tumoral està associada a un menor número de cèl·lules CD8 activades en el lloc de la implantació del

tumor, suggerint una resposta CD4 Th1 deficient. En resum, els nostres resultats indiquen que NFAT5 és capaç de regular un conjunt de gens que en diferents condicions de polarització dels macròfags contribueixen preferentment en la inducció de funcions inflamatòries, com ara la inducció de respostes Th1 i la restricció de la progressió tumoral.

PREFACE

Nowadays there is a great interest in developing immunotherapies as alternative treatments to fight diseases that prove difficult to be cured with conventional therapies. To this end, current research in the field is focused on studying the interplay between the immune system and diseases such as cancer, to take advantage of our own biological weapons to fight them. To accomplish this, it is necessary to unravel the molecular mechanisms that govern leukocyte responses to several disorders. My project seeks to improve our knowledge on the molecular mechanisms that control macrophage responses. Macrophages are cells from the innate immune system that are seeded in every organ to maintain body homeostasis. In this regard, macrophages sense perturbations in their microenvironment and acquire pro- or anti-inflammatory functions necessary to restore homeostasis or cause diseases. My work has identified NFAT5 as a transcriptional regulator of macrophage functional polarisation that is particularly important for the acquisition of a pro-inflammatory state. In addition, my work has unravelled NFAT5-specific functions in macrophages that contribute to control tumour progression.

ABBREVIATIONS

AED	Auxiliary export domain
Akt (PKB)	Protein kinase B
ALR	AIM2-like receptor
ANG2	Angiopoietin 2
AP-1	Activator protein 1
APC	Antigen presenting cell
AR	Aldose reductase
ATM	Ataxia telangiectasia-mutated
BAFF	B cell-activating factor
BCG	Bacillus Calmette–Guérin
Bcl2	B-cell lymphoma 2
BGT1	Betaine transporter
BMDM	Bone marrow-derived macrophage
C/EBPβ	CCAAT/enhancer binding protein beta
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDP	Common dendric cell progenitor
CLR	C-type lectin receptor
COX	Cyclooxygenase
CSF1R	Colony-stimulating factor 1 receptor
CTLA	Cytolytic T lymphocyte-associated antigen
Cxcl	CXC chemokine ligand
DAMP	Damage-associated molecular pattern
DBD	DNA-binding domain
DC	Dendritic cell
DEN	Diethylnitrosamine
DN	Double negative
DP	Double positive
dsRNA	Double-strand RNA
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
FOXP3	Forkhead box P3

GAS	Gamma-activated sequence
GATA6	GATA binding protein 6
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1 protein
HRG	Histidine-rich glycoprotein
HSP	Heat shock protein
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNAR	Interferon-α/β receptor
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPS-1	IFN-β-promoter stimulator 1
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IRS2	Insulin receptor substrate 2
ISG	Interferon stimulated gene
IVIg	Intravenous immunoglobulin
IκBα	NF-κB inhibitor alpha
JAK	Janus kinase
Jmjd3	Jumonji domain containing-3
JNK	c-Jun N-terminal kinase
KLF4	Krüppel-like factor 4
KO	Knock-out
LBP	LPS-binding protein
LDI	Low dose irradiation
LDL	Low density lipoprotein
LLC	Lewis lung carcinoma

LPS	Lipopolysaccharide
LT-α	Lymphotoxin alpha
LXRα	Liver X receptor alpha
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MDP	Macrophage/dendritic cell progenitor
MerTK	Mer tyrosine kinase receptor
MHC	Major histocompatibility complex
miRNA	Micro RNA
MMP	Metalloproteinase
MMTV	Mouse mammary tumour virus
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MTb	Mycobacterium tuberculosis
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT cell	Natural killer T cell
NLR	Nod-like receptor
NLS	Nuclear localisation signal
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood leukocytes
PD-1	Programmed cell death protein 1
PDGF	Platelet-derived growth-factor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIGF	Placental growth factor
PMBC	Peripheral blood mononuclear cells
PPARγ	Peroxisome proliferator activated receptor gamma
PRR	Pathogen recognition receptor
PTX	Paclitaxel
PyMT	Polyoma middle T oncoprotein

RANKL	Receptor activator of nuclear factor-κB ligand
RHR	Rel-like homology region
RLR	RIG-like receptor
ROR	retinoid-related orphan receptor
RT-qPCR	Real-time quantitative polymerase chain reaction
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
SDF-1	Stromal-derived factor-1
SGK1	Serum and glucocorticoid-regulated kinase 1
siRNA	Small interfering RNA
SMIT	Sodium-myo-inositol transporter
ssRNA	Single-strand RNA
STAT	Signal transducer and activator of transcription
TAB	TAK1-binding protein
TAD	Transactivation domain
TAK1	TGF-β-activated kinase 1
TAM	Tumour associated macrophage
TauT	Taurine transporter
T-bet	T-box transcription factor TBX21
TCR	T cell receptor
TEM	Tie2 expressing perivascular macrophage
TGF-β	Transforming growth factor beta
Th	T helper cell
Tie2	Angiopoietin receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TRIF	TIR domain–containing adaptor inducing interferon beta
VEGF	Vascular endothelial growth factor
WT	Wild-type
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1. Macrophages

1.1. Macrophages, innate immune cells with different ontogenies

Macrophages are multifunctional myeloid cells that can be found in all tissues, accounting for up to 10% of total cells in organs (Jenkins & Hume, 2014). These cells present well-established roles in the primary response to pathogens, tissue homeostasis, inflammation, resolution, and repair (Martinez et al. 2009; Chow et al. 2011; Ginhoux et al. 2015).

Macrophages belong to the innate immune system, the first line of defence that acts upon injury or pathogen recognition, which also includes haematopoietic cells such as monocytes, dendritic cells (DCs), natural killer (NK) cells and granulocytes (neutrophils, eosinophils and mast cells) (Chaplin 2010; Warrington et al. 2011). This system responds immediately and in a pattern specific manner to insults, and can be sufficient to defeat the pathogenic attack in most cases. However, when the damage persists, the immune response requires the participation of the adaptive branch of the immune system. The acquired immune system includes cell-mediated immunity directed by T cells and humoral immunity driven by B lymphocytes. These immune responses depend on the ability of the innate system to present antigens to T lymphocytes, which is a key step in the process, since this promotes the clonal expansion of specific selected lymphocytes that recognise a particular pathogen. Moreover, the adaptive immune system has the advantage of generating immunological memory after the initial response, which facilitates an enhanced and faster response to

subsequent encounters with the same pathogen (Chaplin 2010; Warrington et al. 2011).

The macrophage system was first described by Elie Metchnikoff in the late 19th century. Metchnikoff named these cells, ancient in metazoan phylogeny (Wynn et al. 2013), as “macrophages” from the Greek “big eaters”, and this term was accurately chosen as it refers to their great phagocytic ability, observation by which he was awarded the Nobel prize in 1908 (Varol et al. 2015). He defined the phagocytic ability of these mammalian cells comparing them to the phagocytes present during the development of invertebrate organisms. He also proposed that macrophages would have not only developmental functions, but also important roles in innate immunity, such as pathogen clearance or tissue repair.

In the early 1970’s, van Furth and Cohn defined the Mononuclear Phagocyte System (MPS) (van Furth et al. 1972), which became a central dogma in immunology for several decades (Lavin et al. 2015). This system included highly phagocytic myeloid cells such as monocytes and macrophages, and dendritic cells, which were discovered by Steinman and Cohn in the mid-1970’s (Steinman & Cohn 1973). The MPS refers to a linear system in which macrophages are considered as fully differentiated cells with no proliferative potential that derive from blood monocytes which became committed in the bone marrow from specific precursors. This was shown by studies performed in mice using radiolabelled monocytes that were recruited to the sites of inflammation to replenish de pool of tissue macrophages (Lavin et al. 2015).

The MPS has been the prevalent model for years. However, it has been demonstrated that a variable fraction of tissue macrophages

do not replenish from circulating monocytes but instead, they have self-renewal capacity (Gomez Perdiguero et al. 2014; Rosas et al. 2014). Moreover, dendritic cells derive from monocytic-independent specific precursors and they are not highly phagocytic cells but instead are professional antigen presenting cells (Gautier et al. 2012; Merad et al. 2013).

The revised concept of the MPS now includes tissue macrophages of two distinct origins, the ones that are established in tissues prenatally and those that come from blood monocytes upon specific conditions (van de Laar et al. 2016; Varol et al. 2015).

1.1.1 Prenatally established tissue macrophages

The foetal origin of tissue resident macrophages has been widely studied in the murine system. In the yolk sac, in early phases of the embryonic development, primitive erythrocytes and myeloid precursors are produced, and then seeded into some tissues, such as brain, lung and skin, giving rise to foetal proliferative macrophages. Some of these yolk-sac derived progenitors migrate to the foetal liver through the recently formed blood circulation to initiate the first wave of haematopoiesis. Then, a second wave of haematopoiesis arises in the mouse embryo coming from major arterial vessels to generate foetal liver monocytes that will replace foetal macrophages (except for microglia), together with multipotent hematopoietic stem cells (HSCs) that will expand in the liver and colonise the spleen and also the bone marrow, which becomes the main site of haematopoiesis in the adult life (definitive haematopoiesis) (Perdiguero & Geissmann 2015; Lavin et al. 2015; Davies et al. 2013a).

There is plenty of evidence supporting that most of the tissue resident macrophages barely rely on blood monocytes for their renewal because they can self-renew through local proliferation. This has been shown using fate-mapping and parabiotic studies. Hashimoto *et al.* observed, performing long-term parabiotic experiments (up to one year long), that in mice sharing the same circulation system, the monocytic chimerism found in blood was not present in microglia, Langerhans cells, alveolar macrophages or peritoneal macrophages (Hashimoto et al. 2013; Merad et al. 2002; Ginhoux et al. 2010). Surprisingly, they observed substantial tissue macrophage turnover over the course of 21 days, with the exception of spleen red pulp macrophages, suggesting that tissue resident macrophages constantly proliferate and have a relatively short half-life (Hashimoto et al. 2013).

Tissue macrophages are maintained by local production of growth factors and cytokines. Almost all macrophages rely on macrophage-colony stimulating factor (M-CSF), a growth factor that is produced by the tissular niche (the exact mechanisms of its production remain unknown) and that promotes survival and proliferation of macrophages (Epelman et al. 2014; Ginhoux & Guilliams 2016). M-CSF signals through CSF1R (CD115), a membrane receptor that is highly expressed in macrophages and also in monocytes. CSF1R has an alternative ligand, IL-34, produced exclusively by some neurons and keratinocytes in the skin, thus allowing the proliferation of microglia and Langerhans cells, respectively (Lavin et al. 2015; Wang et al. 2012). According to this, *Csf1r* null mice present a more severe deficiency in tissue macrophages compared to *Csf1* null mice. The fact that some macrophages depend on granulocyte-macrophage colony

stimulating factor (GM-CSF) for proliferation and survival explains that mouse models lacking Csf1r signalling still present some tissue macrophages in the spleen or in lungs (Lavin et al. 2015).

1.1.2 Monocyte-derived macrophages

With the exception of the lamina propria in the intestine, which mainly maintains the pool of macrophages through the recruitment of blood $CCR2^{+}$ monocytes (Varol et al. 2015), tissue macrophages maintain constant numbers in homeostatic conditions through self-renewal (Hashimoto et al. 2013), Indeed, monocytopenic patients or mice do not show major alterations in the numbers of Langerhans cells or other tissue resident macrophages (Takahashi 2000). The need for monocyte-derived macrophages is thus associated with injury or homeostatic imbalance.

In pathology-associated inflammation, there is a large infiltration of monocytes that enter injured tissues and differentiate to macrophages. In mice, monocytes are divided in two main subsets. $Ly6C^{hi}$ $CX3CR1^{int}$ $CCR2^{+}$ inflammatory monocytes exit the bone marrow to the site of inflammation to subsequently commit into macrophages. CCR2 is the receptor for the chemokines CCL2 and CCL7, which allow the egress of monocytes from the bone marrow to the bloodstream. It has been suggested that mesenchymal cells in the bone marrow respond to traces of bacterial products and type I interferons by producing CCL2 which causes the release of monocytes into the blood stream (Shi & Pamer 2011). $Ly6C^{lo}$ $CX3CR1^{hi}$ $CCR2^{-}$ monocytes are a subpopulation of blood monocytes that derive from $Ly6C^{hi}$ monocytes and can be considered as differentiated blood-resident monocytes that are

important for patrolling and monitoring endothelial integrity (Auffray et al. 2007).

To what extent do monocyte-derived macrophages contribute to tissue resident macrophages still remains poorly characterised. In the naïve peritoneum, there is a subset of monocyte-derived macrophages that are suggested to come from a residual recruitment of monocytes (Ghosn et al. 2010). In inflammation, though, tissue resident macrophages undergo a substantial contraction likely due to tissue adherence, emigration or death, a phenomenon known as the “macrophage disappearance reaction”. This is accompanied by a substantial monocyte influx, but the key event leading to the restoration of tissue resident macrophages is probably the induction of a proliferative burst in the tissue resident macrophages that remain after the initial inflammatory response (Davies et al. 2011; Ginhoux & Guilliams 2016; Jenkins et al. 2013; Jenkins et al. 2011).

Nonetheless, there is also evidence supporting that monocyte-derived macrophages can proliferate and hence contribute to some extent to the repopulation of the tissue macrophages (Davies et al. 2013b). It is not yet clear whether these recruited monocyte-derived macrophages can completely replace the original tissue resident macrophages in their function. Van der Laar *et al* showed that adult bone-marrow monocytes can efficiently colonise the alveolar niche generating alveolar macrophages that are transcriptionally almost identical to the originally established alveolar macrophages (van de Laar et al. 2016). However, Gundra et al found that upon type 2 responses, macrophages derived from monocytes were phenotypically and functionally distinct from tissue

resident macrophages and defined a subset of markers to differentiate between them (Gundra et al. 2014).

1.1.3 Transcriptional control of macrophage identity

Although there is a core of transcription factors common to all macrophages, the transcriptional programme responsible for their phenotype is tissue specific and depends on the environmental signals they encounter (Gautier et al. 2012; Lavin et al. 2014).

Macrophage differentiation requires the participation of hierarchically organised transcriptional modules (Lawrence & Natoli 2011). In the first place, the activity of the Runt-related transcription factor 1 (RUNX1) is necessary for the regulation of the ETS family transcription factor PU.1. High levels of PU.1 maintain macrophage differentiation, and it functions in a genome-wide manner enhancing the accessibility of macrophage-specific regulatory genomic regions to specific transcription factors. Moreover, PU.1 is responsible for directly inducing the expression of certain genes that encode for essential macrophage regulators such as CSF1R, which provides a positive feedback by promoting in turn high levels of PU.1 (Zhang et al. 1994; Aikawa et al. 2010).

Another level of transcription control is related to tissue-specific signals. The niche in each tissue provides macrophages with particular cues that promote the activation of different signalling cascades and subsequent transcriptional programs enabling the acquisition of tissue-specific phenotypes. For example, peritoneal macrophages rely on C/EBPβ and Gata6 as their subset-specific master regulators. Gata6 is induced by retinoic acid and is involved in limiting their proliferative capacity (Okabe & Medzhitov 2014;

Rosas et al. 2014), localisation and synthesis of TGF-β, leading to the secretion of IgA by B-1 lymphocytes in the lamina propria (Okabe & Medzhitov 2014). Other subtype-specific transcription factors include PPARγ in lung alveolar macrophages (Schneider et al. 2014), Spi-C in splenic red pulp macrophages (Kohyama et al. 2009) and LXRα in splenic marginal zone macrophages (A-Gonzalez et al. 2013).

The fourth level of regulation involves transcription factors activated upon functional requirements (Lawrence & Natoli 2011). These functional demands can be diverse, including, for example, the response to pathogens, which leads to the activation of different signalling cascades such as the JAK-STAT pathways (STAT1, STAT3 and STAT6), Interferon regulatory factors (IRF8, IRF5 or IRF4) and NF-κB-family proteins, among others.

1.2 Macrophage plasticity: regulation of polarisation

Macrophages have a remarkable functional and phenotypic plasticity that allows them to efficiently respond to continuous changes in their microenvironment (Mosser & Edwards 2008). Macrophages can acquire several activation states depending on the cell maturation status, the tissue in which they are located and the presence of environmental cues such as cytokines or pathogen-associated molecular patterns. The stimuli that can act on macrophage physiology can be divided into danger signals on the one hand, and homeostatic, metabolic and modulatory signals on the other hand (Glass & Natoli 2015). The ability of macrophages to efficiently redirect their phenotype in response to changes in their microenvironment relies on membrane and cytosolic receptors that recognise a vast diversity of molecules.

The phenotypes macrophages acquire when responding to changes in the surrounding milieu is involved in the outcome of infections and diseases such as cancer, and metabolic and allergic disorders (Murray et al. 2014). Provided that macrophage polarisation gives rise to diverse functional states (Mosser & Edwards 2008), several studies have been performed to define the mechanisms that control this complex process.

1.2.1 Macrophage polarisation and activation: the M1 and M2 paradigm

The complexity of macrophage polarisation and activation is the consequence of the diversity of signalling molecules, transcription factors, epigenetic mechanisms, and posttranscriptional regulators that are activated in response to changes in their environment (Mosser & Edwards 2008; Lawrence & Natoli 2011)(**figure 1**).

The concept of “macrophage activation” was first described by Mackaness and colleagues in the early 1960’s. They described that macrophages from mice infected with intracellular bacteria (*Listeria monocytogenes* or *Mycobacterium tuberculosis*) developed an antibody-independent indiscriminate antimicrobicidal ability against unrelated bacteria. These studies were the basis for the notion of acquired cellular resistance in macrophages (Mackaness 1962; Mackaness 1964), and became the starting-point for defining the molecular basis of macrophage activation.

Interferon gamma (IFNγ) was identified in the 1970’s as the key soluble factor produced by cells such as activated Th1 lymphocytes, which are essential in immune responses against intracellular bacteria and viruses, and could promote extensive changes in macrophage physiology, increasing their antigen

presenting capacity and increasing their expression of pro-inflammatory cytokines and toxic mediators. In the 1980's, interleukin-4 (IL-4), was identified as a cytokine released by Th2-helper, cells that participate in responses against parasites and extracellular bacteria, that promoted antibody production and inhibited Th1-type responses (Rengarajan et al. 2000). It was then shown that IL-4 was a deactivating cytokine for macrophages, as it was able to inhibit the production of inflammatory cytokines and toxic factors (Abramson & Gallin 1990). However the later observation that IL-4 induced the expression of distinctive markers, such as the mannose receptor, and a mild upregulation of major histocompatibility complex II molecules (Stein et al. 1992), lead to the evaluation of the deactivation concept, and it was thereafter conceived as the "alternative" activation of macrophages in comparison to the phenotype obtained by IFNγ. Interleukin-13 (IL-13), a cytokine also produced by Th2 cells, was also reported to induce a similar polarisation state in macrophages compared to the effects of IL-4 (Gordon 2003).

Mills *et al*. considered that macrophage activation could be regarded as the Th1/Th2 dichotomy observed in T lymphocyte differentiation and proposed the use of the terms M1 or M2 to refer to classically activated and alternatively activated macrophages, respectively (Mills et al. 2000). However, it is important to note that these are two discrete phenotypes out of a vast spectrum of functional stages that macrophages can acquire (Mosser & Edwards 2008) to integrate signals coming from a broad range of stimuli (Glass & Natoli 2015).

1.2.1.1 M1 or classically activated macrophages

M1 or classically activated macrophages are commonly associated to type I or cell-mediated immune responses directed against intracellular pathogens. The increased microbicidal activity of macrophages depends mainly on IFNγ, a pleiotropic cytokine produced by activated T helper 1 lymphocytes or NK cells engaged to provide long-term macrophage activation (Mosser & Edwards 2008; Lawrence & Natoli 2011)(**figure 1**).

IFNγ binds to the type II IFN receptor (IFNAR2), leading to the activation of the Janus kinases 1 and 2 (JAK1 and JAK2), which phosphorylate the signal transducer and activator of transcription 1 (STAT1) in tyrosine residue 701. This results in STAT1 homodimerisation, which translocates to the nucleus and binds gamma-activated sequences (GAS) to initiate transcription (Platanias 2005). IFNγ then induces the expression of several genes important for pro-inflammatory functions of M1 macrophages, such as *Nos2*, *Ciita* and *Il12*. *Nos2* encodes for inducible Nitric Oxide Synthase (iNOS), the enzyme responsible for generating nitric oxide (NO) from L-Arginine, an important source of reactive oxygen and nitrogen species (ROS and RNS) that participates in intracellular pathogen killing (Bronte & Zanovello 2005). *Ciita* encodes for the major histocompatibility complex class II transactivator (CIITA), which upregulates the transcription of MHC-II related genes and thus increases antigen presentation (Reith & Boss 2008). IL-12 is a pro-inflammatory cytokine that plays a major role in sustaining Th1-cell mediated responses and cytotoxic $CD8^+$ T cell and NK cell functions, necessary for antimicrobial and antitumoral responses (Sun et al. 2014; Vignali &

Kuchroo 2012). IFNγ also induces the production of the chemokines CXCL9 and CXCL10, involved in T-cell trafficking and in NK and T cell recruitment (Martinez et al. 2008).

In this regard, Dalton and colleagues showed that *Ifng*-deficient mice died from a sub-lethal dose of the intracellular pathogen *Mycobacterium bovis*, due to impaired production of macrophage antimicrobial products and the reduced presentation of major histocompatibility complex class II antigens (Dalton et al. 1993).

Acquisition of a fully differentiated M1 phenotype requires the participation of pathogen recognition receptors (PRRs). These receptors detect a wide range of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) that are related to pathogenic insult or tissue damage, respectively. PRRs include the families of membrane-associated Toll-like receptors (TLRs, containing the members TLR1-7 and TLR9-11) and C-type lectin receptors (CLRs), and families of cytosolic receptors comprising the nucleotide binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-like receptors (RLRs) and the family of AIM2-like receptors (ALRs) (Brubaker et al. 2015).

The endogenous danger signals known as ‘alarmins’, include the chromatin protein HMGB1 (high mobility group box 1 protein), extracellular ATP and heat-shock proteins, among others. They are released during sterile injury or necrosis and signalling through TLRs such as TLR4 and NLRs to trigger responses similar to those induced by microbial ligands, with the aim of repairing the tissue and restoring homeostasis (Glass & Natoli 2015; Martinez 2011; Brubaker et al. 2015).

For the *in vitro* study of M1 macrophage polarisation, PAMP mediated PRR activation is mainly used, being the Gram-negative bacterial wall product lipopolysaccharide (LPS), a TLR4 ligand, the most widely used (El Kasmi et al. 2008; Buxadé et al. 2012; Xu et al. 2012; Brubaker et al. 2015). The TLR4 response to LPS requires a multireceptor complex consisting of the LPS-binding protein (LBP) CD14, and MD-2 (this last important for TLR4 homodimerisation) (Brubaker et al. 2015). TLR4 signalling from the plasma membrane utilises the adaptor myeloid differentiation primary response 88 (MyD88) together with other adaptors and kinases that trigger the activation of the kinases IKKβ, and the MAP kinases p38 and JNK, which ultimately activate the transcription factors NF-κB and AP-1, respectively (Takeuchi & Akira 2010; Akira et al. 2006). These transcription factors trigger the expression of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and IL-23, and enzymes such as iNOS. IL-1, IL-6 and IL-23 promote the expansion of Th17 cells that can also amplify the inflammatory response by releasing IL-17, which promotes the recruitment of inflammatory polymorphonuclear leukocytes (Mosser & Edwards 2008). Moreover, IL-1 prolongs the lifespan and stimulates the effector function of neutrophils, innate cells that are important for the initial inflammatory response, since they are highly phagocytic cells, release lytic enzymes from their granules and produce reactive oxygen intermediates (Mantovani et al. 2011). The MyD88-dependent pathway also induces the upregulation of the costimulatory molecules such as CD80 and CD86, which participate in antigen presentation (Nolan et al. 2009).

TLR4 also signals from the endosomes after undergoing endocytosis and triggers pro-inflammatory gene expression using a

distinct signalling pathway. Endosome-bound TLR4 signalling does not use MyD88 but instead utilises TIR domain–containing adaptor inducing interferon-β (TRIF) as the key adaptor protein participating in the activation of IRF3, the master transcription factor that leads to type I IFN production and TNFα synthesis. TNFα binds its receptor (TNFR1) and in an autocrine manner, leads to IKKβ activation and, thus, to a secondary phase of NF-kB activation, involved in sustaining the pro-inflammatory phenotype (Covert et al. 2005). Type I IFN binds IFNAR and triggers the expression of interferon stimulated genes (ISGs) such as the chemokines CXCL9 and CXCL10 (Platanias 2005).

The pro-inflammatory outcomes of macrophages are different depending on the type of stimulation. The phenotype of macrophages stimulated only with IFNγ, differs from that of macrophages stimulated with both IFNγ and LPS. In fact, IFNγ was first described as the “Macrophage activating factor” and it has been demonstrated that IFNγ treatment primes macrophages to be more susceptible to TLR ligands. IFNγ increases TLR expression and induces the activation of NF-κB and other transcription factors that are essential for the expression of certain TLR responsive genes. In addition, IFNγ acts as a priming factor by inactivating feedback inhibitory pathways. IFNγ abrogates the expression of IL-10 induced by TLR signalling, an anti-inflammatory cytokine involved in fine-tuning TLR-induced gene programs. (Hu & Ivashkiv 2009).

Other pro-inflammatory cytokines are also involved in the classical activation of macrophages. Tumour necrosis factor α (TNFα) cooperates with IFNγ to induce M1 polarisation (Ohmori et al.

1997). This pro-inflammatory cytokine is produced by macrophages themselves upon TLR stimulation and acts in an autocrine manner to promote the M1 phenotype (Mosser & Edwards 2008). IL-1 and type I IFN have also been associated to pro-inflammatory polarisation of macrophages. IL-1 promotes inflammatory responses through the IL-1 receptor (IL-1R1), which recognises both IL-1α and IL-1β and signals through MyD88 in the same way as the TLRs. Both receptors share the TIR domain in its structure, responsible for MyD88 binding (Garlanda et al. 2013).

Besides STAT1, NF-κB and AP-1, other transcription factors are involved in M1 polarisation. These include interferon-regulating factor 5 (IRF5) and Activin A, which are induced upon GM-CSF treatment (Krausgruber et al. 2011; Sierra-Filardi et al. 2011). The transcription factor IRF-8 has a role in the cross-talk between IFNγ and TLR signalling pathways. It is expressed upon IFNγ-induced STAT1 activation and promotes maximal production of pro-inflammatory cytokines inducing increased activation of MAPKs downstream TLRs (Zhao et al. 2006). IRF-8 can also induce M1 gene expression after being upregulated by the Notch signalling pathway, which acts synergistically with TLR4 (Xu et al. 2012).

1.2.1.2 M2 or alternatively activated macrophages

Alternatively activated macrophages are associated with Type 2 immunity or Th2-like responses, and in general they present functional phenotypes in macrophages different to those obtained with pro-inflammatory cytokines such as IFNγ. The molecules driving alternative activation of macrophages can be the Th2-cytokines IL-4 and IL-13, the combination of Ig complexes with TLR ligands or anti-inflammatory cytokines such as IL-10 and TGFβ

(Gordon & Martinez 2010; Martinez et al. 2009). According to this, M2 macrophage polarisation can be subdivided in M2a (reviewed below), M2b and M2c, respectively. In general terms, M2 macrophages are associated with parasite containment, promotion of tissue remodelling and tumour progression but they also have immunoregulatory functions (**figure 1**).

Several cell types produce IL-4 and IL-13, including conventional $CD4^+$ Th2 and $CD8^+$ T cells, NKT cells, basophils, mast cells, and eosinophils, which are all associated with type 2 immunity (Van Dyken & Locksley 2013). IL-4 binds to a heterodimeric receptor comprising the γc and the IL4Rα chains, to activate JAK1 and JAK3, to phosphorylate STAT6 in tyrosine 641, promoting its homodimerisation and subsequent translocation to the nucleus. STAT6 is the transcription factor responsible for the induction of several M2-associated genes such as Arginase-1, the non-opsonic receptors Mannose receptor (*Mrc1* or CD206) and Dectin-1 (*Clec7a*), Chitinase I (*Chi3l3*) and Resistin-like molecule α (*Relma* or Found in inflammatory zone 1, or *Fizz1*). Most of these markers are exclusively related to the M2 phenotype in the murine system, but not in the human one (Raes et al. 2005).

Arginase-1 is the enzyme that converts L-arginine into L-ornithine and proline, molecules involved in the regulation of cell growth and the production of collagen, respectively. Since the substrate for both Arginase-1 (M2) and iNOS (M1) is L-arginine, it has been proposed that substrate competition between these two enzymes is central for the control of macrophage polarisation (Bronte & Zanovello 2005). In fact, the ratio between iNOS and Arg1 determines the state of macrophage polarisation. Arginase-1 plays a critical role inhibiting unrestrained pathologic inflammatory

responses, as it suppresses IL-12-driven intestinal inflammation during acute schistosomiasis and also inhibits T cell proliferation by limiting L-arginine availability, an essential nutrient for their proliferation (Herbert et al. 2010). Arginase I is also part of a feed-back loop that acts to restrain Th2 cell functions (Pesce et al 2009b).

Mannose receptor is a C-type lectin receptor (CLR) important for the homeostasis of serum glycoproteins, and also plays a role in antigen presentation favouring the endocytosis of fungal particles that are rich in mannoproteins (Gazi & Martinez-Pomares 2009; Taylor et al. 2005; Wills-Karp 2010).

Dectin-1 is the principal PRR mediating fungal recognition in mice, but it can also recognise β-glucans from other organisms such as *Listeria* and *Mycobacterium* (Brown 2006). Although Dectin-1 is upregulated by IL-4 and IL-13, it signals to activate MAPK and NF-κB to enhance microbial killing through a respiratory burst (Gordon & Martinez 2010) and increased phagocytosis (Brubaker et al. 2015).

Chi3l3 codifies for chitinase I (YM1), a protein that lacks chitinase activity, necessary to hydrolyse chitin, a polymer found in fungi and parasites. It has been suggested that YM1 acts as a lectin binding protein with moderate affinity towards GlcN oligomers (Chang et al. 2001; Gordon & Martinez 2010).

Resistin-like molecule α (Relmα) is a cysteine-rich secreted protein, also referred to as 'found in inflammatory zone', whose expression increases during allergic reactions and upon infection with parasites that cause the release of IL-4 and IL-13 by Th2 cells.

In these settings Relmα functions to suppress exacerbated Th2-type immunity to prevent, among others, fibrosis (Pesce et al. 2009a).

However, IL-4-induced macrophage polarisation changes the expression of several other genes. It causes downregulation of the receptors for the common fraction chain (FcR) CD16, CD32 and CD64 (Duque Correa & Rojas López 2007) and it increases the expression of integrins such as CD11b and CD11c (Han et al. 2010). IL-4 induces the expression of IL1R2, the decoy receptor for IL-1 (Duque Correa & Rojas López 2007) and also induces the expression of the galactose-type C-type lectins MGL1 and MGL2, involved in the recognition and endocytosis of galactosylated glycoproteins, and stabilin 1, a scavenger receptor that mediates acLDL recognition and responses to pathogens (Martinez et al. 2009). IL-4 upregulates the expression of chemokines such as CCL2, required for the recruitment of $CCR2^+$ cells such as monocytes, and CCL17 and CCL22, chemoattractants for $CCR4^+$ cells such as macrophages, Th2 T cells, and regulatory T cells (Chvatchko et al. 2000; Iellem et al. 2001; Imai et al. 1998). M2 macrophages have an important role in tissue remodelling, something they achieve expressing molecules involved in extracellular matrix degradation, such as collagenase (MMP1) and metaloelastase (MMP12). They also participate in extracellular matrix deposition producing fibronectin or releasing TFGβ to activate collagen deposition by fibroblasts (Duque Correa & Rojas López 2007).

STAT6 is important for IL-4 mediated transcriptional regulation, but other transcription factors also participate in the activation of the

M2 gene program. The CREB-C/EBPβ axis is important for the expression of several M2-related genes (Ruffell et al. 2009), and in the case of arginase I and RELMα, for example, C/EBPβ binds to their promoters together with STAT6 (Gray et al. 2005; Stütz et al. 2003). Krüppel-like factor 4 (KLF4), as shown by Liao *et al*, is upregulated by STAT6 upon IL-4 stimulation, and both cooperate to induce the expression of M2 genes such as *Arg1* or *Retnla*, and nuclear factors such as Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor that regulates many aspects of lipid metabolism and inflammation (Szanto et al. 2010). In addition, KLF4 hampers M1-gene expression by sequestering cofactors important for NF-κB regulation (Liao et al. 2011) and STAT6 serves as a licensing factor for PPARγ, a mechanism that might contribute to regulate its M2-related roles. PPARγ also induces the expression of mir-223, a regulatory molecule necessary for the induction of several M2 genes (Ying et al. 2015). Satoh and colleagues also showed that the IL-4-induced M2 phenotype in macrophages requires the transcription factor IRF4, whose expression is dependent on the histone 3 Lys Jumonji domain containing-3 (Jmjd3), a histone 3 Lys27 (H3K27) demethylase that acts on the *Irf4* gene promoter. As a consequence, Jmjd3-deficient mice show impaired responses to helminth infection and chitin administration (Satoh et al. 2010). PPARγ can also form heterodimers with retinoid X receptor (RXR)-α to control the expression of genes that codify for phagocytic mediators, such as CD36, MerTK or AXL (Roszer et al. 2011). It has also been shown that homodimers of p50, a protein belonging to the NF-κB family, are also involved in M2 macrophage

polarisation through the inhibition of NF-κB-induced IFNβ production (Porta et al. 2009).

When IL-4 binds to the type I IL-4 receptor it also engages the PI3K/Akt/mTOR pathway through the adaptor molecule insulin receptor substrate 2 (IRS2) to promote M2 gene expression (Heller et al. 2008). Indeed, Akt, a kinase that is downstream IRS2, is necessary for the induction of several M2 genes such as *Arg1*, *Relma* and *Chi3l3,* as shown by TSC-1 deficient cells that have low Akt levels as a consequence of a negative feedback lead by constitutively active mTORC1 (Byles et al. 2013). Moreover, TSC1-deficient cells also show reduced levels of the transcription factor C/EBPβ, causing further impaired M2 gene expression (Zhu et al. 2014).

M1 and M2 phenotypes have mechanisms of cross-regulation in order to fine-tune the gene expression profile that is induced upon a specific environment. There is a well-established antagonism between STAT1 and STAT6 in Th1 and Th2 cells that is likely to behave in the same way in macrophage polarisation (Ohmori & Hamilton 1997). PPARγ is a negative regulator of classical macrophage activation through transrepression of NF-κB and AP1 (Ricote et al. 1998) and KLF4 sequesters co-factors for NF-κB activation (Liao et al. 2011). IRF5 induces the expression of pro-inflammatory genes while repressing the induction of anti-inflammatory mediators such as IL-10 (Krausgruber et al. 2011)(**figure 1**).

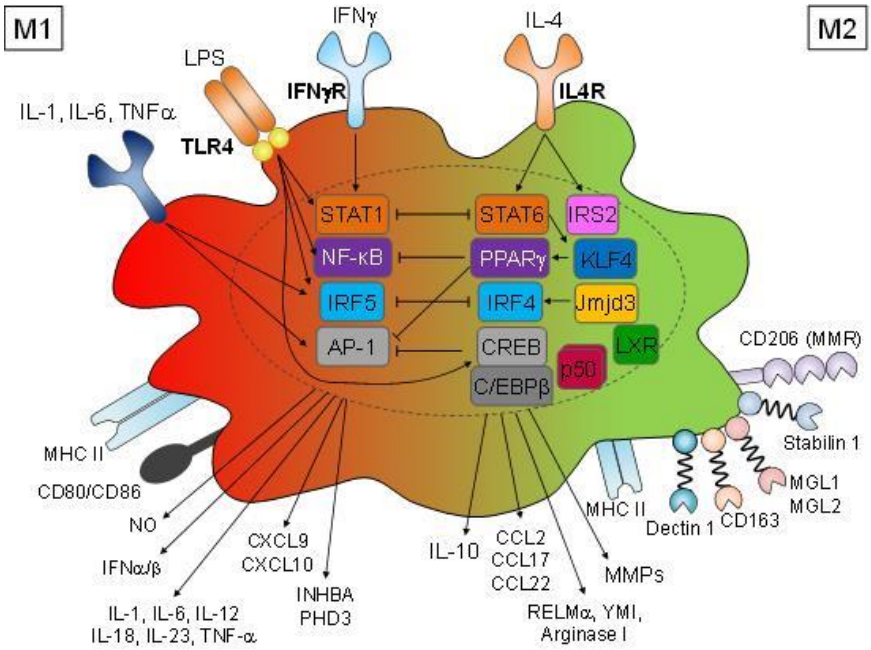


Figure 1. Regulation of macrophage polarisation towards two extrems of the possible all the polarisation phenotypes. The figure illustrates a simplified version of the signals, receptors, transcription factors and subsequently induced molecules in M1 and M2 macrophages.

1.3 Macrophage functions in homeostasis maintenance

Macrophages are virtually present in all tissues and they count on a large variety of receptors that sense changes in their microenvironment to modulate tissue homeostasis.

1.3.1 Organ homeostasis and macrophages

Macrophages are important for the correct function of several organs. Microglia release trophic factors (e.g. insulin-like growth factor-1 (IGF-1)) that support the formation of neuronal circuits in

the brain as well as factors that are important for synaptogenesis. These macrophages are also essential for the phagocytosis of apoptotic cells in both developing and adult brain, a process that takes place without inducing pro-inflammatory cytokines (Nayak et al. 2014).

Macrophages play a critical role in the maintenance of bone homeostasis. While mesenchymal osteoblasts are involved in the synthesis of bone, osteoclasts or bone-resident macrophages are contingent on receptor activator of nuclear factor-κB ligand (RANKL) and they function in bone resorption processes. The equilibrium between these two activities defines proper bone shape. For instance, CSF1 deficient mice ($Csf1^{op/op}$) present osteopetrosis that can even reduce bone-marrow hematopoiesis (Pollard 2009).

Spleen and liver macrophages play a central role in iron homeostasis. Red pulp macrophages in the spleen and Kupffer cells in the liver phagocytose damaged and senescence erythrocytes. In the blood, haemoglobin binds to haptoglobin and then is recognised and cleared by macrophages through the scavenger receptor CD163. Kupffer cells in the liver also regulate plasma cholesterol levels by catabolising LDLs and modified LDLs, and remove material released from dying cells or extracellular matrix components to prevent intravascular coagulation (Varol et al. 2015).

Tissue resident macrophages are constantly monitoring their environment, and this is essential in organs whose function is associated with blood circulation, such as the spleen or the liver, or in mucosal tissues and associated cavities, such as the lungs, the

intestine and the peritoneal cavity. The liver and the spleen serve as blood filters and macrophages in these organs constantly sense microbial products and toxins. In the marginal zone of the spleen, the interface between the red and the white pulp, macrophages monitor arterial blood. Pathogens enter the host via mucosal surfaces, through a wound or breach in the skin and via bites of insect vectors (Iwasaki & Medzhitov 2015), and macrophages in these locations are prepared to mount the aforementioned responses.

1.3.2 Macrophages as sentinel cells in tissues

The ability of macrophages to respond to changes in their surrounding environment stems from two main features. They express a wide range of receptors to accurately sense the type of perturbation and mount a response. In addition, macrophages also have strategic locations in distinct anatomical, tissue and subcellular compartments (Iwasaki & Medzhitov 2015).

Macrophages can sense exogenous and endogenous danger signals through the expression of several pattern recognition receptors (PRR) such as membrane-associated Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and the cytosolic receptors nucleotide binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-like receptors (RLRs) and the AIM2-like receptors (ALRs). These PRRs are specifically designed to bind to pathogen and damage associated molecular patterns (PAMPs and DAMPs). While the CLR Mannose receptor (CD206) functions exclusively in pathogen binding and phagocytosis, signalling PRRs (including the TLRs, NLRs and RLRs) sense microbial products and activate transcriptional

mechanisms that lead to phagocytosis, cellular activation and the release of cytokines, chemokines and growth factors (Murray & Wynn 2011). Phagocytosis leads to microbial killing in the phagosome through mechanisms such as phagosomal pH acidification and release of hydrolytic enzymes and toxic metabolites into the phagosome as a result of the respiratory burst (Brown 2006; Brown et al. 2002).

TLRs are one of the most studied families of pathogen recognition receptors (PRRs) (**figure 2**). They are involved in the recognition of a wide range of pathogen moieties and endogenous signals, and lead to the activation of pro-inflammatory gene expression. TLRs are a family of 12 members in mice, while humans have 10 members. TLRs are defined by a Leucine-rich repeats (LRR)-containing N-terminal domain that is responsible for ligand recognition and binding, and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain, important for the binding of TIR-domain containing adaptors that participate in the signal transduction (Takeuchi & Akira 2010; Akira et al. 2001, O'Neill et al. 2014).

Homodimers of TL4 and TLR5 and heterodimers of TLR1/TLR2 and TLR1/TLR6 can be found in the plasma membrane and recognise moieties associated to the external part of pathogens. Lipopolysaccharide (LPS), a wall constituent of some Gram-negative bacteria, is one of the ligands for TLR4 recognises TLR5 is expressed in intestinal macrophages and binds to flagellin present in some bacteria. TLR1/TLR2 and TLR1/TLR6 heterodimers bind to lipoproteins in bacteria and some viruses.

Homodimers of TLR3, TLR7 and TLR9 are associated to endosomal-membranes. TLR3 and TLR7 bind to viral dsRNA and ssRNA, respectively. TLR9 binds to unmethylated CpG motifs from bacteria and viruses. These TLRs are strategically located and compartmentalised in endosomes to avoid unwanted activation by self-nucleotides.

Signalling cascades activated upon ligand binding to these receptors can be divided in two main groups according to the central adaptor involved in the signal transduction (**figure 2**). On the one hand, MyD88, which does not signal from TLR3, is the key adaptor that leads to the activation of the nuclear factor kappa B (NF-κB) and the AP-1 complex (formed by c-Fos and c-Jun), important for the expression of their target pro-inflammatory cytokines. TLR2 and TLR4 require TIRAP/Mal for interacting with MyD88. MyD88 activates IL-1R-associated kinase 4 (IRAK4), which then binds to and activates IRAK-1 and IRAK-2. This kinase complex uncouples from MyD88 and then activates the E3 ubiquitin ligase TNFR-associated factor 6 (TRAF6). This factor forms an ubiquitin complex that ubiquitinates TRAF6 to recruit and activate the complex formed by TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3, which phosphorylates IkB kinase (IKK)-β and different MAP kinase kinases. IKK-β forms a complex that phosphorylates IκBα, the sequester of NF-κB, and marks it to be degraded by the proteasome to allow NF-κB translocation to the nucleus. The MAPK cascade leads to the activation of AP-1 complex. These two transcription factors participate in the expression of pro-inflammatory genes necessary for eliminating the pathogen, such as iNOS, and pro-inflammatory

cytokines such as IL-1, IL-12 or TNFα that induce Th1 and cytotoxic T cell responses (Takeuchi & Akira 2010).

On the other hand, TRIF is the key adaptor protein required for TLR3 signalling, and that can also signal from TLR4 in a late and endosomal-associated response (Kagan et al. 2008). TRIF can bind to TRAF6, and therefore engage the same signalling pathway as MyD88. TRIF can also interact with TRAF3, which then activates the kinases TBK1 and IKK-ε, required for the activation of IRF3 and IRF7. These transcription factors homodimerise and translocate to the nucleus, inducing the expression of type I IFNs. The TNFR-associated death domain protein (TRADD) can in the same way participate in TRIF signalling pathway, by activating RIP1 that in turn triggers NF-κB activation (Takeuchi & Akira 2010).

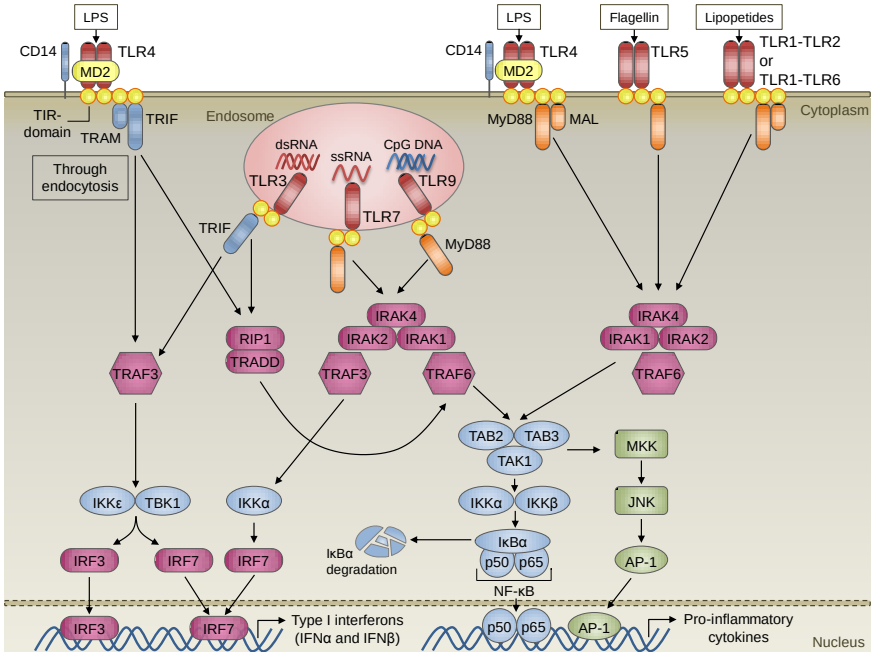


Figure 2. TLR signalling pathways in macrophages. Illustration of the main TLRs in mice, their ligands and the signaling cascades that are activated upon ligand engament. *Adapted from O'Neill et al., 2014, Nature Reviews Immunology.*

CLRs are transmembrane receptors characterised by the presence of a carbohydrate-binding domain that recognises carbohydrate-residues on particular host proteins on microorganisms such as viruses, bacteria, and fungi. Dectin-1 (Clec7a) and Dectin-2 (Clec6a) are two of the main CLRs and they are specialised in detecting fungal β-glucan. CLRs activate intracellular signalling by ITAM domains, present in the CLR itself or in adaptors such as DAP12, that activate Syk tyrosine kinase that is responsible for MAP kinase, NFAT and NF-κB activation, leading to pro-inflammatory gene expression (Geijtenbeek & Gringhuis 2009, Takeuchi & Akira 2010).

The NLR proteins are cytoplasmic sensors activated by potassium efflux or bacterial moieties such as peptidoglycans. They are composed of a central nucleotide-binding domain, C-terminal leucine-rich repeats and N-terminal protein binding domains such as CARD. NOD1 and NOD2, the main members in the family, signal through the adaptor RIP2/RICK to activate NF-κB-mediated transcription (Chen et al. 2009).

The RIG-I-like receptor (RLR) family is composed of RIG-I, MDA5, and LGP2. RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain. RLRs are localised in the cytosol in association to the mitochondria and recognise viral dsRNA and ssRNA without CAP. The

expression of RLRs greatly enhanced in response to type I IFN or virus infection. The CARD domains of RLRs trigger signalling by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1), activating TRAF3 and TRADD pathways, to induce type I interferons and pro-inflammatory cytokines (Kawai & Akira 2009).

Macrophages also detect different types of cell death. Necrosis refers to the pathological cell death that is accompanied by the release of endogenous danger signals, known as 'alarmins', that trigger macrophage pro-inflammatory responses through PRRs such as TLR4. Necrotic cells release inflammatory triggers such as the chromatin protein HMGB1 (high mobility group box 1 protein), extracellular ATP, heat-shock proteins and fibronectin (Cocco & Ucker 2001; Glass & Natoli 2015; Martinez 2011; Brubaker et al. 2015). By contrast, apoptosis is a process of physiological cell death in which the apoptotic bodies are usually phagocytosed by macrophages in a silent manner, without inducing their activation and promoting anti-inflammatory responses such as TGFβ and IL-10 expression (Hochreiter-hufford & Ravichandran 2013; Elliott & Ravichandran 2010). Programmed cell death is associated with homeostatic maintenance of several tissues (e.g. thymus), while necrosis is generally related with infection or cancer.

1.3.3 Macrophage mediated T cell activation and differentiation

T lymphocyte responses require that innate cells such as macrophages or dendritic cells act as a bridge between them and the pathogen or host antigens that have to be cleared. Activated antigen presenting cells (APCs) as MPS cells increase antigen

presentation and cytokine production. MPS cells uptake the pathogen and present antigen peptides to T cells through Major Histocompatilibty Complexes (MHC). MHCII molecules present antigens to T $CD4^+$ helper cells while MHCI molecules to T $CD8^+$ cytotoxic cells. The antigen is recognised by the cognate T-cell receptor (TCR), being the first signal for T cell activation. Then the immune synapse is formed around the MHC-TCR complex, which involves the participation of several molecules, including costimulatory molecules and integrins, such as LFA-1 in T cells and VCAM-1 in APCs. The costimulatory molecules CD80 and CD86 in the APC membrane bind to CD28 in the lymphocytes providing T cells with the co-activating signal. Activated T cells undergo growth and clonal expansion.

Pathogen sensing by MPS cells defines the phenotype that T cells will acquire. In this regard, T cells are committed to Th1 cells by IL-12 (produced by APCs) and IFNγ (produced by NK cells), through the activation of the signal transducer and activator of transcription 4 (STAT4) and STAT1 respectively. The master regulator that governs Th1 responses is T box transcription factor Tbx21 (T-bet, encoded by the Tbx21 gene), and the signature cytokines are IFNγ, tumour necrosis factor-α (TNFα) and lymphotoxin-α (LT-α). IL-2 is also produced by these cells, but it is not a Th1-exclusive cytokine (Zhu & Paul 2008). IFNγ produced by T cells is necessary to activate M1 macrophages and to trigger cellular immunity against intracellular microorganisms.

Th2 cells participate in the clearance of extracellular parasites and their differentiation relies on STAT6-dependent GATA3 transcription factor activation by the IL-4R (Zhu et al. 2001). Th2

cells produce IL-4, IL-5 and IL-13, which are required for humoral immunity (Ig class switching) to control helminths and other extracellular pathogens and also to promote alternative activation of macrophages through IL-4.

The balance between Th1 and Th2 responses controls immune responses against tumour progression. Indeed, enhanced Th1 responses positively correlate with limited tumour development, as they promote cytotoxic effector functions (Knutson & Disis 2005), while adaptive responses skewed towards Th2 responses have been related with poor prognosis in cancers such as pancreatic or breast cancer (De Monte et al. 2011, Xu et al. 2011, Zhang et al. 2015).

Th17 cells produce IL-17 (IL-17A), IL-17F, and IL-22, playing important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Th17 cell differentiation is based on the master transcription factor retinoid-related orphan receptor (ROR)γt activity, a transcription factor that is induced by synergistic action of different cytokines including TGF-β and the pro-inflammatory cytokines IL-1β, IL-6, IL-21, and IL-23 (DuPage & Bluestone 2016; Zhou et al. 2009).

iTreg (Treg) cells differentiate from naïve T cells upon antigen presentation in the periphery and mediate the suppression of the immune response, being the negative regulators of immune inflammation (Josefowicz et al. 2012). TGF-β induces Treg cell differentiation upon TCR activation, together with IL-2, and this phenotype is controlled by the master regulator forkhead box P3 (Foxp3). Upon TCR stimulation, Treg cells induce high levels of CD25, the alpha subunit of the high affinity receptor for IL-2, to

consume high quantities of IL-2, and thus reducing its availability for other T cells (Boyman & Sprent 2012). Treg cells produce cytokines such as IL-17, IL-10, IL-35 and TGF-β (Zhu & Paul 2010). Tregs have important functions in the tumour microenvironment due to their immunosuppressive phenotype. IL-10 and TGF-β are well-known cytokines involved in immunosuppression, which facilitate tumour progression by immune evasion. Given the phenotype of Treg cells, their absence can lead to autoimmunity, as these cells are a specialised subset of lymphocytes that restrain pathogenic immune responses. In addition to consuming IL-2 and secreting anti-inflammatory cytokines, these cells also express molecules such as the surface protein CTLA-4, which is implicated in limiting responses of activated T cells (Josefowicz et al. 2012).

1.4 Macrophages in pathology

Macrophages play crucial roles in maintaining tissue homeostasis. They assist in combating infection, resolving acute inflammation and regulating responses to tissue stress. However, macrophage function can also lead to several pathological conditions including diabetes, atherosclerosis and cancer.

1.4.1 Chronic inflammation leads to cancer

90% of total cancers are related to somatic mutations and environmental factors, rather than inherited mutations (Grivennikov et al. 2010). Among the environmental factors that lead to cancer we find pathogens that cause chronic infections, and carcinogens that have been related to inflammation, such as tobacco smoking,

inhaled pollutants (silica and asbestos) and dietary factors (Aggarwal et al. 2009).

An example of infection-induced cancer is the persistent *Helicobacter pylori* infection that causes gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. Infections with hepatitis B (HBV) or C (HCV) viruses increase the risk of hepatocellular carcinoma (HCC). These tumorigenic pathogens can evade/undermine host immunity and sustain persistent inflammation that is mild but chronic (Karin & Greten 2005).

This inflammatory environment can increase both the mutation and the proliferation rate of cells. Neutrophils and macrophages secrete reactive oxygen and nitrogen species during acute inflammation that can induce DNA damage and increase genomic instability (Grivennikov et al. 2010). DNA damage can also lead to inflammation that promotes tumorigenesis. This is the case of HCC induced by the carcinogen diethylnitrosamine (DEN). DEN causes DNA damage in hepatocytes after being bioactivated by cytochrome P450 (CYP), resulting in necrotic cell death that in turn triggers an inflammatory reaction that promotes compensatory proliferation of surviving transformed hepatocytes and leads to hepatocellular carcinoma. Although the pathogenesis in this mouse model differs from human HCC, it has a histology and genetic signature resembling poor prognosis HCC, and also recapitulates the dependence on inflammation and gender disparity seen in human HCC (Maeda et al. 2005). Naugler and colleagues demonstrated that liver injury and compensatory proliferation were strongly dependent on IL-6, whose production is higher in males than in females since oestrogens inhibit IL-6 expression. The production of this cytokine by Kupffer cells is contingent on MyD88

(Naugler et al. 2007), the main adaptor molecule downstream TLRs and IL-1 receptor (Gay et al. 2014; Brubaker et al. 2015). Indeed, Sakurai and colleagues showed that DEN-induced hepatocyte necrosis results in the release of IL-1α and subsequent activation of IL-1R signalling, leading to IL-6 induction and compensatory proliferation, essential for hepatocarcinogenesis (Sakurai et al. 2008). Other pro-inflammatory cytokines such as TNFα or lymphotoxin β (TNFβ) are also related to hepatocarcinogenesis in the case of obesity-related HCC (Park et al. 2010). It is important to note that this type of tumorigenic inflammation is different to that induced by solid malignancies, in which the pro-inflammatory and tumour-promoting response provides cancer cells with neoangiogenesis and growth factors produced by newly recruited immune cells (Grivennikov et al. 2010).

1.4.2 Cancer and the immune system

In the early 1960's, Burnet and Thomas defined the concept of "Cancer immunosurveillance" as the immunological mechanism that eliminates potentially dangerous mutant somatic cells after the accumulation of several genetic changes. During the following decades much effort was made to define the specific role of the distinct immune cell types in protecting the host from the formation of tumour malignancies. Intense research in the field served to conclude that the immune system exerts not only host-protecting but also tumour-sculpting effects on developing tumours. As a consequence, the new concept of "Cancer Immunoediting" was proposed by Schreiber and colleagues (Dunn et al. 2002) to describe the participation of the immune system not only to prevent but also to shape the immunogenicity of neoplastic cells. Cancer

immunoediting consists of three main phases: elimination, equilibrium and escape. Immunosurveillance occurs during the elimination phase. Equilibrium arises as the consequence of the pressure that the immune system exerts to eliminate transformed cells, which leads to the Darwinian selection of genetically unstable tumour variants that developed mechanisms of immune evasion. In the escape phase, clinically detectable tumours appear because malignant cells modulated the immune system to retain supportive elements while escaping from the hostile ones (Mittal et al. 2014).

As a result, acquiring cell-intrinsic traits to become a malignant cell, such as immortalization, tumour suppressor gene inactivation, uncontrolled growth, metabolic alterations and resistance to cell death, usually leads to the development of dormant or benign tumours. Only when these processes are accompanied by immune-shaped cell-extrinsic traits such as angiogenesis, promotion of invasion and metastasis and immune escape, tumours can fully evolve. This is why cancer is a disease that could be defined by a progressive disruption of the immune system rather than a disorder of exacerbated cell growth and survival (Prendergast 2008).

1.4.2.1 Macrophage impact on tumour development

When tumours reach a certain size, they begin to grow invasively and they require an enhanced blood supply induced by stromagenic and angiogenic proteins. This growth disrupts the surrounding tissue generating inflammatory signals that lead to the recruitment of innate immune cells such as NKT, NK, γδT cells, macrophages and dendritic cells. These signals include type I IFNs, DAMPs and stress ligands that are recognised together with

tumour antigens by infiltrating lymphocytes, leading to their activation and production of IFN-γ. This IFN-γ induces a limited amount of tumour death through antiproliferative and apoptotic mechanisms, and also induces the production of the chemokines CXCL10 and CXCL9 that block the formation of new blood vessels to further promote tumour cell death. Chemokines produced during this inflammatory process recruit more NK cells and macrophages. These cells activate one another through the production of IFN-γ and IL-12, respectively. Activated NK cells and M1-like macrophages release perforin and reactive oxygen and nitrogen species to kill tumour cells. DCs migrate from the tumour site to the draining lymph node and present tumour antigens to specific $CD4^+$ T helper cells. These will then express IFN-γ that in turn will facilitate the development of cytotoxic responses by $CD8^+$ T cells. Both types of lymphocytes then migrate to the tumour site where cytotoxic cells destroy antigen-bearing tumour cells via perforin, granzyme B, TRAIL, or FasL-dependent mechanisms (Grivennikov et al. 2010; Vesely et al. 2011).

When elimination is not completely effective, tumour cells can enter the equilibrium phase, where the adaptive immune system prevents tumour outgrowth and sculpts the immunogenicity of the tumour cells. Lymphocytes exert pressure to contain tumour cells that is enough, but malignant cells can lead to the formation of a pool of genetically unstable and rapidly mutating dormant tumour cells. This phase is usually the longest one in tumour development. Progression from the equilibrium to the escape phase occurs because the tumour cell population changes in response to the immune system's editing functions and/or due to increased cancer-induced immunosuppression. Immune editing involves the loss of

tumour antigen generation due to reduced expression of strong rejection antigens or through loss of major histocompatibility complex (MHC) class I proteins and increased resistance to cytotoxic immunity through induction of anti-apoptotic mechanisms. Tumour-induced immunosuppression consists of establishing a tumour environment that engages immune cells in favouring tumour progression, as it is the case of tumour associated macrophages.

Tumour associated macrophages (TAMs) represent up to a 50% of the stromal cells in established solid tumours (Ball et al. 2016) and usually correlate with poor prognosis (Kitamura et al. 2015). Solid tumours are characterised by hypoxia and abundant cell death, factors that lead to macrophage modulation in an attempt to achieve homeostatic restoration. However, the macrophage response generally drives tumour growth by promoting angiogenesis, tissue remodelling, ectopic survival of malignant cells and the development of an immunosuppressive microenvironment that abrogates cytotoxic T cell activities. Macrophage polarisation in the tumour is an active process driven by the reciprocal interaction between malignant and stromal cells (Ruffell et al. 2012; Sica et al. 2008) (**figure 3**).

Tumour macrophages derive predominantly from circulating monocytes recruited by a set of chemokines released by tumours, as for the case of sarcomas, gliomas, lung tumours, ovary, cervix and breast carcinomas and melanomas. The main chemokine involved in monocyte recruitment is CCL2, which acts through the chemokine receptor CCR2. CCL2 together with stromal-derived factor-1 (SDF-1; CXCL12), have been demonstrated to have chemoattractant roles in models of glioblastoma, melanoma, and

cervical and prostate cancer (Kratochvill et al. 2015; De Palma & Lewis 2013). Other chemokines produced by malignant or stromal cells include CCL17 and CCL22, which have been associated with Th2 and Treg $CD4^+$ lymphocyte recruitment, or CXCL8 (IL-8), a chemokine that promotes angiogenesis and potentiates the proliferation and migration of cancer cells (Waugh & Wilson 2008).

High levels of M-CSF in the tumour area contribute to macrophage recruitment, survival and proliferation, and they impact in tumour progression. Studies using M-CSF deficient mice (*Mcsf*$^{op/op}$) show impaired tumour development in several tumour models such as the Lewis lung carcinoma model (Ruffell et al. 2012) or the spontaneous MMTV-PyMT mammary carcinoma model, in which M-CSF depletion reduced progression to metastasis (De Palma & Lewis 2013).

TAMs acquire an M2-like phenotype in response to stimuli from the tumour microenvironment. In tumours, such as mammary carcinoma infiltrating T cells show a type 2 response and produce IL-4 (DeNardo et al. 2009), IL-13 and IL-10 that contribute to M2 polarisation of TAMs (Gordon & Martinez 2010). Other immune cells recruited to tumour areas, such as eosinophils, also produce M2-polarising cytokines such as IL-13 (Kratochvill et al. 2015). Murray and colleagues showed that TAMs in EG7 thymomas or LLC tumours expressed prototypical M2 genes such as *Arg1*, *Mrc1*, *Relma* or *Ccl17* (Kratochvill et al. 2015). TAMs usually show an IL-10^{high} IL-12^{low} cytokine profile, reflecting an M2-like phenotype (Mantovani et al. 2002; Condeelis & Pollard 2006).The anti-inflammatory cytokines IL-10 and TGFβ can also be produced by a variety of tumour cells apart from TAMs themselves (Sica et al. 2008). IL-10 promotes the differentiation of monocytes to mature

macrophages and blocks their differentiation to DCs. IL-10 also renders TAMs with almost no cytotoxic activity, becoming poor producers of reactive oxygen and nitrogen species such as NO. In response to these cytokines, TAMs can secrete chemokines including CCL2, CCL22 and CCL18. CCL18 is responsible for recruiting naïve T cells into the tumour, but by entering in an environment dominated by M2 cells, they usually become anergic. TAMs participate in immunosuppressive responses by hampering T-cell activation and proliferation, since the secretion of IL-10, TGF-β and prostaglandins by TAMs negatively affects T cell activation. These cytokines can lead to T-cell exhaustion, a state of T-cell dysfunction characterised by the expression of inhibitory receptors such as programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte antigen-4 (CTLA-4) and a progressive decrease in the release of pro-inflammatory cytokines and mediators such as interleukin-2 (IL-2), TNFα, IFNγ and granzyme B (GzmB) (Jiang et al. 2015; Chen & Mellman 2013). Macrophages and DCs can also suppress T cell responses by depleting essential nutrients from the environment. APCs and even tumour cells can express indoleamine 2,3-dioxygenase (IDO) (Prendergast 2008) and macrophages also express arginase I (Gordon & Martinez, 2010. These molecules deplete tryptophan and L-arginine, respectively, inhibiting T cell proliferation and activation (Bronte et al. 2003).

New blood vessel formation is essential to sustain tumour growth as it guarantees nutrient and oxygen availability across the tumour mass. Macrophages play an important role in the “angiogenic switch”, a key step that usually precedes the transition to malignancy. TAMs participate in the generation of new vessels by secreting specific growth factors that stimulate angiogenesis, such

as the vascular endothelial growth factor (VEGF)-A, a major player in control of the “angiogenic switch”. The bioavailability of this growth factor is dependent on the enzymatic activity of matrix metalloproteinase MMP-9, which releases VEGF-A trapped in the extracellular matrix (Bergers et al. 2010). VEGF-A can also be produced by the tumour itself (Goel & Mercurio 2013) and besides its role in angiogenesis, it also acts as a monocyte chemoattractant and induces vascular permeabilisation to recruit leukocytes. Inadequate vascular perfusion due to the formation of disorganised and immature blood vessels within the tumour causes hypoxia and upregulates VEGF-A through the transcription factors hypoxia-inducible factor (HIF)-1α and HIF-2α. Pro-inflammatory cytokines such as IL-1α, IL-6 and TNF-α can also induce VEGF-A expression (Ferrara et al. 2003; Olsson et al. 2006a). VEGF-A signals through VEGFR-1 and VEGFR-2, and interestingly, VEGFR-1 can also bind the ligand placental growth factor (PIGF), a growth factor released by TAMs (Ferrara et al. 2003; Olsson et al. 2006b). Macrophages closely associated to the vasculature are positive for the angiopoietin receptor Tie2, which recognises the vascular growth factor angiopoietin 2 (ANG2) secreted by endothelial cells. These macrophages facilitate angiogenesis, as they express angiogenesis-promoting factors. In fact, specific deletion of these $Tie2^{+}$ macrophages inhibits tumour growth and metastasis in metastatic mammary carcinomas and pancreatic insulinomas (Mazzieri et al. 2011). Besides VEGF-A, TAMs secrete several factors that also participate in angiogenesis, such as TNF-α, IL-1β, IL-8, PDGF, FGF, plasminogen activator, urokinase (uPA) and angiogenesis-modulating enzymes such as matrix metalloproteinase 7 (MMP7), MMP9, MMP12 and cyclooxygenase

2 (COX2) (Murdoch et al. 2008; Lewis & Pollard 2006; Bergers et al. 2010).

Tumour associated macrophages are important in promoting invasion and metastasis of transformed cells. Tumour cells secrete CSF-1, which stimulates macrophage recruitment and production of epidermal growth factor (EGF), which in turn activates migration of the tumour cells to be released into the circulation, as demonstrated by Wyckoff and colleagues in mammary tumours (Wyckoff et al. 2004; Wyckoff et al. 2007). Tumour cell migration requires modifications in the structure of the extracellular matrix (ECM). For that, TAMs regulate the proteolytic destruction of the matrix to allow the migration of tumour cells through the dense stroma. This involves the production of proteases such as matrix metalloproteinases (MMP)-9 and MMP-2, cathepsins B and S and serine proteases (Qian & Pollard 2010). Moreover, macrophages modify the ECM composition by releasing specific ECM components such as fibrillar collagen I, which facilitates tumour cell migration (Qian & Pollard 2010; Quail & Joyce 2013).

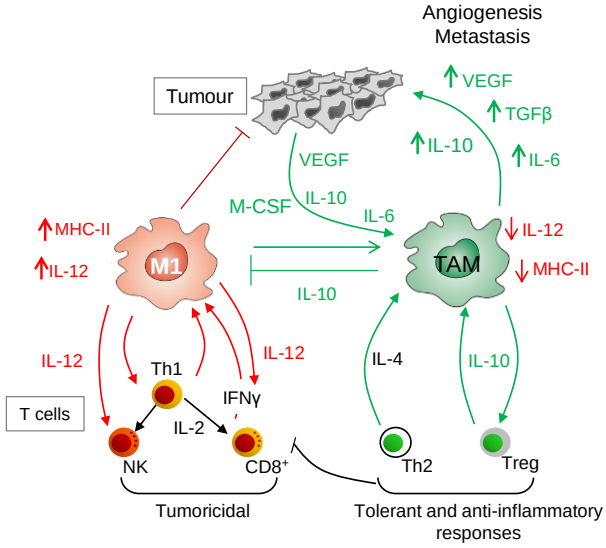


Figure 3. Macrophage-tumour cell cross-talk influences macrophage polarisation and tumour development. Simplified illustration describing the interactions between cancer cells, M1 and M2-like macrophages and other cells from the immune system in the tumour microenvironment.

1.4.2.2 Cancer therapies targeting tumour-associated macrophages

Considering the functional plasticity of macrophages and their role in tumour development, there has been an increasing interest in developing cancer therapies that target them, either by boosting their pro-inflammatory and cytotoxic properties (Hagemann et al. 2008) or by inhibiting their recruitment or eliminating the tumour-promoting macrophages from established tumours. These therapies are predominantly being used as adjuvants of chemotherapy or radiotherapy (Mantovani & Allavena 2015, Ruffell & Coussens 2015, Noy & Pollard 2014).

Repolarisation of TAMs to M1-like macrophages can be achieved by different agents used as anticancer treatments as described next. The Th1 cytokine IFNγ was used to treat minimal residual ovarian cancer (Pujade-Lauraine et al. 1996, Colombo et al. 1992). Bacterial products such as intravesical Bacillus Calmette–Guérin (BCG) has been used for bladder cancer (Redelman-Sidi et al. 2014). CpG oligonucleotides, which are TLR ligands, are undergoing clinical evaluation, as they cause pro-inflammatory cytokine production (e.g. TNFα) in tumour-infiltrating myeloid cells, such as in subcutaneous tumours (Iida et al. 2013). The agonistic antibody against the costimulatory molecule CD40, induces up-regulation of MHC class II and CD86 when administered to a

mouse model of pancreatic cancer (Beatty et al. 2011). The plasma protein histidine-rich glycoprotein (HRG) changes TAM polarisation to antitumor cells by down-regulating PlGF (Rolny et al. 2011).

TAM repolarisation to an M1-like phenotype is also shown to be achieved by a local low dose irradiation (LDI). LDI polarised M2-like TAMs to $iNOS^+$ M1-like macrophages that subsequently promoted vascular normalisation and recruitment of tumour-reactive $CD4^+$ and $CD8^+$ effector and memory T cells (Klug et al. 2013).

Intravenous immunoglobulins (IVIg) promote the repolarisation of macrophages depending on their initial functional state, as it induces a pro-inflammatory phenotype in M2-like macrophages and limits the pro-inflammatory actions of M1-like macrophages. The immunomodulatory effects of IVIg on cancer rely on their binding to activating Fc receptors in M2-like macrophages, skewing TAMs towards a pro-inflammatory phenotype that prevents tumour progression and metastasis (Dominguez-Soto et al. 2014).

The localisation of TAMs within the tumour reflects their functional properties, with pro-tumoural TAMs being more prevalent in hypoxic areas and showing a superior proangiogenic activity (Movahedi et al. 2010). Thus, relocating TAMs to normoxic tumour regions could be a strategy to inhibit their protumour phenotype. The inactivation of Nrp1, a protein involved in triggering VEGFR1 phosphorylation and macrophage attraction, enhances TAM localisation in normoxic areas of mammary tumours, reducing their immunosuppressive and angiogenic capacity (Laoui et al. 2014; Casazza et al. 2013).

Mazzieri and colleagues showed that the inhibition of the ANG2/Tie2 axis impaired tumour growth and metastasis in mouse

models of mammary carcinomas and pancreatic insulinomas. Blocking Angiopoietin 2 (ANG2) secretion by endothelial cells impedes the upregulation of Tie2 in monocytes recruited to the lesion. This results in reduced numbers of Tie2 expressing perivascular macrophages (TEMs) interacting with endothelial cells, which causes a decrease in their proangiogenic program and consequently, a reduction in tumour size (Mazzieri et al. 2011).

Tumours secrete CSF-1 that is involved in the proliferation and survival of macrophages. Indeed, several chemotherapies that target the CSF-1/CSF1R axis by using antisense oligonucleotides, monoclonal antibodies, or kinase inhibitors have proven useful to eliminate TAMs. Ries *et al* generated a monoclonal antibody (RG7155) that inhibits CSF-1R activation resulting in cell death of CSF-1-differentiated macrophages, accompanied by an increase in the $CD8^+/CD4^+$ T cell ratio *in vivo*. Administration of this antibody to patients with diffuse-type giant cell tumour, a disease driven by overexpression of CSF-1 and increased recruitment of myeloid cells, led to clinical responses due to $CSF\text{-}1R^+CD163^+$ macrophage reduction in tumour tissues (Ries et al. 2014). Moreover, Pyonteck et al showed that glioma treatment with a chemical inhibitor of CSF-1R promoted TAM repolarisation by decreasing their M2-like phenotype, preventing tumour progression (Pyonteck et al. 2013). The clinical outcome of the treatment of MMTV-PyMT tumour bearing mice with αCSF-1 mAb in combination with paclitaxel (PTX) is contingent on IL-10. Indeed, combination of PTX with an αIL-10 mAb showed the same clinical result (Ruffell et al. 2014). This observation was reported to be a consequence of the depletion of IL-10 secreted by macrophages, which inhibits IL-12

production by DCs, and in turn, IL-12 engages cytotoxic CD8 T cell responses (Ruffell et al. 2014).

Provided that TAMs originate from blood monocytes, blocking monocyte recruitment is a strategy to reduce TAM numbers. CCL2 is the main chemoattractant responsible for inducing the influx of circulating monocytes to tumour tissues. Antibodies to CCL2, such as Carlumab are now being tested in clinical trials and they show preliminary antitumor activity in patients with advanced cancer (Pienta et al. 2013; Sandhu et al. 2013). Nonetheless, anti-CCL2 therapies might require further investigation since Bonapace and colleagues reported that interruption of this treatment in a breast cancer model caused an increase in monocyte egress from the bone marrow, mobilisation and infiltration of cancer cells, and augmented angiogenesis driven by IL-6 and VEGF (Bonapace et al. 2014).

2. The transcription factor NFAT5

Nuclear factor of activated T cells 5 (NFAT5) is a transcription factor that is primarily known as being the unique regulator of osmotic stress responses in mammalian cells (Miyakawa et al. 1999, Ko et al. 2000).

NFAT5 is the latest addition to the Rel-family of proteins, which comprises the NFAT1-4 and NF-κB proteins. NFAT5 was cloned in 1999 by two independent groups, who followed different strategies. Cristina López Rodríguez and its colleagues discovered NFAT5 by means of the homology of its DNA-binding domain (DBD) to that of the NFATc proteins (Lopez-Rodríguez et al. 1999), whereas Miyakawa and his group, using a yeast two-hybrid assay, cloned

the protein that bound to the tonicity-responsive enhancer (TonE) elements in genes involved in osmostress responses (Miyakawa et al. 1999).

NFAT5 is probably the member that diverged earlier among the Rel family of proteins, a family that has a long evolutionary history. Structural homology analysis shows that the Rel-proteins can be found from arthropods to mammals, including humans, while are absent in nematodes and unicellular eukaryotes (Aramburu et al. 2006). While the NFATc proteins are solely found in vertebrates (Graef et al. 2001; Hogan et al. 2003), cells from the arthropod *Drosophila melanogaster* express a single NFAT-like factor, called dNFAT, which has around 1400 aminoacids and shows a DBD that has high homology to that of NFAT5 (Adams et al. 2000, Aramburu et al. 2006).

Not only does NFAT5 function as an osmostress regulating factor, as it was first described by Miyakawa *et al.* in 1999, but it also has several roles which are independent of hypertonicity. Some of these functions are related to the development and activation of immune cells, highlighting the potential of NFAT5 as a *bona-fide* member of the Rel-family of proteins, a family of transcription factors that are important in immune cell function (Rao et al. 1997).

2.1 Structure of NFAT5

NFAT5 was classified into this group of proteins due to its homology with the Rel-like homology region (RHR), which contains the DNA-binding domain (DBD) followed by the dimerisation domain (Lopez-Rodríguez et al. 1999)(**figure 4**). NFAT5 can be seen as a hybrid between the NF-κB and the NFATc proteins. On

the one hand, similar to NF-κB proteins, its DBD can form a stable dimer even in the absence of DNA, but this dimerisation is essential for its DNA binding and transcriptional activity (López-Rodríguez et al. 2001). Although they share a similar dimerisation surface, NFAT5 shows an additional dimer interface (E'F) loop that is responsible for encircling DNA providing a high stability for NFAT5 into its target sites (Stroud et al. 2002). However, its aminoacid sequence does not show the contact residues that account for Jun binding in NFATc proteins, suggesting that it does not require AP-1 to bind to DNA cooperatively (Lopez-Rodríguez et al. 1999)(**figure 5**).

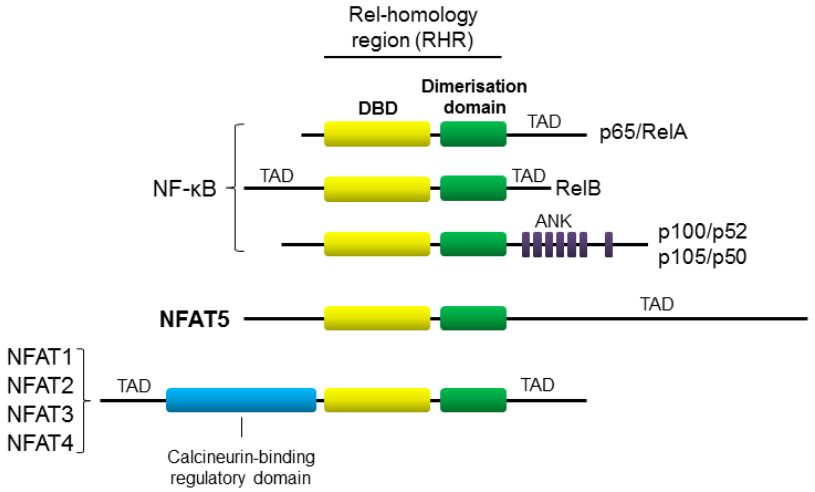


Figure 4. Schematic representation of the protein domains of the members of the Rel family of proteins. All the members share the Rel-homology region, comprised of the DNA-binding domain (DBD) in yellow and the dimerization domain in green. Other domains include the transactivation domain (TAD), the calcineurin-binding domain in blue and the ankirin-repeats (ANK) in purple. *Adapted from Aramburu et al., 2006, Biochemical Pharmacology.*

On the other hand, in terms of DNA binding specificity, NFAT5 has greater resemblance to NFATc proteins. The DNA consensus site that is recognised by NFAT5, which is (A/T)GGAAANN(C/T)N(C/T), contains the NFATc core binding site (GGAA) (Lopez-Rodríguez et al. 1999). This sequence clearly differs from the consensus sequences defined for NF-κB, which are palindromic elements (GGGRNNYYCC, where R is A/G and Y is C/T) to which the two halves of the dimer bind symmetrically. In contrast, NFAT5 only uses one half of the dimer to contact the consensus DNA site, while the other half binds the DNA backbone in a base-inespecific manner (Stroud et al. 2002).

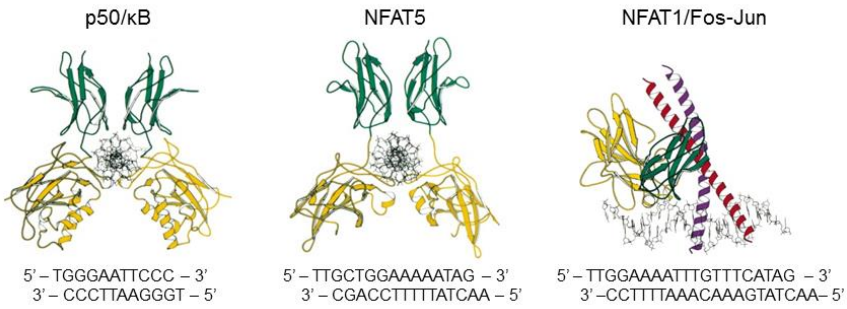


Figure 5. Rel-family members binding their DNA consensus site. Representation of the DNA consensus site and the Rel-homology region of NF-κB, NFAT5 and NFAT1 binding DNA. *Adapted from Hogan et al., 2010, Genes and Development.*

Outside the Rel-homology region, NFAT5 does not show any structural similarities to the other members in the family (Aramburu et al. 2006). Whereas the conventional NFATc factors are activated by calcium in response to different stimuli, in a calcineurin-dependent manner, the N-terminal region of NFAT5 lacks the calcineurin docking sites and phosphorylation residues found in the

N-terminal regulatory region of the NFATc proteins. NFAT5 is formed by almost 1500 aminoacids, with more than 900 aminoacids giving rise to the long C-terminal transactivation domain (TAD) (Aramburu et al. 2006).

2.2 Regulation of NFAT5 expression

NFAT5 mRNA is ubiquitously expressed, as it can be detected in all tissues, with highest levels skeletal muscle, brain, heart, and peripheral blood leukocytes (PBL) (Lopez-Rodríguez et al. 1999). However, NFAT5 protein is only detected in tissues such as the thymus and the testis (Trama et al. 2000). Regulation of NFAT5 expression occurs both at the transcriptional and translation levels (Aramburu et al. 2006).

In response to tonicity, the increase in NFAT5 synthesis contributes to sustain the genetic programs in which it is involved. Both mRNA and protein levels of NFAT5 augment, but the mechanisms underlying this process are only partially known. Nonetheless, it has been demonstrated that some miRNAs participate in the regulation of the stability of NFAT5 mRNA. Specifically, miR-200b and miR-717 are two important molecules involved in NFAT5 repression. These regulatory RNAs interact with the 3'-UTR of NFAT5 mRNA and cause a decrease in both its mRNA and protein levels. In response to hypertonicity, this process is abrogated since the levels of these regulatory RNAs are strongly downregulated (Huang et al. 2011).

Although a substantial proportion of NFAT5 is constitutively nuclear in most cell lines analysed, hypertonicity promotes complete translocation of NFAT5 to the nucleus (López-Rodríguez et al.

2001). This translocation is mainly regulated by the exposure of a nuclear localisation signal (NLS), whereas the export from the nucleus in isotonic or hypotonic conditions is controlled by a nuclear export signal (NES) and by the auxiliary export domain (AED) (Aramburu et al. 2006). Estrada-Gelonch et al. also unravelled a molecular mechanism that restrains the association of NFAT5 with chromatin regardless of the extracellular tonicity and therefore controls its nuclear localisation. This process involves a switch in the transactivating module of NFAT5 from a stimulus-specific activator of transcription in interphase to a stimulus-independent repressor of binding to DNA in mitosis (Estrada-Gelonch et al. 2009).

In response to elevated extracellular osmolalities, NFAT5 increases its transactivation activity. The mechanism beneath this process is not completely understood because of controversial results obtained in various works using different cell types. However, it is well established that it relies on the phosphorylation of some Tyr and Ser residues found in the transactivation domain (TAD) by several kinases belonging to different signalling pathways. These kinases include the mitogen-activated protein kinase (MAPK) p38, DNA damage response related protein Ataxia telangiectasia-mutated (ATM) and the Src kinase family-member Fyn (involved in cell adhesion and motility), among others (Aramburu et al. 2006; Zhou 2016).

Other than changes in tonicity, TLR stimulation upregulates NFAT5 mRNA levels in macrophages, leading to a progressive accumulation of its protein. The increase in NFAT5 protein is mainly and is regulated by NF-κB, which binds the *Nfat5* promoter

(Buxadé et al. 2012) upon nuclear translocation triggered by IKKβ-dependent phosphorylation and degradation of IκBα (Gilmore 2006). Moreover, IKKβ also regulates the accessibility of NFAT5 to target genes as Nos2 (Buxadé et al. 2012).

Similarly, IKKβ-NF-κB pathway also targets NFAT5 in thymocytes. In these cells, NFAT5 acts as a survival and differentiation factor under the pre-TCR (Berga-Bolaños et al. 2013). Indeed, both IKKβ *knock-out* mice and conditional knock-out mouse model that deletes NFAT5 in double negative thymocytes present reduced cellularity in thymocytes compared to *wild-type* mice (Berga-Bolaños et al. 2013).

In mature T lymphocytes, NFAT5 levels can be upregulated by increasing extracellular tonicity *in vitro*, and this leads to the expression of osmoadaptive-related genes such as aldose reductase (AR) (Drews-Elger et al. 2009). Interestingly, when T cells are activated by TCR-engagement or through mitogens in isotonic conditions, they also show an increase in NFAT5 mRNA and protein levels, albeit in these circumstances the upregulation is dependent on calcineurin and there is no induction of any osmoadaptive-related gene (Trama et al. 2000; López-Rodríguez et al. 2001).

2.3 Functions of NFAT5

2.3.1 NFAT5 functions in hypertonicity

Cells shrink in response to hypertonicity due to the loss of water followed by an increase in intracellular ions, aiming to compensate the differences in solute concentrations. These variations in cell volume and solute concentrations cause intracellular molecular

crowding that can have deleterious consequences, such as denaturing of macromolecules as DNA and proteins, and even apoptosis (Cheung & Ko 2013). NFAT5 activates an adaptive response to hypertonicity to restore the osmotic balance through the gradual replacement of intracellular electrolytes by uncharged small organic osmolytes which do not show those harmful effects. More specifically, NFAT5 drives the expression of the enzyme aldose reductase (AR), which mediates the synthesis of sorbitol; and of some transporters of organic molecules such as the betaine transporter (BGT1), the coupled sodium-myo-inositol transporter (SMIT), and the taurine transporter (TauT). Besides regulating the electrolytic balance, NFAT5 also induces the expression of certain heat shock proteins, such as HSP70 (Woo et al. 2002).

This osmoregulatory process is essential in cells of the renal medulla, which are constantly exposed to high levels of hypertonicity in the extracellular medium, due to the urine concentrating mechanism. In fact, NFAT5 null mice show severe kidney atrophy associated with cellular loss (López-Rodríguez et al. 2004). Considering the kidney aside, even if overall extracellular tonicity is widely maintained within a tightly defined and homeostatically regulated range (Go et al. 2004), there are fluctuations in osmolality that can explain the wide variety of cells that can respond to hypertonic conditions. For example, some organs and tissues, such as thymus, spleen and liver (Halterman et al. 2012), can show moderate and variable levels of hypertonicity in homeostatic conditions, although it has not been clearly demonstrated whether these can be high enough to induce the activation of NFAT5.

Moreover, some inflammatory pathologies can also be associated with hyperosmolality. For example, hypernatremia caused by severe dehydration or heart stroke, uremia, diabetis mellitus or inflammatory bowel disease show systemic or local hypertonicity to varying levels (Neuhofer 2010). Nonetheless, due to technical limitations, the direct association of NFAT5 with these diseases has only been proven for some conditions, such as diabetic microvascular lesions, where NFAT5 drives aldose reductase and inflammatory cytokine expression in peripheral blood mononuclear cells (PMBCs) (Neuhofer 2010).

As mentioned earlier, hypertonicity can be related to immune organs such as the spleen or the thymus and to inflammatory pathologies such as diabetis mellitus. Moreover, it has been shown that NFAT5 promotes the expression of pro-inflammatory cytokines such as lymphotoxin β and TNF-α in Jurkat cells exposed to osmotic stress (human T lymphocyte immortalized line)(López-Rodríguez et al. 2001). As a consequence, there has been an increasing interest on studying the role of NFAT5 in diverse immune cell types under osmotic stress conditions, and several papers have been published in the recent years. In T lymphocytes, Drews-Elger *et al*. show that NFAT5 facilitates the progression throughout the cell cycle in response to hypertonicity-induced genotoxic stress *in vitro*, due to its ability to induce the expression of the cyclins that regulate the G1 to G2/M progression (Drews-Elger et al. 2009). On the other hand, Bolaños *et al.* observed that NFAT5-null mice, which suffer from systemic hypernatremia, show T cell lymphopenia (Go et al. 2004; Berga-Bolaños et al. 2010) together with a biased memory phenotype in the $CD8^+$ T cell compartment and impaired ability to reject allogeneic tumours

(Berga-Bolaños et al. 2010). This phenotype is in part due to a defect in CD24 expression, a protein that is necessary for T cell proliferation and conversion to memory cells (Berga-Bolaños et al. 2010).

NFAT5 is also involved in the acquisition of a pro-inflammatory phenotype of mature T cells exposed to elevated osmolalities. According to Kleinewietfeld *et al.*, high salt concentrations promote exacerbated Th17 differentiation driven by the p38-NFAT5-SGK1 axis, with high levels of expression of IL-17A, GM-CSF, TNFα and IL-2 compared to cells cultured in isotonic conditions. They observed that mice fed with high-salt diet presented severe forms of experimental autoimmune encephalomyelitis, the animal model for multiple sclerosis, an autoimmune and inflammatory disease that affects the central nervous system (CNS). High-salt diet fed animals show earlier onset of the disease, with increased severity and higher infiltration of IL-17A expressing $CD4^+$ T cells in the CNS (Kleinewietfeld et al. 2013). In line with this, Alberdi *et al.* showed, using T cell-specific NFAT5 *knock-out* mice, that IL-17 production by $CD4^+$ lymphocytes in hypertonic conditions required NFAT5-dependent RORγT induction (Alberdi et al. 2016).

Kino and colleagues studied the role NFAT5 in B lymphocyte biology. They demonstrated that Brx, a guanine nucleotide exchanging factor, directly interacts with an activator of p38 MAPK, a well-known regulator of NFAT5 in hypertonicity (Morancho et al. 2008). The authors show that Brx haploinsufficient mice, which express low levels of NFAT5, show smaller spleens with fewer splenocytes and aberrant follicular structures, and impaired differentiation of terminal B cells, that produce lower amounts of

immunoglobulins. They also observed that induction of B-cell activating factor BAFF by splenocytes upregulated upon hypertonic treatment and in a NFAT5-dependent manner (Kino et al. 2009).

Regarding innate immune cells, high levels of salt can boost pro-inflammatory and antimicrobial responses in macrophages. High-salt diet fed animals accumulate sodium in the skin and this is beneficial to fight cutaneous infections by *Leishmania major* (Jantsch et al. 2015). In response to increased tonicity in the skin, macrophages upregulate the expression of pro-inflammatory cytokines such as TNF-α, and also increase the levels of the enzyme inducible nitric oxide synthase (iNOS). This enzyme is involved in the production of nitric oxide, a molecule that is essential for eliminating this pathogen. Machnik and colleagues also show that NFAT5 in MPS cells can be important in hypertension. They demonstrate that upon interstitial skin sodium accumulation in high-salt diet fed animals, NFAT5 induction in MPS cells promotes the expression of vascular endothelial growth factor-C (VEGF-C). The release of this growth factor has two main beneficial effects: it causes hyperplasia of the lymph capillary network to help recover interstitial electrolyte and volume homeostasis, and it also promotes the upregulation of endothelial nitric oxide synthase (eNOS) to maintain blood pressure through its vasodilatory function (Machnik et al. 2009).

2.3.2 NFAT5 functions independent of osmotic stress

Although NFAT5 is a factor involved in osmoregulation, several works have already described different roles for this protein that are independent of osmotic stress. Some of these functions are important in immune responses and will be reviewed here.

NFAT5 is a survival factor that acts under the pre-TCR in thymocyte development to regulate their differentiation in the transition from double negative (DN) to double positive (DP) cells. Mice that delete NFAT5 during the DN2 stage of thymocyte differentiation (*Nfat5*$^{fl/fl,\ Lck\text{-}Cre}$) show smaller thymi, accumulation of DN3 thymocytes and decreased cellularity of double negative and double positive thymocytes, followed by a reduction in the numbers of mature $CD4^+$ and $CD8^+$ lymphocytes in spleen and lymph-nodes (Berga-Bolaños et al. 2013). NFAT5 is a target of IKKβ downstream the pre-TCR to upregulate its expression and, in turn, control the β-chain allelic exclusion and regulate the balance between specific antiapoptotic Bcl2 family members and the p53/Noxa axis (Berga-Bolaños et al. 2013).

Regarding mature T lymphocytes, Alberdi et al. showed that *in vivo* activation of NFAT5-deficient $CD4^+$ T cells by anti-CD3 injection resulted in a gene profile different to that observed in experiments done in hypertonic conditions. In these new settings, NFAT5-deficient T cells were skewed towards enhanced IFNγ and IL-17 expression and attenuated Treg responses. This work also showed that T cell-specific NFAT5-deficient mice suffered from exacerbated intestinal inflammation in an experimental model of dextran sodium sulfate-induced colitis, with enhanced expression of IFNγ in draining lymph nodes and colon (Alberdi et al. 2016).

NFAT5 plays a key role in the macrophage response to pathogens. Buxadé *et al.* show that NFAT5 is necessary for the expression of genes codifying for cytokines and chemokines, extracellular matrix or protease-related proteins, regulators of nitric oxide production and certain repressors of inflammation, all of them relevant in

various aspects of the response to pathogens (Buxadé et al. 2012). These genes are induced in response to TLR stimulation, and their dependence on NFAT5 is overall higher when macrophages sense low doses of the stimulatory signal. Thus, NFAT5 could be an important transcription factor involved in coupling the strength of signal input to the specificity of gene expression. These stimuli include the bacterial lypopolisaccharide LPS, that is recognised by TLR4, the dsRNA mimetic polyIC, which binds to TLR3, and Zymosan A, a wall component of *Saccharomyces cerevisiae* that is recognised by TLR2-TLR1 complexes. Chromatin immunoprecipitation (ChIP) analysis demonstrated that NFAT5 binds to the promoters of genes encoding for pro-inflammatory cytokines such as *Il6* and *Il12b*, and antimicrobial molecules such as *Nos2* (iNOS). The association of NFAT5 with its target genes differs among primary and secondary response genes. On the one hand, NFAT5 is constitutively bound to the promoter of a subset of primary response genes such as *Tnfa, Il1a* or *Ccl2* in unstimulated macrophages and its binding is not modified upon TLR activation. On the other hand, the recruitment of NFAT5 to secondary response genes such as *Il12b* or *Nos2* is dependent on TLR-mediated activation. Upon TLR-stimulation, the binding of NFAT5 to the promoters of its secondary target genes is probably controlled by increased chromatin accessibility. This process is most likely regulated by the IKKβ-NF-κB axis and is dependent on *de novo* protein synthesis (but not of NFAT5 itself) and the activity of histone acetyl transferases. IKKβ could be acting through diverse mechanisms, such as causing posttranslational modifications in NFAT5 or regulatory factors, or even inducing, via NF-κB, the *de novo* expression of a chromatin modifier or a potential NFAT5 partner. *In vivo*, the immunomodulatory role of

NFAT5 in TLR-stimulated macrophages has an impact in the control of *Leishmania major* infection, a parasite whose clearance strongly depends on nitric oxide produced by the enzyme iNOS and TLR activation. In fact, upon TLR stimulation, NF-κB induces the accumulation of NFAT5, which can therefore collaborate in sustaining the expression of genes with slow induction kinetics, such as *Nos2* or *Il6* (Buxadé et al. 2012).

In a similar way, Kim *et al.* showed that NFAT5 regulates the expression of TLR-dependent pro-inflammatory genes in the macrophage cell line Raw264.7, downstream p38-MAPK and the enzyme xanthine oxidase (XO) (Kim et al. 2014). They confirm their findings using a mouse model of rheumatoid arthritis, a chronic inflammatory and autoimmune disease characterised by a prolonged secretion of pro-inflammatory mediators such as TNF-α, IL-6 or NO by immune cells that leads to synovial joint destruction. In this mouse model, NFAT5 haploinsufficiency or treatment with allopurinol, a XO pharmacological inhibitor, reduced the severity of the disease, and this correlated with decreased NFAT5 expression in macrophages isolated from the lesions.

It has been recently described that NFAT5 impairs the expression of M2 genes in the macrophage cell line Raw264.7 by downmodulating *Il10*, a pleiotropic cytokine that dampens pro-inflammatory gene expression promoting an anti-inflammatory phenotype. The authors show that NFAT5 impairs the recruitment of the transcription factor Sp1 to the promoter of *Il10* gene by reducing chromatin accessibility at this genomic region. As a consequence, upon NFAT5 *knock-down* using siRNA, they observe

an upregulation of M2-related genes such as *Arg1* and *Cd206* (mannose receptor) (Choi et al. 2016).

Macrophages can also act as a reservoir for the Human Immunodeficiency Virus (HIV), and in these cells, NFAT5 acts as a host factor that facilitates the replication of the viral genome, as it interacts with a specific enhancer binding site conserved in several HIV virus subtypes (Ranjbar et al. 2006). Moreover, co-infection with *Mycobacterium tuberculosis* (MTb) further promotes this replication function, because MTb recognised by TLRs causes the upregulation of NFAT5 protein levels (Ranjbar et al. 2012).

CHAPTER 2

OBJECTIVES

OBJECTIVES

Macrophages are cells of the innate immune system found in every tissue where they act as sentinel cells, essential to maintain whole-body homeostasis. Macrophage responses are remarkably dynamic and plastic and are mainly controlled at the transcriptional level. In this regard, our group has identified the transcription factor NFAT5 as a key regulator of pro-inflammatory gene expression in macrophages in response to mild TLR stimulation. As a result, NFAT5-deficient showed impaired *in vivo* anti-pathogen responses.

To further characterise the role of NFAT5 in macrophage function, in this thesis project we aimed at elucidating its role in macrophage polarisation. To this purpose, we defined the following specific objectives:

1. Determine the contribution of NFAT5 to the acquisition of pro-inflammatory and anti-inflammatory functional phenotypes by macrophages *in vitro*, by characterising the impact of NFAT5 in the expression of polarisation markers.

2. Explore the role of NFAT5 in polarised macrophage responses, by analysing different polarisation functions *in vitro* and by defining the phenotype and function of NFAT5-deficient macrophages in models of tumour progression *in vivo*.

CHAPTER 3

MATERIALS AND METHODS

Mice

1. NFAT5 knock-out mice

Mice heterozygous for *Nfat5* were described previously (López-Rodríguez et al., 2004). Because NFAT5-null mice in a mixed 129/sv-C57BL/6 background had a severe mortality rate from late embryonic development to early perinatal stages (Go et al. 2004; López-Rodríguez et al. 2004), we bred them for >10 generations to a pure 129/sv background and observed that the rate of survival of NFAT5-null mice (*Nfat5*$^{-/-}$) increased, with >30% of the expected Mendelian ratio of *Nfat5*$^{-/-}$ mice reaching adulthood. *Nfat5*$^{+/-}$ mice were maintained in an isogenic 129/sv background and were crossed to obtain *Nfat5*$^{-/-}$ mice and control *Nfat5*$^{+/+}$ littermates. All mice were analysed between 6 and 10 weeks of age. Mice were bred and maintained in specific pathogen-free conditions, and animal handling was performed according to institutional guidelines approved by the ethics committee of the PRBB/UPF Animal Care and Use Committee and carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

2. Conditional NFAT5 knock-out mice

Conditional NFAT5 knock-out mice were generated by crossing mice that have the exon 6 of the *Nfat5* gene floxed with mice expressing the Cre recombinase in a tissue specific manner. In this way we obtained three different conditional knock-out mice in the C57BL/6 background that can be used to study the effect of *Nfat5* deletion in myeloid cells or in BMDMs: *Nfat5*$^{fl/fl,\ MxCre}$, *Nfat5*$^{fl/fl,\ LysMcre}$ and *Nfat5*$^{fl/fl,\ Vavcre}$.

2.1. MxCre knock-out mice

MxCre is an inducible and conditional knock-out mouse model in which mice express the Cre recombinase under the control of the Myxovirus resistance-1 (Mx1-Cre) gene promoter, which is a vital part of the viral defence mechanism that is highly induced in response to type I interferons (Kuhn et al. 1995). The Cre recombinase expression can be induced in cells that respond to interferon-α/β in response to double stranded RNA [poly(I): poly(C)]. To induce Cre expression *in vivo*, mice were intraperitoneally injected 3 times every other day with high-molecular weight poly(I): poly(C) (pIC) (Invivogen, catalogue tlrl-pic) at 15 mg/kg of mouse and mice were sacrificed 11 days after the last injection. This knock-out model was used exclusively to generate BMDMs.

2.2. VavCre knock-out mice

Vav1 cre conditional knock-out mice was kindly provided by Thomas Graf. The constitutively active Vav-Cre mice achieve gene deletion in the hematopoietic system and in endothelial cells, mediated by Cre recombinase expression under the control of the murine vav gene regulatory elements (Stadtfeld & Graf 2005). In this mouse model, there is NFAT5 deletion in all haematopoietic cells, including all myeloid subsets (**figure 6**).

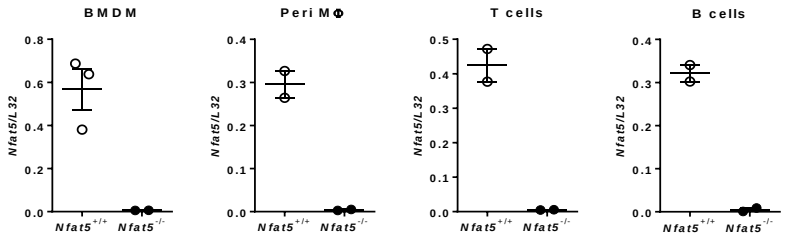


Figure 6. NFAT5 expression in bone marrow-derived macrophages, peritoneal macrophages (Peri MØ) and T and B lymphocytes from WT and *Nfat5*$^{fl/fl,\ Vavcre}$ mice. NFAT5 mRNA expression was analysed relative to L32 mRNA levels. Each dot represents an individual sample.

2.3. LysMcre knock-out

Lys-M-Cre mice (Clausen et al. 1999) were obtained from Jackson Laboratory (B6.129P2-Lyz2tm1(cre)Ifo/J). The Cre recombinase is under the control of the Lyz2 (M lysozyme) gene promoter, which is expressed in all myeloid cells, including monocytes, macrophages and neutrophils during development, in resident tissue macrophages and in response to infectious agents. Mice have two copies of the Cre recombinase, and *Nfat5* floxed/floxed LysMcre mice rendered a reduction of more than 85% in the expression of NFAT5, as seen in peritoneal resident macrophages (figure 7).

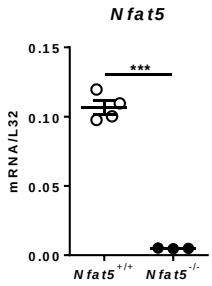


Figure 7. NFAT5 mRNA expression in peritoneal macrophages from WT and *Nfat5*$^{fl/fl,LysMcre}$ mice. *Nfat5* mRNA expression was analysed relative to L32 mRNA levels. Each dot represents an individual sample. Bars show mean ± SEM and significance was determined using unpaired t test, ***<p=0.001.

Bone marrow-derived macrophage (BMDM) culture

To obtain bone marrow-derived macrophages (BMDMs), 6 to 10 week-old mice were sacrificed and the femoral and tibial marrow flushed with DMEM supplemented with 2 mM glutamine, 50 µM β-mercaptoethanol, 1 mM sodium pyruvate and penicillin/streptomycin (from now on incomplete medium) (all from Thermofisher) using 25G syringes. Cells were then resuspended in complete medium (incomplete plus 10% heat inactivated foetal bovine serum (FBS)) and counted using Türk solution (Cristal violet and 1% acetic acid to lyse erythrocytes). 10 x 10^6 bone marrow precursors were plated with 25% (vol/vol) of L929-conditioned medium (as a source of macrophage-colony stimulating factor (M-CSF)) in 150 mm diameter sterile polystyrene petri dishes (Sarstedt, catalogue 82.1184.500) and incubated for 6-7 days at 37ºC in a humidified tissue culture incubator (5% CO_2).

In vitro stimulation of BMDMs

Differentiated macrophages were harvested by washing once with 1X PBS and then incubating for 10 minutes with ice cold 1X PBS with 5 mM EDTA. Detached macrophages were collected by gentle pipetting, centrifuged for 5 minutes at 1200 rpm and then resuspended in complete medium. Macrophages were counted with tripan blue solution and plated in sterile tissue culture-treated

multiwell plates. For Western blot, ELISA and mRNA analysis, 6 well multiwell (Nunc, Thermofisher, catalogue 140675) plates were used with 1 x 10^6 cells/well in 3 ml of complete medium. For FACS and T $CD4^+$ co-cultures, 12 well multiwell plates (Labclinics, catalogue PLC30012) were used with 0.7 x 10^6 cells/well in 2 ml of complete medium. For tumour cell-macrophage co-culture analysis, BMDMs were plated in cell culture treated 100 mm diameter plates (Thermofisher, catalogue 172958) with 4 x 10^6 cells in 12 ml. For macrophage polarisation analysis, replated macrophages were treated with 100 U/ml of rm-IFNγ or 10 ng/ml of rm-IL-4 (Immunotools, catalogue 12343536 and 12340043, respectively) for 24 hours to induce M1 and M2 polarisation, respectively. Cells were then activated with LPS at 0.3 ng/ml (from *E.Coli* 055:B5, Sigma, catalogue L2880) for the indicated time points. For Western blots analysis of STAT6 and pSTAT6, BMDMs were also stimulated with rm-IL-4 for 1, 2 and 4 hours.

Gene expression analysis

Total RNA from BMDMs (1 x 10^6) was isolated using the High Pure RNA Isolation kit (Roche, catalogue 11828665001) and quantified in a NanoDrop (ND-1000) spectrophotometer. 100-600 ng of total RNA was retro-transcribed to cDNA using the First Strand cDNA synthesis kit with random primers (Roche, catalogue 04 897 030 001). For real-time quantitative PCR (RT-qPCR), LightCycler 480 SYBR Green I Master Mix (Roche, catalogue 04 887 352 001), LightCycler 480 Multiwell Plates (Roche, catalogue 4729749001) and the LightCycler 480 Real-Time PCR System (Roche) were used according to the manufacturer's instructions.

To extract RNA from sorted samples containing low amounts of cells (10^3-10^5 cells), the RNeasy Microkit (Quiagen, catalogue 74004) was used and the RNA was retrotranscribed using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, catalogue 18080-051).

In all cases, samples were normalised to L32 (L32 ribosomal protein gene) mRNA levels using the Light Cycler Software, version 1.5. Primer sequences for the quantitative PCR reactions are listed in table 1.

Immunoblotting assays

For Western blotting, BMDMs were lysed in Triton X-100 lysis buffer (1 x 10^6 cells in 100 µl; 1% Triton-X-100, 40 mM Hepes pH 7.4, 120 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µg/ml pepstatin A, 1 mM NaF, 1 mM sodium orthovanadate, 10 mM NaPPi and 10 mM β-glycerophophate). Protein concentration was quantified using the BCA assay (Thermo Scientific, catalogue 23227) and lysates were boiled in reducing 1X Laemmli buffer. 10–50 µg of total protein were subjected to SDS-PAGE electrophoresis for 90-120 minutes at 25 mA per gel and transferred to PROTRAN (BA83; Schleicher & Schuell) membranes in 25 mM Tris pH 8.4, 192 mM glycine and 20% methanol. Membranes were blocked with 5% dry milk in TBS (Tris-buffered saline) (or 5% BSA for immunoblotting against phosphorylated proteins) for 1 hour and immunoblotted with specific antibodies (listed below) overnight at 4ºC TBS Tween 0.05% (TBST). Membranes were then washed 3 times with TBST (5 minutes per wash) and incubated for 1 hour with the specific horseradish peroxidase (HRP)-conjugated secondary antibody. Finally,

membranes were washed three times with TBST and developed using ECL (ECL Western Blotting Detection reagents, Amersham, catalogue RPN2106) and photographic films (AGFA).

The antibodies used were: rabbit polyclonal NFAT5-specific antibody (Affinity BioReagents, catalogue PAI-123) recognises the last 17 carboxyterminal amino acids, rabbit anti-iNOS (Santa Cruz, catalogue sc-651), rabbit anti-Arg1 (Cell Signalling, catalogue 9819), mouse-anti-STAT6 (catalogue 611290), mouse-anti-pSTAT6 (Y642) (catalogue 558241), rabbit anti-STAT1 (catalogue 610119) and mouse-anti pSTAT1 (Y701) (catalogue 612132), from BD biosciences.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from polarised BMDM cultures were harvested and centrifuged for 3 minutes at 3000 rpm to obtain cell-free supernatants that were stored at -80ºC. Detection of IL-12p40 was done in duplicate in 96 well-plates (Costar, catalogue 3590) using the ELISA mouse IL-12/IL-23 non allele specific duo kit (R&D, catalogue DY2398-05) following the manufacturer's instructions.

Flow cytometry

2 x 10^5 cells were blocked for 20 minutes in 1X PBS containing 10% FBS and 0.1% sodium azide (PSA buffer) plus 0.2 µg of an antibody against the Fc receptor CD16/32 (Biogelend, catalogue 101302). Cells were then incubated with surface marker-specific antibodies in the same solution (1 µg of antibody per 10^6 cells) for 30 minutes in the dark at 4ºC. After washing them twice with 1X PBS they were resuspended in PSA and analysed with

FACScalibur or LSR II flow cytometers. Data analysis was done using the FlowJo software (TreeStar).

Macrophage and $CD4^+$ T cell co-cultures

Wild-type and NFAT5-deficient BMDMs were seeded in 12 well multiplates in triplicate (0.7 x 10^6 cells/well) and polarised to M1 and M2 phenotypes for 18 hours and treated with LPS 0.3 ng/ml for 12 hours before adding of $CD4^+$ T cells. $CD4^+$ T cells were isolated from the spleen and peripheral lymph-nodes of wild-type C57BL/6 mice by negative selection using the Magnisort Mouse CD4 T cell enrichment kit (eBioscience, catalogue 8804-6821-74) according to manufacturer's instructions. Upon isolation, cells were incubated with 1 µg of anti-CD3 per million of cells (BD Pharmingen, catalogue 553058) for 1 hour at 4ºC, washed with DMEM medium and added to the BMDM cultures in a 1:1 ratio for 48 hours. $CD4^+$ cells were harvested from the co-culture supernatant by gentle pipetting and centrifuged for 5 minutes at 1200 rpm. The supernatant was saved at -80ºC for cytokine detection by ELISA. The pellet was resuspended in PSA for 45 minutes and then isolated by positive selection (Dynabeads CD4 positive Isolation system, catalogue 11331D, Thermo Fisher Scientific) and lysed in RNA lysis buffer (Roche) for total RNA extraction.

Phagocytosis and bacteria killing assay

E.coli (Amp^R) saturated cultures where opsonised with mouse serum before incubating them for 30 minutes at 37ºC with wild-type and NFAT5-deficient M1 or M2 polarised macrophages using a MOI of 1. As a negative control of phagocytosis a parallel assay was maintained at 4ºC. For the killing assay, infected macrophages

were first treated for 1 hour with a high dose of gentamycin (50 µg/ml) to kill extracellular (not uptaken) bacteria, washed and maintained in a low dose of gentamycin (6 µg/ml) for 0, 3 and 6 hours. To determine phagocytosis and killing abilities, BMDMs were lysed at the indicated time points with 1% deoxycholate in 1X PBS and the lysates cultured in ampicilin-containing agar plates to measure the bacterial load.

In vivo tumour models

We used two different isogenic tumour cell lines: the epithelial ovarian carcinoma cell line ID8 and the Lewis Lung Carcinoma (LLC) cell line.

1. Lewis Lung Carcinoma model

The Lewis lung carcinoma cells (LLC) are derived from C57BL/6 mice and were kindly provided by PhD, M.D. Ignacio Melero (CIMA, Pamplona). LLC cells were grown in complete medium in sterile tissue culture dishes (100 x 20 mm Thermofisher, catalogue 172958 or 150 x 20 mm, Ecogen catalogue cc-7682-3614) and maintained at subconfluency by passing them every day or every other day without trypsin, by gentle pipetting.

For solid tumour development, a 1:1 mixture of 1×10^5 of subconfluent LLC cells and wild-type or NFAT5-deficient macrophages were subcutaneously injected in the back right flank of 6 to 8 week-old C57BL/6 female mice. Tumour growth was periodically measured using a caliper and the tumour volume was calculated using the formula $L \times W^2 \times 0.52$, where "L"= maximal length and "W"= maximal width. Alternatively, 0.5×10^6 subconfluent

LLC cells were subcutaneously injected into the right flank of wild-type and *Nfat5*$^{fl/fl\text{-}LysMcre}$ 6 to 10-week-old female mice and tumour development measured as described earlier. Mice were sacrificed at the time points indicated in the figure legends, and solid tumours, spleens and inguinal draining lymph-nodes were excised and weighted. Where indicated, tumours were minced using a scalpel and digested with 0.5 mg/ml of collagenase A (Roche, catalogue 10103578001) plus 0.01% of DNaseI (Sigma, catalogue D4263-5VL) in complete DMEM medium without β-mercaptoethanol during 1 hour at 37ºC in rotation. Samples were then filtered through a 70 μm cell strainer to remove undigested fragments and the filter washed by adding 20 ml of DMEM. Filtered cells were then centrifuged 8 minutes at 1200 rpm and pellets resuspended in 500 μl of PSA. Cells were then analysed by flow cytometry to determine the percentages of lymphocytes and myeloid cells, or were subjected to positive selection using Dynabeads Sheep anti-rat IgG (Life Technologies, catalogue 11035) coated with the anti-CD11b M1/70.15 monoclonal antibody supernatant, for RNA extraction and gene expression analysis by RT-qPCR.

2. Ovarian carcinoma (ID8) model

ID8 cells were derived from an isogenic C57BL/6 ovarian epithelial carcinoma and were used as a serous tumour model (Roby et al. 2000). ID8 cells and ID8 cells expressing stable luciferase (ID8-luc) were a kind gift of Drs. Katherine F. Roby (University of Kansas), José R. Conejo-García (The Wistar Institute), Laurence Zitvogel and Paula Roberti (Institut Gustave Roussy). The cells were grown in sterile cell culture treated dishes in DMEM supplemented with 5% FBS and 1X Insulin-transferrin-sodium selenite media

supplement (ITS 100X, Sigma, catalogue I1884) and passed using 0.05% Trypsin 0.53 mM EDTA (Thermofisher, catalogue 25300-054).

For *in vivo* tumour development, 5 x 10^6 subconfluent cells in 200 μl were injected intraperitoneally in 6-8 week-old C57BL/6 female mice. Tumour growth was assessed at day 30 post-injection by intraperitoneal injection of 200 μl of luciferin at 12,5 mg/ml (Goldbio, catalogue LUCK-500) and bioluminescent image detection and analysis was done using the IVIS200 (PerkinElmer). Mice bearing tumours were intraperitoneally injected with 9 x 10^6 wild-type or NFAT5-deficient macrophages and tumour growth was assessed by bioluminescence measurement at the indicated time points after the injection. Mice were sacrificed after the last measurement and peritoneal cells were harvested by peritoneal lavage performed twice with 5 ml of ice cold 1X PBS. Immune cell populations were then analysed by flow cytometry and a fraction of the lavage was subjected to positive isolation with CD11b magnetic beads to obtain myeloid cells for gene expression analysis.

3. DEN-induced hepatocellular carcinoma (HCC)

To induce hepatocellular carcinoma (HCC) in mice, diethylnitrosamine (DEN) (Sigma, catalogue N0756-10ML) diluted in PBS was intraperitoneally injected in 14-day-old WT and *Nfat5*$^{fl/fl,\ LysMcre}$ male mice at a dose of 10 mg/kg mouse. Serum was obtained from blood samples collected at 6 and 10 months post injection, by tail snip and cardiac punction, respectively. In these samples, the extent of liver damage was assessed by measuring the levels of the hepatic enzyme alanine aminotransferase (ALT) in collaboration with Joan Serret (Universitat de Barcelona). Livers

were obtained from sacrificed mice and and the amount and size of tumours in the liver was quantified. Livers were then fixed in 4% paraformaldehyde in PBS for 24 hours and stored in 40% ethanol at 4ºC for subsequent immunohistochemistry analysis.

Reagents

Sodium chloride, Trizma base, glycine, EDTA, sodium orthovanadate, β-glycerophospate, phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin A, aprotinin, sodium dodecyl sulphate (SDS), sodium pyrophosphate (NaPPi), methanol, bovine serum albumin (BSA), Triton X-100 (TX-100), Tween 20 and LB broth were purchased from Sigma-Aldrich. Sodium fluoride (NaF) was from Merck. Hepes was from Lonza.

Statistical analysis

Statistical analyses were done using the GraphPad Prism 5 software. Significance of the differences between sets of experimental data was determined with unpaired Student's t tests or non-parametric Mann Whitney tests. Student's t test was used, for example, when analysing gene expression profile of *in vitro* polarised macrophages. Mann-Whitney analysis was used to analyse tumour growth in experiments of co-inoculation of LLC cells with macrophages derived from NFAT5-conditional Vav-cre mice.

Table 1. Primers for RT-qPCR.

Target gene		**Sequence**
Arg1	Forward	CAC ACT GAC ATC AAC ACT CC
Arg1	Reverse	TCT CGC AAG CCA ATG TAC AC
Ccl2	Forward	CTC AGC CAG ATG CAG TTA ACG
Ccl2	Reverse	CAG ACC TCT CTC TTG AGC TTG G
Cd163	Forward	CTG ATG GAG CAG ATC TGG AAC
Cd163	Reverse	CAG ATC CAC ATC CAA GCT GAC
Chi3l3	Forward	TCC ATG ATC CTA AGG ATG GC
Chi3l3	Reverse	ATG AGC TTC TCA GAA GCT GC
Csf1r	Forward	GCA ATA CCT ACG TGT GCA AGA C
Csf1r	Reverse	GTG TCT CCA GGT TTG AGG ATA AC
Egln3	Forward	GCA ATG GTG GCT TGC TAT CC
Egln3	Reverse	CTC CGT GTA ACT TGG CGT CC
Gas6	Forward	GAG GAC ATC TTA CCA TGT GTG C
Gas6	Reverse	TGA AGC CTC TTG AAG CGT AG
H2-Ab	Forward	CCA TTA CCT GTG CCT TAG AG
H2-Ab	Reverse	GAA CTG GTA CAC GAA ATG CC
Hgf	Forward	TGT TAT CGT GGC AAT GGG AA
Hgf	Reverse	GTG TAG CAC CAA GGT CCA TG
Htr2b	Forward	AAT AGG CAT CGC CAT CCC AG
Htr2b	Reverse	CAT GAT GGT GAG AGG TGC GAA G
Ifng	Forward	CTC AAG TGG CAT AGA TGT GG
Ifng	Reverse	CAG GTG TGA TTC AAT GAC GC
Il10	Forward	TAC CTG GTA GAA GTG ATG CC
Il10	Reverse	TGT AGA CAC CTT GGT CTT GG
Il12b	Forward	AGA TGA AGG AGA CAG AGG AG
Il12b	Reverse	ACT TGC TGC ATG AGG AAT TG
Il17a	Forward	TCA GAC TAC CTC AAC CGT TC
Il17a	Reverse	AAT TCA TGT GGT GGT CCA GC
Il1b	Forward	TGA AGA AGA GCC CAT CCT CTG
Il1b	Reverse	AGC TTT CAG CTC ATA TGG GTC
Il2	Forward	AGC TGT TGA TGG ACC TAC AG
Il2	Reverse	AAA TCC AGA ACA TGC CGC AG
Il4	Forward	CTC ACA GCA ACG AAG AAC ACC

Il4	Reverse	GCT TAT CGA TGA ATC CAG GC
Il6	Forward	GCC AGA GTC CTC CAG AGA GAT AC
Il6	Reverse	CCA CTC CTT CTG TGA CTC CAG C
Inhba	Forward	AGG CAC AGC CAG GAA GAC AC
Inhba	Reverse	ACG GAT GGT GAC TTT GGT CC
Itgal (F4/80)	Forward	ATG AAT GTG TGA CTC GAG ACG
Itgal (F4/80)	Reverse	ACT CAT CCA CAT CTT CAC AGG
Itgam (Cd11b)	Forward	AGA GAA TGT CCT CAG CAG GAG
Itgam (Cd11b)	Reverse	TCC GAG TAC TGC ATC AAA GAG
L32	Forward	ACC AGT CAG ACC GAT ATG TG
L32	Reverse	ATT GTG GAC CAG GAA CTT GC
Mrc1	Forward	GGA CTC TGG ATT GGA CTC AAC AG
Mrc1	Reverse	GCT CTG ATG ATG GAC TTC CTG G
Nfat5	Forward	CAG CCA AAA GGG AAC TGG AG
Nfat5	Reverse	GAA AGC CTT GCT GTG TTC TG
Nos2	Forward	AGC TGG GCT GTA CAA ACC TT
Nos2	Reverse	CTC CCA TGT TGC ATT GGA AG
Relma	Forward	CAG CTG ATG GTC CCA GTG AAT A
Relma	Reverse	GGC CCA TCT GTT CAT AGT CTT GAC
Stab1	Forward	AAT GAC TTG CAG CAT CTG TGG
Stab1	Reverse	GAG TGC AAT GGA GTT GTC CAG
Tgfb1	Forward	GAA CCA AGG AGA CGG AAT ACA G
Tgfb1	Reverse	CTG ATC CCG TTG ATT TCC AC
Tnfa	Forward	TCG TAG CAA ACC ACC AAG TG
Tnfa	Reverse	GGA GTA GAC AAG GTA CAA CC
Vegfa	Forward	CCA GCG AAG CTA CTG CCG TC
Vegfa	Reverse	GAT CCG CAT GAT CTG CAT GG

Table 2 Antibodies used for flow cytometry analysis

Antigen	**Label**	**Isotype**	**Clone**	**Manufacturer**
CD11b	APC	IgG2b, κ	M1/70	eBioscience

CD11b	FITC	IgG2b, κ	M1/70	eBioscience
CD11b	PE	IgG2b, κ	M1/70	eBioscience
CD11b	APC	IgG2b, κ	M1/70	BD Pharmingen
CD11c	FITC	IgG	N418	eBioscience
CD11c	PE	IgG1, λ2	HL3	BD Pharmingen
CD206	FITC	IgG2a, κ	MR5D3	Biolegend
CD3	FITC	IgG	145-2C11	eBioscience
CD3	PE-Cy7	IgG	145-2C11	Biolegend
CD4	PE-Cy5	IgG2a, κ	RM4-5	eBioscience
CD44	FITC	IgG2b, κ	IM7	eBioscience
CD45.2	FITC	IgG2a, κ	104	eBioscience
CD45.2	PerCP-Cy5.5	IgG2a, κ	104	eBioscience
CD62L	PE	IgG2a, κ	MEL-14	eBioscience
CD8	APC	IgG2a, κ	53-6.7	eBioscience
CD86	FITC	IgG2a, κ	GL1	eBioscience
F4/80	APCefluor780	IgG2a, κ	BM8	eBioscience
F4/80	APC-Cy7	IgG2a, κ	BM8	Biolegend
Ly6C	PE	IgG2c, κ	HK1.4	eBioscience
Ly6C	PE	IgM, κ	AL-21	BD Pharmingen
Ly6G	PE-Cy7	IgG2a, κ	1A8	Biolegend
MHCII (I-A/I-E)	APC	IgG2b, κ	M5/114.15.2	eBioscience
MHCII (I-A/I-E)	PE	IgG2b, κ	M5/114.15.2	eBioscience
NK1.1	PE	IgG2a, κ	PK136	eBioscience

CHAPTER 4

RESULTS

1. NFAT5 role in macrophage polarisation in vitro

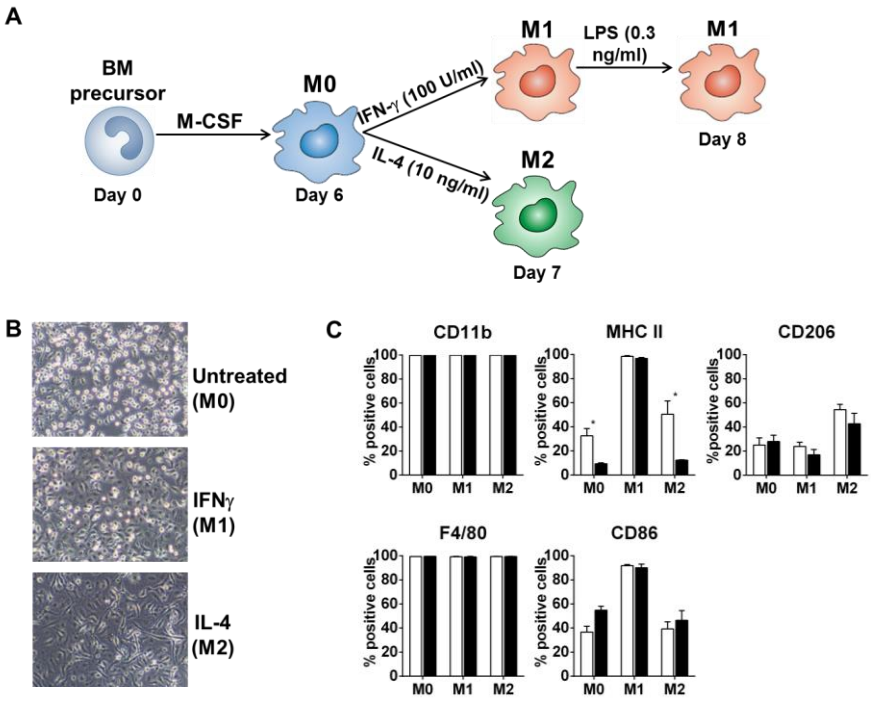
As previously published by our group, NFAT5 is required for the expression of several pro-inflammatory genes expressed in macrophages upon TLR stimulation (Buxadé et al. 2012). Macrophages are characterised by their functional plasticity, changing their phenotype in response to their microenvironment. A proper regulation of this functional plasticity is essential to maintain homeostasis and to avoid pathologies such as autoimmunity, chronic inflammation and cancer. Provided that this phenotypic plasticity is mainly regulated at the transcriptional level and that NFAT5 is required for the expression of pro-inflammatory genes (Buxadé et al. 2012), we decided to determine its role in macrophage polarisation.

We assessed the function of NFAT5 in polarisation by subjecting macrophages to opposed polarising conditions. For that purpose, we used WT and NFAT5-deficient macrophages generated by treating bone marrow cells with M-CSF (from L929-conditioned medium), from total knock-out (KO) or from *Nfat5*$^{fl/fl,\ Vav\text{-}cre}$ and *Nfat5*$^{fl/fk,\ Mx\text{-}cre}$ conditional knock-out mice. First, we established a protocol to generate polarised macrophages. To obtain M1 or classically activated macrophages, macrophages were treated with 100 U/ml of IFNγ for 24 hours followed by 24 hours of activation with a low dose of 0.3 ng/ml of LPS, which is a TLR4 ligand. To generate M2 macrophages or classically activated macrophages, we stimulated the macrophages with 10 ng/ml of IL-4 for 24 hours (**figure 1A**).

To test the differentiation capacity of macrophage cultures, we analysed the surface expression of CD11b and F4/80 by flow

cytometry, two characteristic receptors of the macrophage lineage. As shown in **figure 1B**, both WT and NFAT5-deficient polarised macrophages expressed high and comparable levels of CD11b and F4/80. Polarised cultures showed differences in their morphology by bright field microscopy compared with untreated cells (**figure 1C**). To validate our polarisation strategy, we analysed by flow cytometry the surface expression of characteristic polarisation markers. We studied the expression of the antigen presenting molecule MHC-II and the costimulatory molecule CD86 as M1 markers, while we looked at mannose receptor (CD206) as an M2 marker. IFNγ-stimulated M1 polarised macrophages expressed higher levels of MHC-II and CD86 compared with M2 macrophages. On the other hand, the mannose receptor was enriched in M2-polarised macrophages and its expression showed no dependency on NFAT5. Only MHC-II was NFAT5-dependent in untreated and IL-4 treated macrophages. (**figure 1B)**.

Figure 1. ***In vitro*** **polarised macrophage cultures (next page).** A) Diagram of the culture conditions used to obtain polarised macrophages. B) Bright field microscopy images of polarised macrophages. M0 refers to untreated macrophages, while M1 and M2 macrophages were treated for 24h with 100 U/ml of IFNγ and 10 ng/ml of IL-4, respectively. C) Flow cytometry analysis of markers related to macrophage lineage and macrophage polarisation. Culture conditions are the same as in B. The panels show the mean of the percentage of positive cells ± SEM of at least 3 independent experiments.



Higher NFAT5 expression levels in M1 polarised macrophages

We determined the expression of NFAT5 in M1 and M2 *in vitro* polarised macrophages. As shown in **figure 2A**, comparable levels of NFAT5 mRNA were detected in untreated macrophages (M0), as well as in macrophages stimulated with IFNγ (M1) and IL-4 (M2). Upon activation with a low dose of LPS of 0.3 ng/ml, both NFAT5 protein and mRNA levels increased over time (**figure 2**). Highest levels of NFAT5 induction were obtained in IFNγ plus LPS-treated macrophages compared with IL-4 plus LPS-stimulated cells.

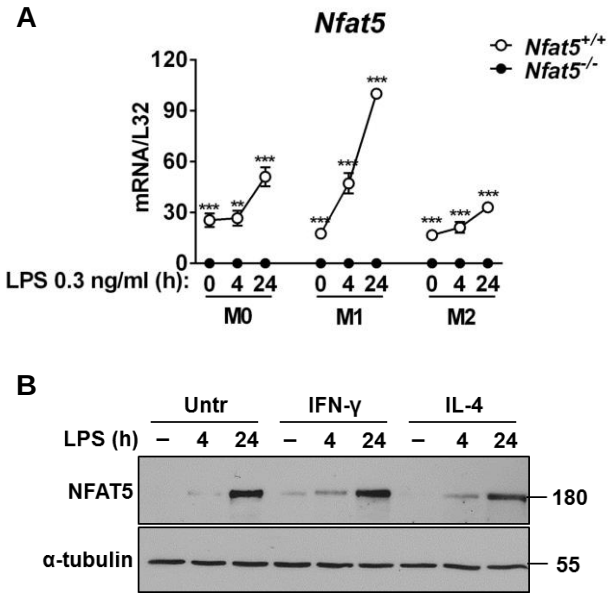


Figure 2. **NFAT5 expression in polarised macrophage cultures.** A) NFAT5 mRNA levels assessed by RT-qPCR. Relative mRNA abundance values were normalised to the respective L32 mRNA levels. Results show mean ± SEM of at least 3 independent experiments. Significance was determined by an unpaired t test (*p<0.05; **p<0.01 ***p<0.001). B) NFAT5 protein levels determined by Western blot. α-tubulin, is used as a loading control. Results are representative of at least two independent experiments.

M1 and M2 gene expression profile in polarised macrophages

We analysed the expression of several M1 and M2 markers at the mRNA level to further validate the selected polarising conditions and analysed the dependence on NFAT5 for the expression of these genes. As observed in **figure 3**, polarisation of macrophages using IFNγ plus LPS generated M1 macrophages, as determined by the expression of pro-inflammatory genes encoding for

cytokines, such as *Il1b*, *Il6*, *Il12b* and *Tnf*, the enzyme inducible nitric oxide synthase (*Nos2*), the α chain of the major histocompatibility complex (*H2-Aa*), activin A (*Inhba*) and hypoxia-inducible factor prolyl hydroxylase 3 (*Egln3*). Remarkably, NFAT5-deficient M1 macrophages showed a substantial decrease in the expression of these markers, with the exception of *Tnf* and *H2-Aa*.

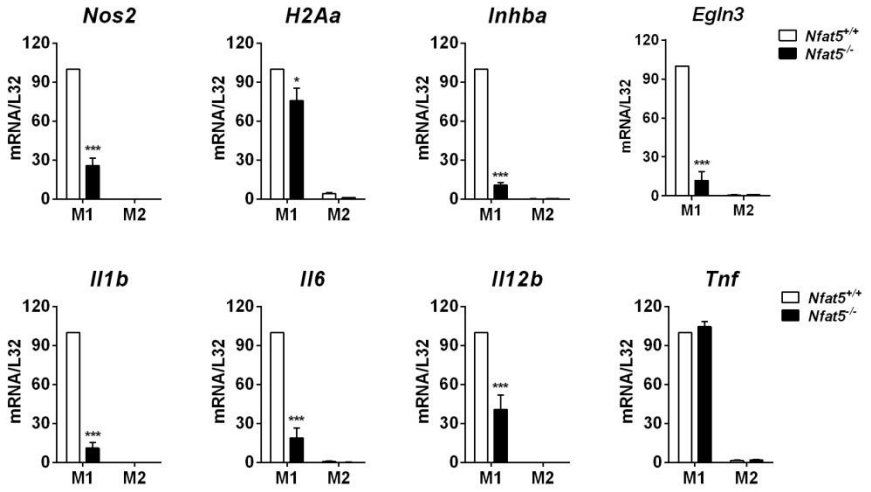


Figure 3. M1 gene expression in NFAT5-deficient polarised macrophages. Gene expression profile of pro-inflammatory genes in IFNγ plus LPS stimulated (M1) and IL-4-treated (M2) macrophages was assessed by RT-qPCR. Relative mRNA abundance values were normalised to their respective L32 mRNA levels. Results show the mean ± SEM of at least 3 independent experiments. Significance was determined by an unpaired t test (*p<0.05; **p<0.01; ***p<0.001).

Polarisation of macrophages with IL-4 generated M2 or alternatively activated macrophages, as shown by their expression of the mannose receptor (*Mrc1)*, arginase I (*Arg1*), resistin-like molecule alpha1 (*Relma*) and chitinase I (*Chi3l3*), as well as the scavenger receptor CD163 (Cd163), growth arrest-specific 6 (*Gas6*), stabilin 1 (*Stab1*) and serotonin receptor 2B (*Htr2b*) (**figure 4**). The dependence on NFAT5 for the expression of M2 markers was gene specific. NFAT5 was necessary for the expression of genes such as *Relma*, *Chi3l3*, *Arg1* and *Cd163*, but it did not affect the expression of *Mrc1* and *Gas6,* and it negatively regulated *Stab1* and *Htr2b* expression.

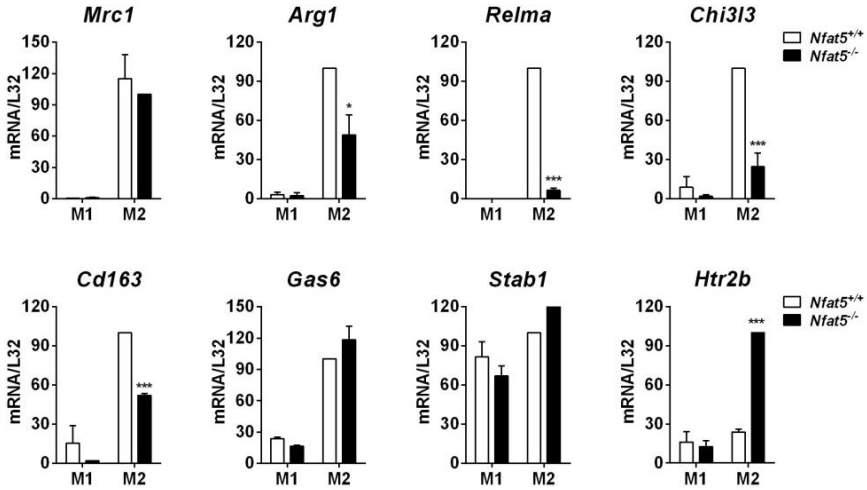


Figure 4. Analysis of M2 genes in NFAT5-deficient polarised macrophages. Gene expression profile of M2 genes in M1 and M2 polarised macrophages was assessed by RT-qPCR. Relative mRNA abundance values were normalised to their respective L32 mRNA levels. Results show mean ± SEM of at least 3 independent experiments. Significance was determined by an unpaired t test (*p<0.05; **p<0.01; ***p<0.001).

Gene expression profile in LPS activated M1 and M2 macrophages

Polarised macrophages were activated with 0.3 ng/ml of LPS for 4 and 24 hours to study the response of WT and NFAT5-deficient macrophages to a pro-inflammatory signal that further upregulated later NFAT5 expression. All M1 markers analysed were induced by LPS treatment and this induction was impaired in NFAT5-deficient macrophages (**figure 5**). *Nos2*, *H2Aa*, *Inhba*, *Egln3* and *Il1b* expression was higher at 24 hours of stimulation and strongly dependent on NFAT5, with the exception of *H2Aa*, which was less affected in *Nfat5*$^{-/-}$ macrophages. The mRNA level of the cytokines *Il12b* and *Tnf* was higher at 4 hours of LPS stimulation and this induction was abrogated in NFAT5-deficient macrophages, (**figure 5**). Genes such as *H2Aa*, *Inhba*, *Il1b*, *Il6*, *Il12b* and *Tnf,* could also be induced, although to a lower extent, in M2-polarised macrophages activated with LPS and they also displayed NFAT5-dependence.

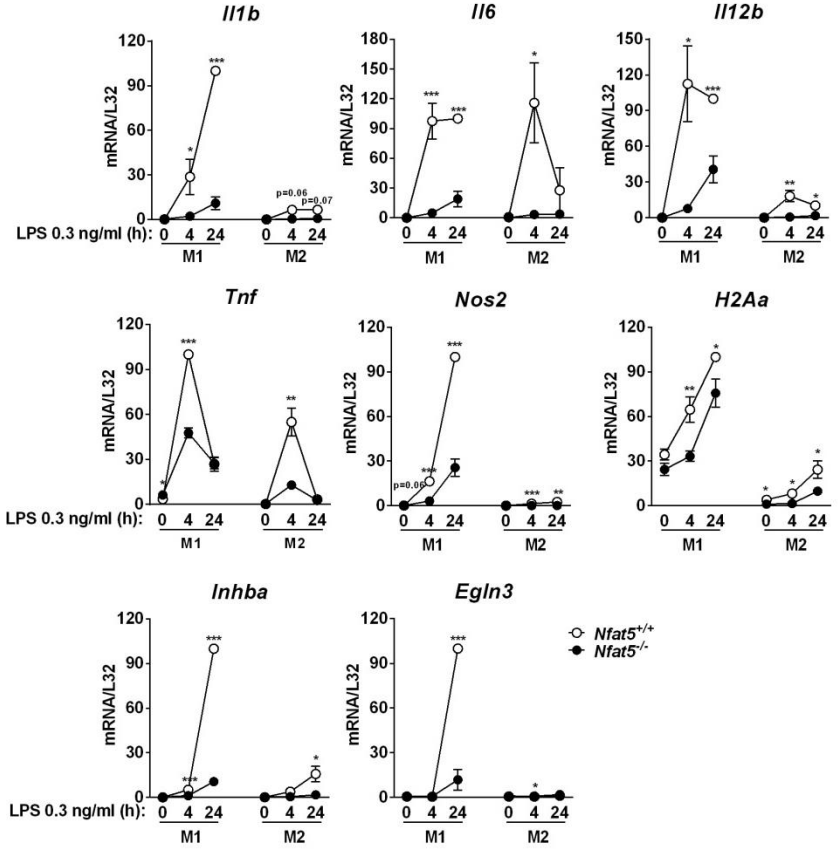


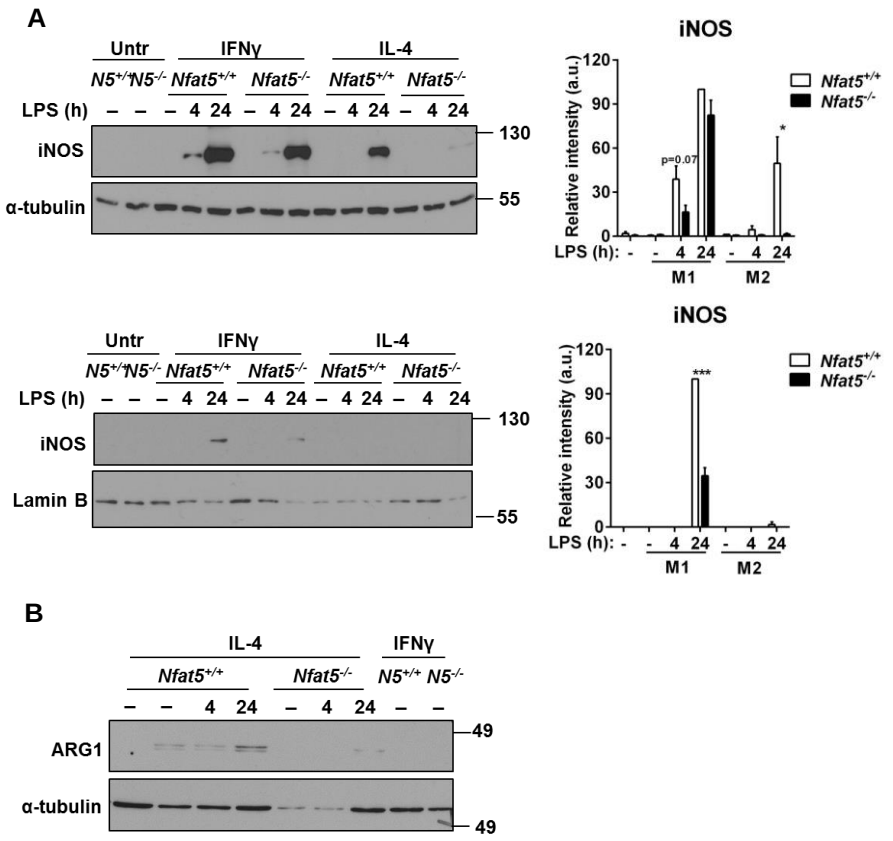
Figure 5. Pro-inflammatory gene expression in LPS-activated M1 and M2-polarised macrophages. IFNγ (100 U/ml) (M1) and IL-4 (10 ng/ml) (M2) stimulated macrophages were activated with 0.3 ng/ml of LPS for 4 and 24 hours and the expression of pro-inflammatory cytokines was analysed by RT-qPCR and was represented relative to their respective L32 mRNA levels. Results show mean ± SEM of at least 3 independent experiments. Significance was determined by an unpaired t test (*p<0.05; **p<0.01; ***p<0.001).

iNOS and arginase I protein expression are impaired in NFAT5-deficient macrophages

The expression of iNOS and arginase I was also determined at the protein level in M1 and M2 polarised macrophages stimulated with LPS. These two enzymes are well-known M1 and M2 markers, respectively, and both compete for L-arginine as their enzymatic substrate. Although iNOS is predominantly expressed in IFNγ differentiated macrophages, it was further induced by LPS treatment of both M1 and M2 macrophages. iNOS induction was NFAT5-dependent in both M1 and M2 LPS-activated macrophages (**figure 6A**).

As expected, arginase I expression was observed in IL-4-stimulated macrophages, but not in IFNγ-differentiated cells, and this expression required further LPS stimulation. Intriguingly, LPS-induced arginase I expression was also NFAT5-dependent **(figure 6B)**.

Figure 6. iNOS and Arginase I proteins are reduced in NFAT5-deficient macrophages (next page). Western blot analysis of the M1 marker iNOS (A) and the M2 marker Arginase I (B) in polarised and LPS-activated WT and *Nfat5*$^{-/-}$ macrophages. In (A) the top western blot panels and the graph showing their band quantification correspond to low protein loading (5-8ug per lane) while the western blot and the graph in the bottom panels correspond to high protein loading (30 ug per line). Quantification graphs show the mean ± SEM of 3-4 independent experiments. α-tubulin or lamin B were used as the loading controls.



IL-12 production is impaired in NFAT5-deficient macrophages

The production of the pro-inflammatory cytokine IL-12, which induces Th1 cell differentiation (Rengarajan et al. 2000), was analysed by ELISA. At the protein level, IL-12 was not impaired in NFAT5-deficient macrophages treated with IFNγ plus LPS, but was significantly reduced in IL-4 plus LPS stimulated *Nfat5*$^{-/-}$ macrophages (**figure 7**).

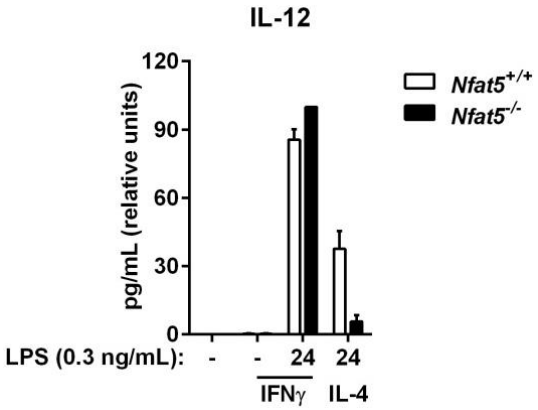


Figure 7. IL-12 production is NFAT5-dependent in mild pro-inflammatory conditions. IL-12 production was determined in culture supernatants of untreated macrophages, or macrophages that were treated with 100 U/ml of IFNγ with or without 0.3 ng/ml of LPS and with 10 ng/ml of IL-4 plus 0.3 ng/ml of LPS. Values are shown normalised to the point with maximal IL-12 production. Significance was determined by an unpaired t test (*p<0.05). n=3 independent experiments.

STAT1 and STAT6 activation are not affected by NFAT5-deficiency

Since the expression of M1 and some M2 genes depend on NFAT5, we checked the signalling pathways triggered by macrophage polarisation in NFAT5-deficient macrophages. To this end, we analysed the phosphorylation of STAT1 and STAT6, which are the transcription factors activated by the IFNγ-receptor (IFNGR) and the IL-4 receptor (IL4R), respectively. As shown in **figure 8A**, STAT1 was expressed in M1 and M2 macrophages and its phosphorylation was predominantly observed in cells stimulated with IFNγ. No significant differences were detected between WT and NFAT5-deficient macrophages.

Regarding STAT6, despite being similarly expressed in both types of polarised macrophages, its activation was only observed in M2 polarised macrophages (**figure 8B)** We analysed STAT6 activation at earlier time points of IL-4 stimulation without LPS treatment and did not observe clear differences in STAT6 phosphorylation between WT and NFAT5-deficient macrophages (**figure 8C)**,

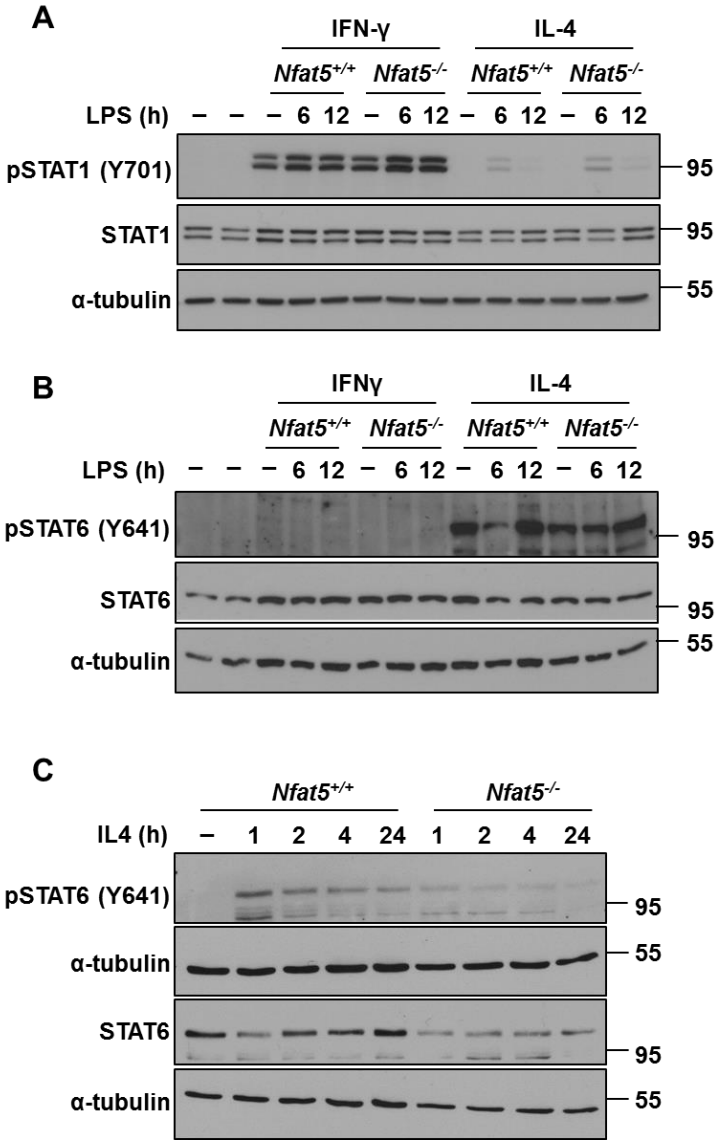


Figure 8. STAT1 and STAT6 activation are not affected by NFAT5 deficiency. STAT pathway activation was assessed by Western blot. A) STAT1 phosphorylation and total STAT1 were determined by measuring the protein levels of pSTAT1 (Y701) and STAT1 in M1 and M2 macrophages activated with 0.3 ng/ml of LPS for the indicated time points. STAT6 phosphorylation and total STAT6 were analysed in M1 and M2 macrophages activated with 0.3 ng/ml for the indicated time points (B) or in macrophages treated with 10 ng/ml of IL-4 for 1, 2, 4 and 24 hours (C). Results are representative of at least two independent experiments.

2. Analysis of the role of NFAT5 in macrophage responses

NFAT5-deficient macrophages have reduced phagocytic and bacteria killing abilities

In order to determine the role of NFAT5 in specific macrophage functions, we analysed the ability of WT and NFAT5-deficient polarised macrophages to phagocytose and kill intracellular bacteria. We infected IFNγ- and IL-4-treated macrophage cultures with *Escherichia coli* and determined the number of phagocytosed and surviving bacteria by plating macrophage lysates after their infection. At short time points, M1 and M2 macrophages had comparable bacterial uptake that was similarly reduced in NFAT5-deficient macrophages, pointing to a role for NFAT5 in facilitating bacterial phagocytosis (**figure 9A**). Regarding our analysis of the killing ability of macrophages, as expected, M1 macrophages were more efficient in bacterial clearance, and NFAT5 facilitated this process in both types of polarised cells. However, its contribution was more relevant in M2-polarised macrophages (**figure 9B).** Despite the differences at 3 hours post-infection, NFAT5-deficient macrophages were capable of controlling the infection at late (6 hours) time points.

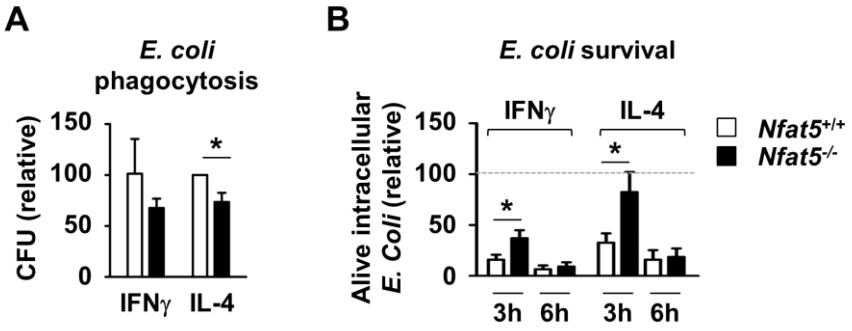


Figure 9. Bacterial phagocytosis and killing in WT and NFAT5-deficient macrophages. M1 (IFNγ) and M2 (IL-4) polarised WT and NFAT5-deficient macrophage cultures were infected at 37ºC with *E.coli* at a multiplicity of infection of 1. A) Bacterial counts in their lysates were done 1 hour post-infection after plating the lysates in agar plates for 24 hours. Values were corrected to the bacterial content in cultures incubated at 4ºC to normalise for differences in bacterial adhesion. B) To determine bacterial killing, bacterial counts were done considering 2 hours post-infection as time 0 for bacterial clearance, as cultures were incubated with the bacteria for 1 hour to allow phagocytosis and then cultures were treated for an additional hour with a high dose of the antibiotic gentamycin (50 mg/ml) to eliminate non-phagocytosed bacteria and then the medium was washed three times to remove gentamycin. The bacterial counts in lysates of polarised macrophages at 3 and 6 hours post gentamycin wash were normalised to their respective starting value of phagocytosis, which was given a value of 100% of bacterial content. Results show mean ± SEM of at least three independent experiments. Statistical significance was determined by an unpaired t test (*p <0.05).

Impaired pro-inflammatory polarisation of $CD4^+$ T lymphocytes differentiated in the presence of NFAT5-deficient polarised macrophages

As macrophages are involved in T lymphocyte activation and differentiation, we examined the phenotype acquired by $CD4^+$ T cells co-cultured with M1 and M2 polarised NFAT5-deficient macrophages. As observed in **figure 10**, $CD4^+$ T cells expressed more *Ifng* when cultured with M1 macrophages compared to M2 macrophages, and this upregulation was NFAT5-dependent on both types of polarised macrophages. *Il17a* and *Il2*, which are also T cell-derived cytokines that induce pro-inflammatory responses,

were also less expressed when $CD4^+$ T cells were co-cultured with M2-polarised and LPS-activated NFAT5-deficient macrophages. In parallel, and regarding the anti-inflammatory cytokine IL-4, which is counter-regulated by IFNγ, we observed that T lymphocytes cultured with M2 NFAT5-deficient macrophages almost doubled their expression of IL-4 mRNA compared with T cells incubated with WT M2 macrophages.

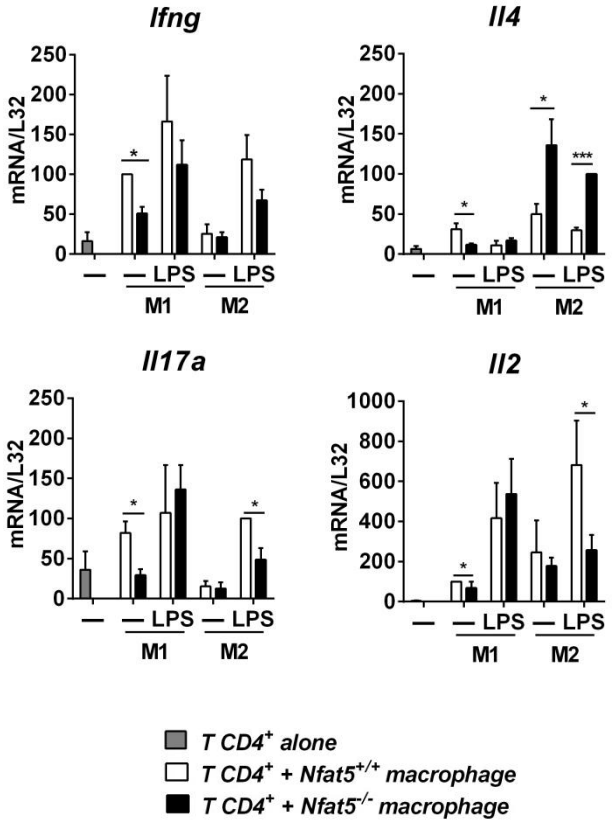


Figure 10. $CD4^+$ T lymphocytes cultured with NFAT5-deficient macrophages are less inflammatory. $CD4^+$ T cells isolated from spleen and peripheral lymph nodes of WT mice were cultured during 48 hours with polarised and LPS activated M1 and M2 WT and *Nfat5*$^{-/-}$ macrophages in the presence of αCD3 antibody (1 µg/10^6 cells). $CD4^+$ T cells were isolated from the co-cultures and their gene expression profile was analysed by RT-qPCR. Values are represented relative to their respective L32 mRNA levels and normalising each experiment to the point showing the highest mRNA level. Results show mean ± SEM of at least 4 independent experiments. Significance was determined by an unpaired t test (*p<0.05; **p<0.01; ***p<0.001).

NFAT5 expression in macrophages restrains tumour progression

The tumour niche is composed of malignant tumour cells, stromal cells and immune cells (Albini & Sporn 2007; Balkwill et al. 2012), which creates a microenvironment rich in cytokines, growth factors, danger signals and cell-cell interactions that facilitate tumour progression (Grivennikov et al. 2010). Macrophages have a vast ability to respond to changes in their surrounding milieu to fine-tune their phenotype according to the changing environment, and this phenotypic plasticity is mainly regulated at the transcriptional level. Moreover, re-education of macrophages towards a pro-inflammatory and cytotoxic phenotype is nowadays a valuable immunotherapeutic strategy used to fight different types of tumours. Therefore, learning about the molecular players involved in macrophage polarisation is an active area of research that is already providing new biomarkers and molecular targets useful to inhibit tumour progression. In this regard, we wanted to assess the role of NFAT5 in macrophage-specific anti-tumour responses.

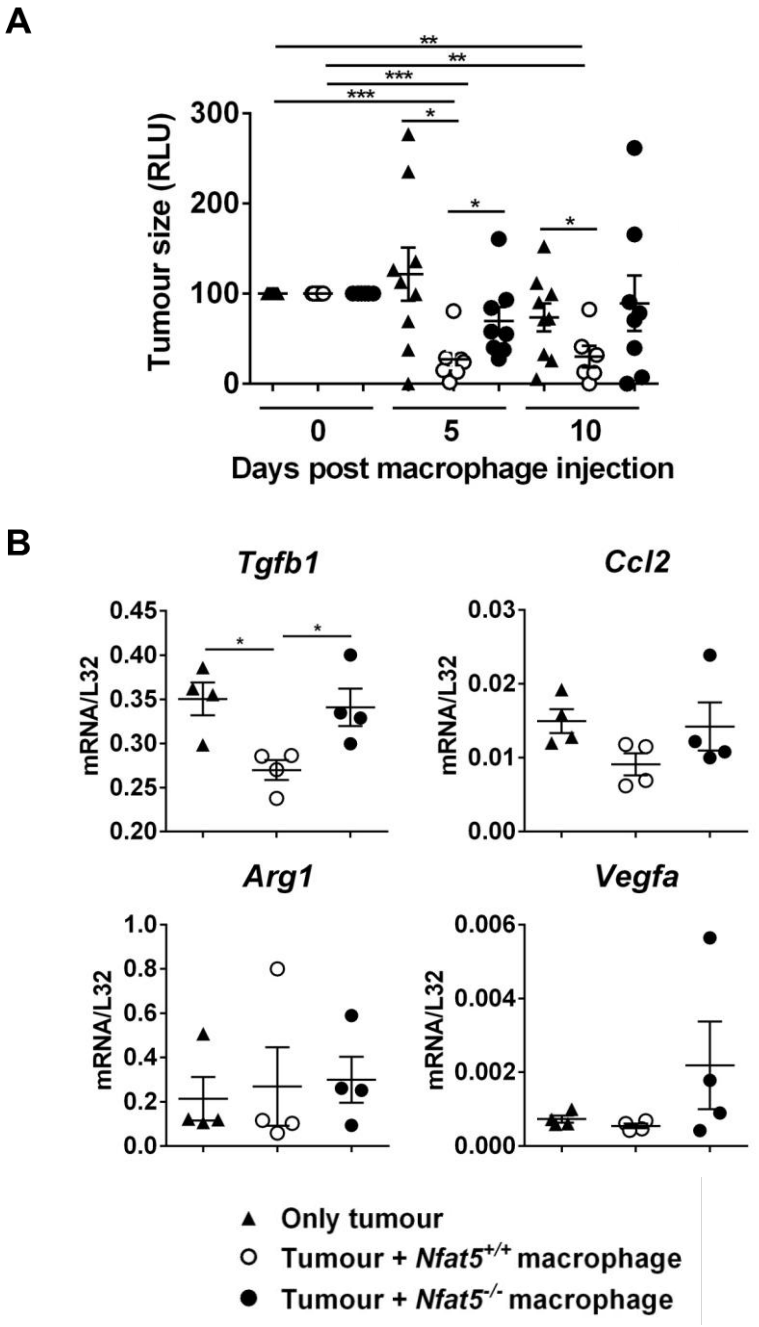
Control of serous tumour growth by NFAT5-deficient macrophages

We first used the ID8 ovarian carcinoma cell line because it is a syngeneic tumour model (H2-Db haplotype) in C57BL/6 background and its growth in the peritoneum resembles that of the effusions induced by human tumours (Roby et al. 2000). Also, development of this type of tumour is known to be highly dependent on macrophage polarisation and T lymphocyte activation (Hagemann et al. 2008) We took advantage of the luciferase-expressing variant of the tumour cell line (ID8-luc) to monitor tumour progression *in vivo* by optical imaging. We injected 5 x 10^6 ID8-luc cells intraperitoneally in WT mice and analysed tumour growth 30 days later. Since we detected variable levels of bioluminescent signal among individual mice (data not shown), we carried on the experiment with mice bearing tumours of similar size. At day 31 we injected these mice with 9 x 10^6 WT or NFAT5-deficient macrophages and left a group with no tumour or no macrophages as controls. We then measured luciferase activity 5 and 10 days after macrophage inoculation. As shown in **figure 11A**, tumour growth into each group was normalised to their growth at the day of macrophage injection, which is given an arbitrary value of 100. What we observed is that 5 days after macrophage injection, both WT and NFAT5-deficient macrophages reduced the tumour size compared to untreated mice. Remarkably, this reduction was significantly higher in mice that had received WT macrophages. 10 days after macrophage inoculation, the tumours showed bioluminescent signals similar to those at day 5, suggesting a stabilisation of tumour growth. Notably again, only mice that received WT macrophages, but not those received NFAT5-deficient ones, could control the tumour size, suggesting

that the anti-tumoural role provided by NFAT5-competent macrophages is quite stable in time.

To characterise myeloid cells from the tumour microenvironment, we performed peritoneal lavages at day 10 and isolated $CD11b^+$ cells. The gene expression profile of these cells showed similar levels of NFAT5 regardless of the group analysed, suggesting that we were isolating not only exogenously injected macrophages, but also macrophages that were recruited. Interestingly, we found that M2 marker genes such as *Tgfb1*, *Arg1*, *Vegfa* and *Ccl2* were expressed at higher levels in myeloid cells isolated from mice injected with NFAT5-deficient macrophages (**figure 11B**). Altogether, these observations indicate that NFAT5 expression in macrophages counters an anti-inflammatory and pro-tumoural microenvironment.

Figure 11. NFAT5 expression in macrophages restrains tumour progression (next page). A) ID8 tumour growth was measured *in vivo* by bioluminescent imaging. Tumours were allowed to grow until day 31, when they were injected with WT or NFAT5-deficient macrophages (day 0 post macrophage injection). Luciferase values were given an arbitrary value of 100, and the development of tumour growth was determined at days 5 and 10 after macrophage injection. Values are represented relative to day 0 and each dot represents an individual mouse in each condition. B) Gene expression profile determined by RT-qPCR of peritoneal $CD11b^+$ cells isolated from tumour-bearing mice at day 10 after macrophage injection. Each dot represents the specific mRNA levels of one individual mouse in each condition and it is represented relative to its respective L32 mRNA level. The graphs show the mean ± SEM of the values obtained and their significance was determined by an unpaired t test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).


A
Tumour size (RLU)
300
200
100
0
**
**
***
***
*
*
*
0
5
10
Days post macrophage injection
B
Tgfb1
Ccl2
Arg1
Vegfa
mRNA/L32
0.45
0.40
0.35
0.30
0.25
0.20
0.03
0.02
0.01
0.00
1.0
0.8
0.6
0.4
0.2
0.0
0.006
0.004
0.002
0.000
Only tumour
Tumour + Nfat5+/+ macrophage
Tumour + Nfat5-/- macrophage

To characterise earlier events in tumour development that could account for the differences in tumour size observed between mice injected with WT and NFAT5-deficient macrophages, we performed the experiment and sacrificed the mice at day 4 after macrophage injection. We again observed a reduction in the size of the tumours from mice that were injected with WT macrophages but an increase in the size of the tumours treated with NFAT5-deficient macrophages (**figure 12**). Tumour-bearing mice injected with NFAT5-deficient macrophages also had increased spleen weight compared to untreated or WT macrophage-injected mice, which usually correlates with tumour size and progression in different mouse tumour models (Cubillos-Ruiz et al. 2015; Liu et al. 2014) (**figure 12A**). Interestingly, and despite that the gene expression profile of peritoneal $CD11b^+$ at day 4 did not show clear differences between mice inoculated with WT or NFAT5-deficient macrophages (data not shown), flow cytometry analysis did reveal more naïve and fewer activated $CD8^+$ T cells into the peritoneal cavity of mice injected with NFAT5-deficient macrophages. However, no significant differences were observed when analysing the infiltrating $CD4^+$ T cells (**figure 12B**). Total numbers of $CD4^+$ and $CD8^+$ T cells were similar between WT and NFAT5-deficient macrophage-injected mice (data not shown).

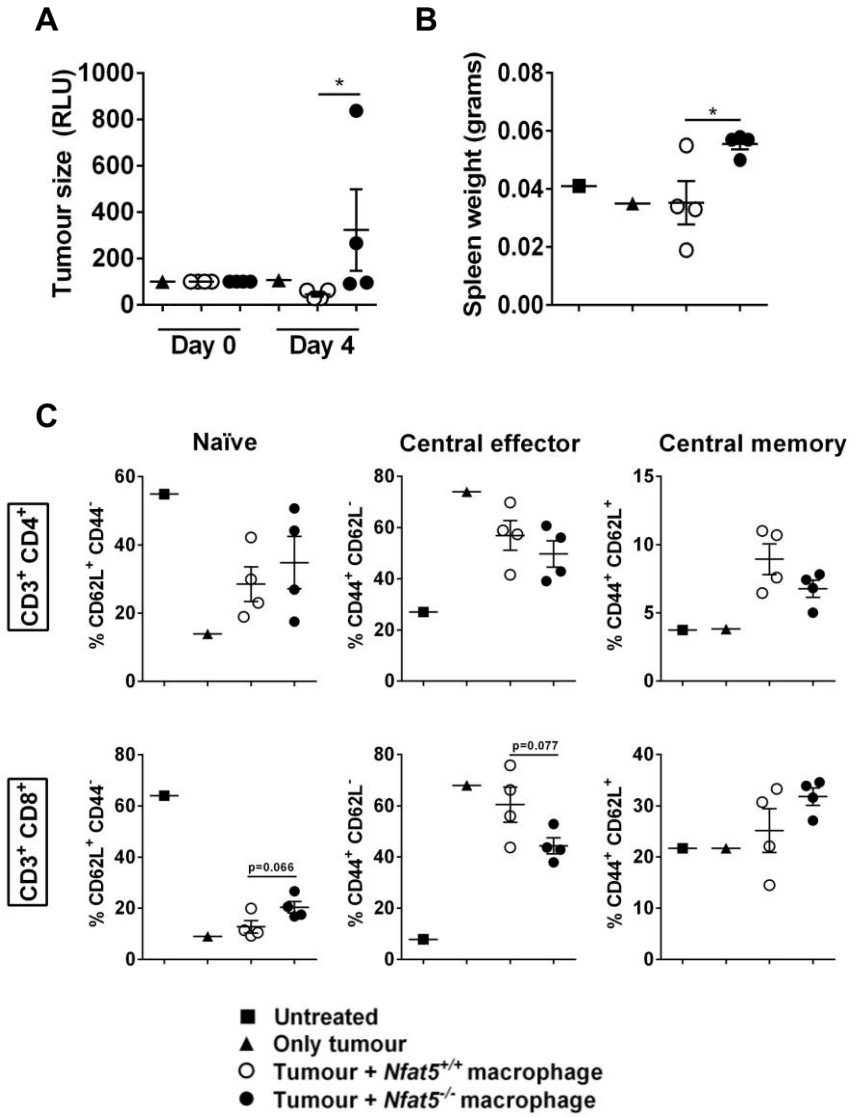


Figure 12. NFAT5 expression in macrophages restrains tumour progression. A) ID8 tumour growth was determined by bioluminescent imaging. Tumours were allowed to grow until day 31, when they were injected with WT or NFAT5-deficient macrophages (day 0 post macrophage injection). Luciferase values were given an arbitrary value of 100, and the development of tumour growth was determined at day 4 post-macrophage injection and the values were represented relative to day 0 (continues in the next page).

B) Spleens of mice were resected and weighted at day 4 post-macrophage injection. Each dot represents one individual mouse in each condition Control (-) represents the group of mice without tumour and tumour (-) represents the group that was injected with tumour cells but not macrophages. C) Flow cytometry analysis of the activation state of $CD4^+$ and $CD8^+$ T cells in the peritoneal cavity of tumour bearing mice. $CD62L^+$ are naïve cells, $CD44^+$ are effector cells and $CD62L^+$ $CD44^+$ are effector memory T cells. The graphs show mean ± SEM of the values obtained and the significance was determined by an unpaired t test (*$p<0.05$).

In order to determine the phenotype that NFAT5-deficient macrophages acquire in response to these tumour cells, we performed *in vitro* co-cultures of macrophages with ID8 cells and analysed the gene expression profile of these macrophages. We observed that ID8 cells induced the upregulation of *Arg1* and *Ccl2*, two genes associated with M2 polarisation, in both WT and NFAT5-deficient macrophages. Noteworthy, this increase was more evident in NFAT5-deficient macrophages. On the contrary, M1 marker genes such as *H2Aa* were downregulated by co-culture with tumour cells and this decrease was more substantial for NFAT5-deficient macrophages. Since we could detect the expression of *Nfat5* in NFAT5-deficient macrophage cultures, this indicates that isolated CD11b macrophages from the co-cultures carried some contamination of ID8 cells (**figure 13A**). Nonetheless, ID8 cells do not express any of the other M1 and M2 marker genes analysed here (**figure 13B**).

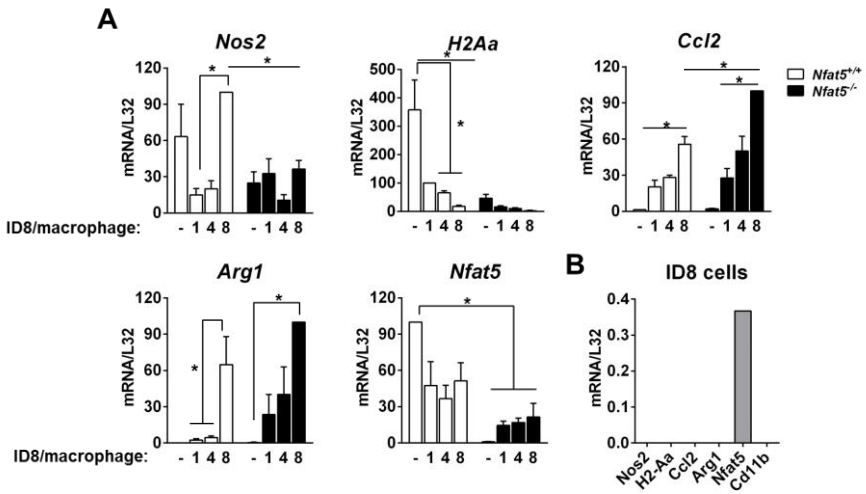


Figure 13. Phenotype of NFAT5-deficient BMDMs upon co-culture with ID8 tumour cells. A) Gene expression profile of WT and NFAT5-deficient macrophages isolated from co-cultures with ID8 cells for 24 hours. The first column in each group represents macrophages alone and the next three columns show increasing ratios of ID8/macrophage (ratios 1, 4 and 8). Analysis shows normalised values of the mRNA levels of target genes analysed by RT-qPCR, relative to their respective L32 mRNA levels. B) Gene expression profile of ID8 cells in culture. Results show mean ± SEM of 4 independent experiments and *<p=0.01.

Control of solid tumour growth by NFAT5-deficient macrophages

To validate our findings on the anti-tumour role of NFAT5 in macrophages, we used a different tumour model that generates subcutaneous solid tumours that are highly infiltrated by myeloid cells, the Lewis lung carcinoma (LLC) cells (Kratochvill et al. 2015). We co-injected WT and NFAT5-deficient macrophages with LLC cells into WT recipient mice and followed tumour growth for up to

15 days by using a calliper. As shown in **figure 14A**, mice injected with NFAT5-deficient macrophages developed larger tumours compared with those that received WT macrophages. Tumour weight was also bigger in mice that received in NFAT5-deficient macrophages (**figure 14B**).

We resected the solid tumours at day 16 after their inoculation and isolated the tumour-associated $CD11b^+$ cells to analyse their gene expression profile. At this time point we did not expect to recover the exogenously injected macrophages, but rather the myeloid cells that were recruited under the influence of the microenvironment controlled by WT or NFAT5-deficient macrophages. Gene expression analysis revealed that $CD11b^+$ cells isolated from the tumours of mice that received NFAT5-deficient macrophages had significantly higher levels of *Vegfa* and *Tnf,* being VEGF-A especially relevant in angiogenesis (Murdoch et al. 2008)(**figure 14C**). They also expressed increased levels of *Ccl2*, a chemokine involved in the recruitment of $CCR2^+$ cells such as monocytes that are known to support pro-tumoural responses (Chen et al. 2016; Loberg et al. 2007). *Vegfa* and *Ccl2* were also upregulated in $CD11b^+$ cells isolated from ID8-tumour bearing mice injected with NFAT5-deficient macrophages (**figure 11B**), Intriguingly, we could also observe that certain pro-inflammatory cytokines, such as *Il1b* or *Il12b*, were also upregulated in $CD11b^+$ cells isolated from tumours inoculated with NFAT5-deficient macrophages (**figure 14 C**). This finding could indicate a delayed pro-inflammatory response going on in the microenvironment of the tumours of these mice.

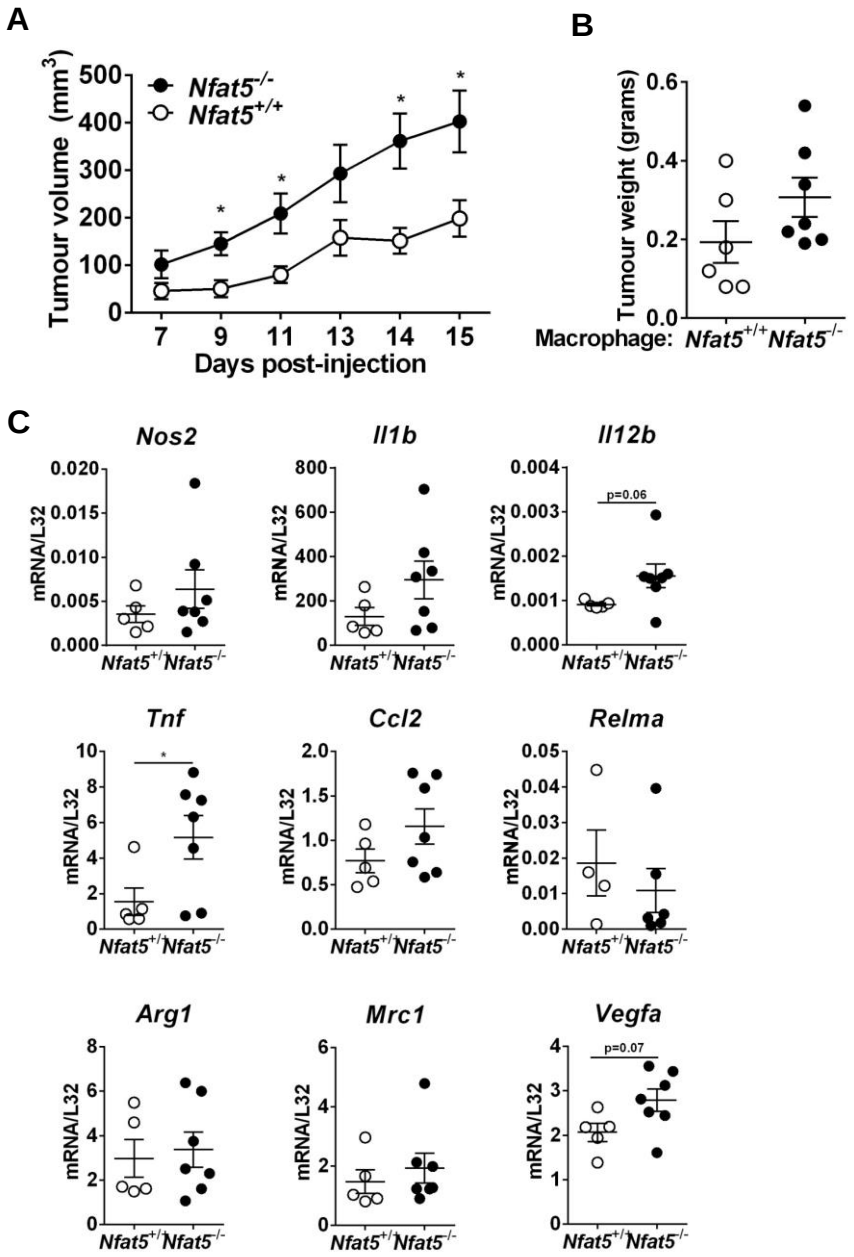

A
Tumour volume (mm3)
Nfat5-/-
Nfat5+/+
Days post-injection
B
Tumour weight (grams)
Macrophage: Nfat5+/+ Nfat5-/-
C
Nos2
Il1b
Il12b
p=0.06
Tnf
Ccl2
Relma
Arg1
Mrc1
Vegfa
p=0.07
mRNA/L32

Figure 14. NFAT5-deficient macrophages promote solid tumour growth. Time course of tumour volume (A) and tumour weight measured at day 15 (B) after WT and NFAT5-deficient macrophages were co-inoculated with LLC cells. Each dot represents the mean ± SEM of 7-10 mice per group. C) Gene expression profile of $CD11b^+$ cells isolated at day 15 from tumours co-injected with WT and NFAT5-deficient macrophages. Each dot represents an individual tumour. Results show mean ± SEM values and their significance was determined by the Mann-Whitney test (*$p<0.05$).

We next repeated the experiment following tumour volume over time and also processing the tumours earlier, at day 12. As observed in **figure 15A**, tumour volume was again higher in mice co-injected with NFAT5-deficient macrophages, although this time we did not observe differences in tumour weight (**figure 15B**). What we did notice was that mice bearing tumours injected with NFAT5-deficient macrophages had larger spleens and increased cellularity in inguinal draining lymph nodes (**figure 15C and D**). This association was also observed in our *in vivo* experiments with ID8 tumours (**figure 12A**), and as mentioned before, is reported to be in accordance with increased tumour size.

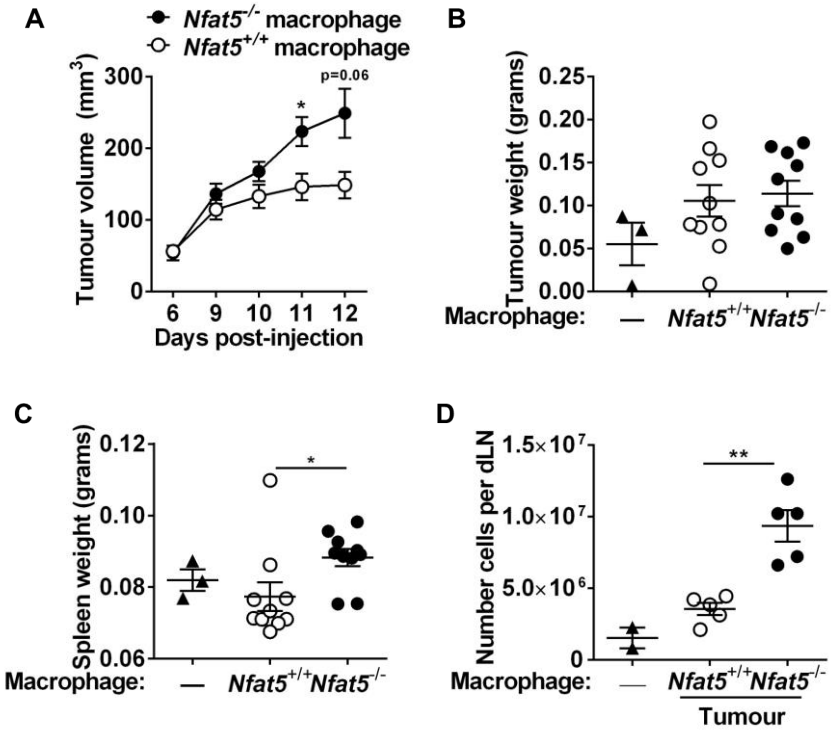
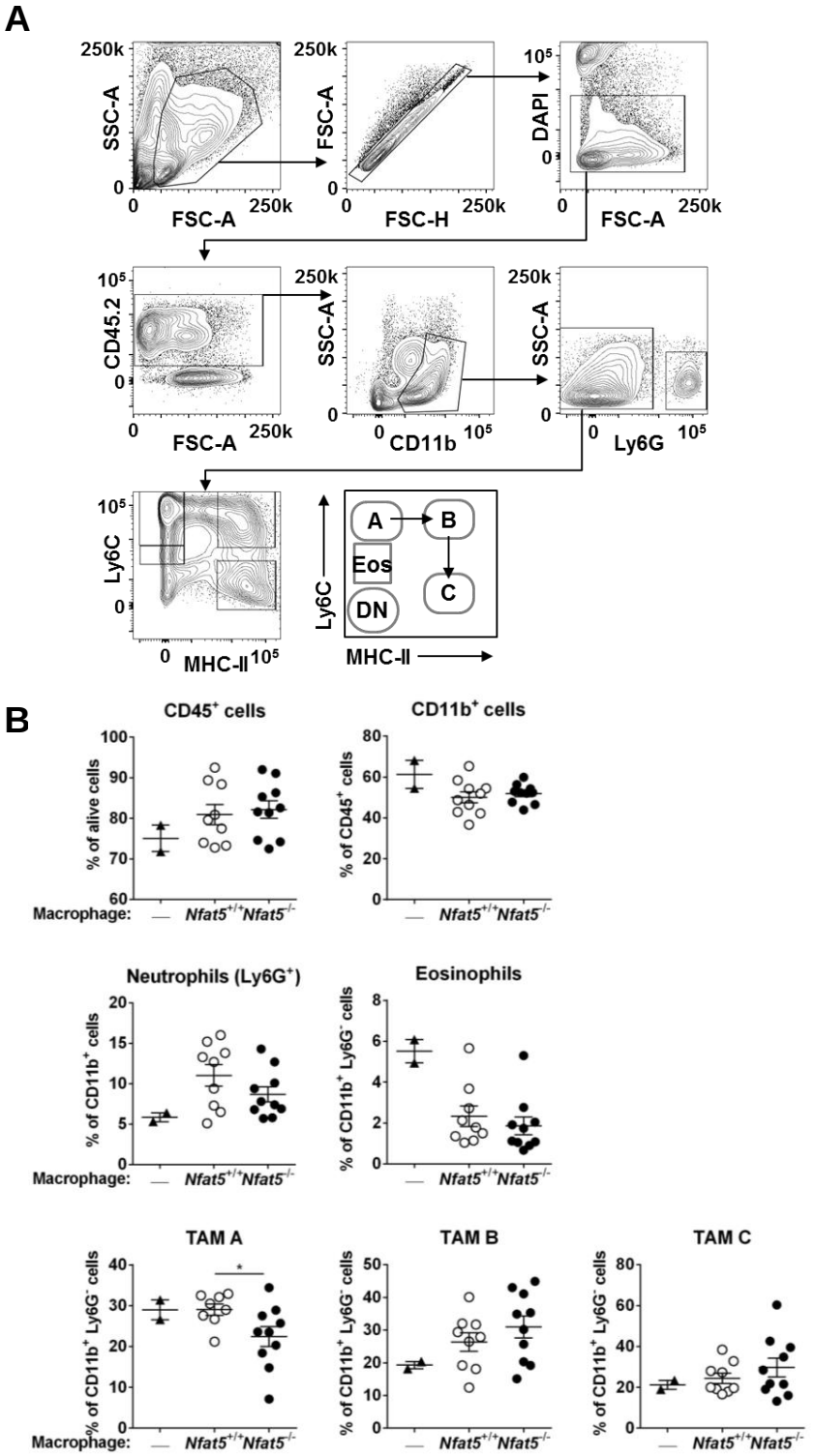


Figure 15. NFAT5-deficient macrophages facilitate solid tumour growth. A) Tumour development in mice co-injected subcutaneously with LLC plus WT or NFAT5-deficient macrophages was assessed by measuring tumour volume over time up to day 12 post-injection. Each dot represents the mean volume of 8 tumours per group and error bars indicate SEM. Tumour weight (B), spleen weight (C) and inguinal draining lymph node (dLN) cellularity (D) were measured 12 days post-injection. Each dot represents one individual mouse and the bars show the mean ± SEM. Significance was determined with the Mann-Whitney test (*p<0.05; **p<0.01). (-) represents data obtained from mice injected with tumour cells without macrophages.

In this experiment we analysed the myeloid populations associated with the solid tumours to determine whether there were differences between the tumour microenvironment in mice that receive WT or NFAT5-deficient macrophages. No differences were observed in total leukocyte ($CD45^+$ cells) or in $CD11b^+$ cell recruitment. However, we detected fewer neutrophils in NFAT5-deficient macrophage injected mice, with a similar percentage of eosinophils between the two groups (**figure 16**). Following the flow cytometry strategy (**figure 16A**) used by Kratochvill and colleagues (Kratochvill et al. 2015) we analysed the percentages of different tumour-associated macrophages (TAMs) defined by their expression levels of Ly6C and MHCII. We observed fewer newly recruited TAM A or immature TAMs in NFAT5-deficient macrophage-injected tumours, and a slight increase in the proportion of TAM B and mature or TAM C (**figure 16B**). Altogether, these findings suggest that the phenotype of macrophages recruited into the tumour microenvironment is altered in tumours initiated in the presence of NFAT5-deficient macrophages.

Figure 16. Myeloid compartment in WT and NFAT5-deficient co-injected tumours (next page). A) Gating strategy to study the myeloid compartment in solid LLC tumours by flow cytometry. Connecting arrows within the last diagram indicate the differentiation of recruited macrophages within the tumour microenvironment. B) Flow cytometry analysis showing the percentages of myeloid cell populations in tumours isolated from mice that were injected only with LLC (triangles), or co-injected with WT (white dots) or NFAT5-deficient macrophages (black dots). Each dot represents the percentage of each specific cell population isolated from an individual tumour. Graphs show the mean with the error bars that represents the SEM. Significance was determined with Mann-Whitney test (*$p<0.05$).


A
SSC-A
FSC-A
FSC-H
DAPI
CD45.2
CD11b
Ly6G
Ly6C
MHC-II
Eos
DN
B
CD45+ cells
CD11b+ cells
% of alive cells
% of CD45+ cells
Neutrophils (Ly6G+)
Eosinophils
% of CD11b+ cells
% of CD11b+ Ly6G- cells
TAM A
TAM B
TAM C
Macrophage:
Nfat5+/+
Nfat5-/-

To determine the impact of LLC cells in the pro-inflammatory state of *wild-type* and NFAT5-deficient macrophages, we co-cultured M1-polarised macrophages with equivalent numbers of LLC cells and analysed their gene expression profile. We observed that LLC cells reduced the mRNA expression of pro-inflammatory genes such as *Il12b* and *Nos2*, being this decrease more abrupt in *wild-type* cells. Interestingly, LLC cells were able to induce Arg1 expression in NFAT5-deficient macrophages but not in wild-type ones (**figure 17**), suggesting that NFAT5-deficient cells are more prone to acquire M2-like phenotypes.

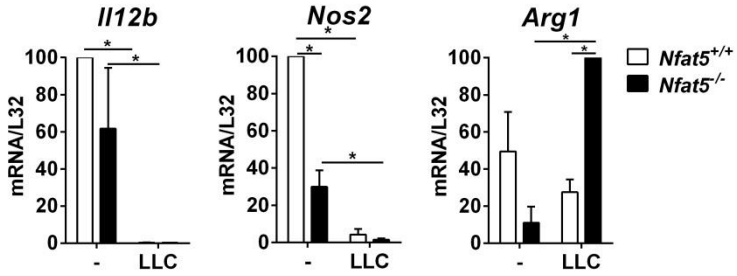


Figure 17. LLC cells induce an M2-like phenotype in M1-polarised macrophages. IFNγ plus LPS stimulated wild-type and NFAT5-deficient macrophages were co-cultured at a ratio 1:1 for 24h. Macrophages were then isolated and gene expression analysed by RT-qPCR**.** Bars show the mRNA levels of target genes, relative to their respective L32 mRNA levels and normalised to the highest value in each case. Results show mean ± SEM of 3-5 independent experiments and *<p 0.01.

Analysis of solid LLC tumour growth in a myeloid-specific NFAT5 knock-out mouse model

We next assessed *in vivo* LLC tumour growth in conditional LysMcre knock-out mice that lack NFAT5 in the myeloid lineage. After injecting 0.5 x 10^6 cells *in* $Nfat5^{wt/wt,\ LysMcre}$ and $Nfat5^{fl/fl,\ LysMcre}$, we followed tumour growth by measuring the volume with a calliper. We could observe that mice lacking NFAT5 in the myeloid compartment presented an early accelerated establishment of solid tumours, which nonetheless reached a similar maximum size in both mouse models (**figure 18**). This observation is in contrast with previous experiments co-injecting LLC cells with NFAT5-deficient macrophages and could reflect that NFAT5 has pro- and anti-tumour effects that differ depending on the myeloid cell

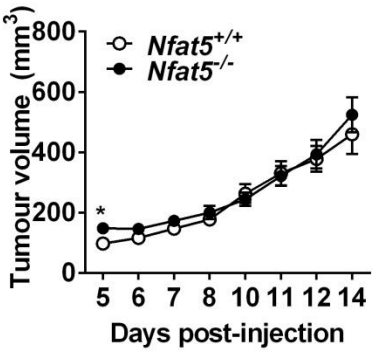


Figure 18. NFAT5 expression in myeloid cells restrains tumour establishment. $Nfat5^{wt/wt,\ LysMcre}$ and $Nfat5^{fl/fl,LysMcre}$ mice were subcutaneously injected with 0.5 x 10^6 cells and tumour development was measured by assessing tumour volume with a calliper. Each dot represents the mean of 7 individual tumours from each group and error bars show SEM. Significance was determined by a Mann-Whitney test (*$p<0.05$). Results are representative of 2 independent experiments.

NFAT5-deficiency in macrophages reduces tumour burden in DEN-induced hepatocellular carcinoma mouse model

Considering that NFAT5 is particularly important for the expression of pro-inflammatory genes in macrophages, we assessed the impact of NFAT5 deficiency in myeloid cell function in a mouse model of inflammation-associated cancer. For this, we used the diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) mouse model. DEN-induced hepatocyte death causes a Kupffer cells-mediated pro-inflammatory response that in turn induces the compensatory proliferation of the surviving transformed hepatocytes, leading finally to carcinoma formation (Naugler et al. 2007). We injected 14-day-old WT and *Nfat5*$^{fl/fl,\ LysMcre}$ male mice with DEN and analysed the extent of HCC formation at 10 months post-injection. Interestingly, we observed that the number of mice that had developed two or more tumours was higher in WT mice than in *Nfat5*$^{fl/fl,\ LysMcre}$ mice (**figure 19A**), with an overall tumour burden reduced in myeloid-specific NFAT5-deficient mice, whereas the average tumour size was similar between the two groups (**figure 19B**).

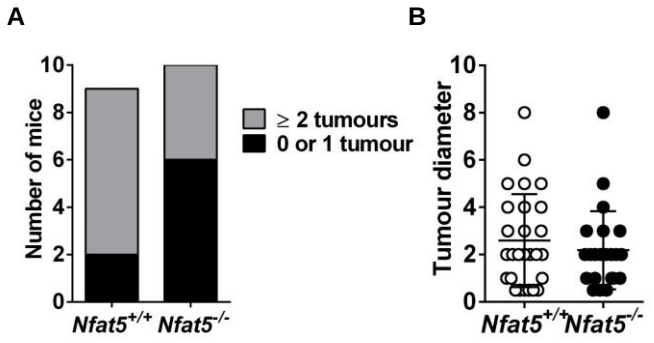


Figure 19. The number of DEN-induced HCCs is reduced in myeloid-specific NFAT5-deficient mice. A) Number of mice with 0 or 1 tumours and mice that had 2 or more tumours in DEN-treated WT and *Nfat5*$^{fl/fl,\ LysMcre}$ mice. B) Diameter of HCC tumours in WT and *Nfat5*$^{fl/fl,\ LysMcre}$ mice. Each dot represents the diameter in mm of one individual tumour. The experiment shows the results corresponding to n=9 WT and n=10 *Nfat5*$^{fl/fl,\ LysMcre}$ mice obtained in three independent experiments.

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CHAPTER 5

DISCUSSION

This work aimed at dissecting the role of NFAT5 in macrophage polarisation in order to unravel whether this transcription factor is essential for macrophages to adopt different functional phenotypes. Our results show that NFAT5 is particularly important for pro-inflammatory macrophage polarisation, as it is necessary for the expression of several pro-inflammatory genes. Indeed, we observed that NFAT5-deficient macrophages have impaired ability to promote type 1 T cell responses and to control tumour progression.

Consistent with its role as a pro-inflammatory regulator, NFAT5 is expressed at higher levels (mRNA and protein) in LPS activated and IFNγ-treated (M1) macrophages, compared to those pre-treated with IL-4 (M2). In line with this, the expression of pro-inflammatory genes in IFNγ-polarised LPS-activated macrophages (**figures 2 and 5**) is impaired in NFAT5-deficient macrophages. This defect in M1 gene expression does not seem to be due to an impaired activation of the STAT1 pathway. Upon IFNγ stimulation, STAT1 is phosphorylated at tyrosine 701 to the same extent in both WT and NFAT5-deficient M1 macrophages (**figure 8**). However, it would also be interesting to check phosphorylation at serine 727 as it is required for maximal transactivation activity of STAT1 (Schroder et al. 2006). Moreover, analysing pSTAT1 translocation to the nucleus and its DNA-binding ability would also be informative. Recruitment of STAT1 to its target genes may be affected in NFAT5-deficient cells, as STAT1 typically cooperates with other transcription factors for the regulation of its target genes (Ramana et al. 2000). We cannot discard the possibility that impaired M1-gene expression in NFAT5-deficient macrophages is also due to functional defects in other well-known pro-inflammatory

transcription factors such as IRF1, IRF3, IRF5 and IRF8 or AP-1 and NF-κB (Lawrence & Natoli 2011). Noteworthy, recent publications indicate that NF-κB and NFAT5 may cooperate for the induction of pro-inflammatory genes (Lee et al. 2016).

Remarkably, LPS-induced genes in IL-4 polarised macrophages are also completely abrogated in the absence of NFAT5. In this regard, while LPS treated M1 (IFNγ polarised) NFAT5-deficient macrophages, can still induce the expression of *Nos2*, *Il1b*, *Il6* or *Il12b*, M2 (IL-4 pretreated) NFAT5-deficient cells cannot (**figure 5**). Accordingly, iNOS protein expression is completely abolished in IL-4-polarised LPS-activated NFAT5-deficient macrophages, while it is only partially decreased in M1-polarised LPS activated cells (figure 6A). Moreover, we also observed that IL-4 polarised macrophages deficient in NFAT5 were impaired in intracellular bacteria killing. These observations may suggest that the transcriptional machinery activated upon IFNγ and LPS treatment (STAT1, IRFs, NF-κB and AP-1) is able to partially bypass the requirement of NFAT5 and still support pro-inflammatory gene expression. On the contrary, IL-4-stimulated macrophages completely rely on NFAT5 to induce the expression of pro-inflammatory genes in response to TLR ligands such as LPS. (**figure 5**). NFAT5 could thus be a key factor for M2 macrophages to repolarise towards a pro-inflammatory phenotype. This could be important in tissue resident macrophages that show an M2-like phenotype in homeostatic conditions but need to acquire M1 functions upon infection or insults that trigger pro-inflammatory responses (Varol et al. 2015; Davies & Taylor 2015).

NFAT5 also participates in the expression of a subset of genes associated with alternative or anti-inflammatory macrophages (IL-4

polarised). In these cells, NFAT5 is required for the expression of genes such as *Arg1*, *Relma*, *Chi3l3 and Cd163*, while it is dispensable for the expression of other M2-related genes such as *Cd206* or *Gas6* (**figure 3**). Regarding our results, the decreased expression of these genes cannot account for defects in STAT6 activation, the master regulator of the M2-gene programme (**figure 8**). The expression of most of these genes depends on the combination of different transcription factors, for example, expression of *Arg1* and *Relma* relies on STAT6 and C/EBPβ cooperation (Sheldon et al. 2013; Stütz et al. 2003; Gray et al. 2005). Considering this, we could hypothesise that the defects observed on these M2 markers in NFAT5-deficient macrophages are due to the impaired function of transcription factors other than STAT6, which are also required to control gene expression in alternatively polarised macrophages. Although C/EBPβ does not seem to be affected because we did not find differences in its mRNA levels between WT and NFAT5-deficient M2 macrophages (data not shown), its protein levels and DNA binding ability could also be analysed. This could also apply for other transcription factors involved in IL-4 polarisation such as KLF4 and Jmjd3. Importantly, mannose receptor (CD206), which is another M2 hallmark gene whose transcription essentially depends on STAT6 (Martinez et al. 2009; Van Dyken & Locksley 2013), is not affected by NFAT5 deficiency. This result might indicate that the defect seen in the expression of some M2-related genes could be due to an impaired activation of the PI3K-mTOR pathway, which is triggered by IL-4 receptor engagement through the adaptor molecule IRS2 (Heller et al. 2008). Indeed, Covarrubias et al. found that the Akt-mTORC1 pathway, independently of the canonical Jak-Stat pathway, regulates a subset of M2 genes through the control

of, among others, histone acetylation (Covarrubias et al. 2016). It could be thus possible that alterations in the activity of Akt-mTORC1 explain the defective expression of specific M2 genes in NFAT5-deficient macrophages. In this line, we plan to study the activation of this pathway in the future.

Our findings with alternatively activated NFAT5-deficient macrophages are clearly opposed to recent results shown by Choi et al. In their work they claim that NFAT5 not only promotes an M1 phenotype but also inhibits M2 gene induction by impairing IL-10 expression (Choi et al. 2016). However, in our hands we have not observed significant differences in *Il10* expression between WT and NFAT5-deficient macrophages (data not shown), and our results show decreased expression of M2 hallmark genes such as *Arg1*, *Relma* and *Chi3l3*. We have to take into account that we generated M2 macrophages by IL-4 treatment (M2a macrophages), while their alternatively-activated macrophages relied mainly on LPS-induced IL-10, a cytokine that promotes a different gene expression program (M2c) compared to IL-4 (Martinez et al. 2009). The discrepancies between these two works could also arise from differences in the experimental systems. While Choi et al. used the macrophage cell line Raw264.7 and siRNA technology to knock-down NFAT5, we worked with primary macrophages (BMDMs) obtained from total or conditional NFAT5 knock-out mice.

Intriguingly, *Arg1* and *Relma*, two well-known M2 markers induced by Th2 cytokines such as IL-4, also function as negative regulators of Th2 responses indirectly by promoting Th1 inflammatory responses (Pesce et al. 2009a, Pesce et al. 2009b). Accordingly, CD4 T cells cultured with NFAT5-deficient M2 macrophages show a more pronounced Th2 phenotype, expressing higher levels of *Il4*

mRNA compared to T cells cultured with *wild-type* macrophages (**figure 10**). This strengthens the idea that NFAT5 is necessary for a complete pro-inflammatory polarisation that facilitates T-dependent pro-inflammatory responses. Indeed, CD4 T lymphocytes co-cultured with NFAT5-deficient M1 macrophages induce lower levels of *Ifng* and *Il2*, two central cytokines in type 1 responses (Cope et al. 2011; Desmedt et al. 1998; Zhu & Paul 2008). In agreement with this, in the ID8 *in vivo* model, tumour bearing mice adoptively transferred with NFAT5-deficient macrophages were unable to limit tumour growth. This defect correlated with lower proportion of activated $CD8^+$ cells in the tumour microenvironment (peritoneal cavity), suggesting that NFAT5-deficient macrophages cannot trigger a proper type 1 anti-tumoural response in vivo (**figure 12**). Indeed, studies done in patients with ovarian carcinoma showed that high infiltration with activated $CD8^+$ T cells correlated with good prognosis (Sato et al. 2005, Nelson 2008).

Consistent with the idea of NFAT5 being important for pro-inflammatory responses, NFAT5-deficient macrophages also facilitated the growth of Lewis lung carcinoma cells *in vivo* (**figures 14A and 15A**), a tumour model whose progression is known to correlate with M2-infiltrating macrophages (Broz et al. 2014; Kratochvill et al. 2015). In accordance to this, *in vitro* experiments showed that NFAT5-deficient macrophages expressed decreased levels of iNOS and IL-12 and induced lower levels of *Ifng* and *Il2* in $CD4^+$ T cells; all of which are important mediators known to promote anti-tumour CD8 cytotoxic responses *in vivo* (Klug et al. 2013; Ruffell et al. 2014). In addition, NFAT5 deficiency also protected mice from an inflammation-induced cancer model.

Conditional myeloid-specific NFAT5-deficient mice developed fewer hepatocellular carcinomas (HCC) than their *wild-type* counterparts when injected with the carcinogen DEN. In this model, DEN induces hepatocyte death and the release of DAMPs that activate Kupffer cells to produce pro-inflammatory cytokines (mainly IL-6 or IL-1) that promote proliferation of the surviving hepatocytes leading to HCC development (Naugler et al. 2007, Sakurai et al. 2008) (**figure 19**).

As we have discussed, although NFAT5 is predominantly a pro-inflammatory factor, it can also regulate M2 gene transcription. The ability of NFAT5 to respond to diverse stimuli by fine-tuning gene expression is also observed for other transcriptional regulators such as Jmjd3, C/EBPβ, Notch/RBP-J and IKKβ. C/EBPβ and Jmjd3 are two important nuclear factors for M2 gene expression (Ruffell et al. 2009; Ye et al. 2012; Satoh et al. 2010) but can also be upregulated by pro-inflammatory stimuli such as IFNγ and/or LPS, contributing to regulate the expression of a subset of M1-associated genes (Udofa et al. 2013; Gorgoni et al. 2002; De Santa et al. 2009). Similar to what we observe for NFAT5, Notch/RBP-J and IKKβ are predominantly related to pro-inflammatory gene expression but can likewise promote M2-gene expression. RBP-J induces pro-inflammatory gene expression by activating the transcription factor IRF8 (Wang et al. 2010; Xu et al. 2012), a key factor for M1 polarisation under IFNγ and TLR signalling pathways (Zhao et al. 2006). Nevertheless, RBP-J is also necessary for the induction of a subset of M2 genes. In this regard, RBP-J deletion is detrimental to mount a proper M2 response in an *in vivo* model of chitin injection (Foldi et al. 2016), while it is beneficial to ameliorate the pathogenesis in a mouse model of Lupus erythematosus (SLE)

(Zhang et al. 2010). IKKβ is necessary for NF-κB activation, a transcription factor that plays a central role in pro-inflammatory gene expression in macrophages. However, IKKβ itself can limit classical M1 polarisation by interfering with the signalling mediated by STAT1, which is involved in the induction of genes such as *Il12* and *Nos2* (Fong et al. 2008). Interestingly, NFAT5 acts downstream Notch1 to promote thymocyte maturation (Berga-Bolaños et al. 2013) and its long-term accumulation in macrophages in response to TLR stimulation is dependent on IKKβ-NF-κB (Buxadé et al. 2012). Considering previous works and results presented here, NFAT5 might act together with these other versatile transcription factors, Notch/RBP-J and IKKβ (which have already been described to cooperate at least in certain cell types such as microglia (Yao et al. 2013)), to fine-tune gene-expression programs induced upon both pro- and anti-inflammatory inputs. M2-like macrophages populate tissues where they are essential to maintain homeostasis but need to rapidly repolarise towards an M1-like phenotype to efficiently respond to tissue damage and infection. The existence of this network of M1-proned transcription factors may allow macrophages to stay poised and maintain a state of readiness to rapidly react to danger signals and disruptors of homeostasis. In summary, the M1/M2 dual responsive capacity of these factors reflects the transcriptional complexity necessary for macrophages to continuously adapt with promptness to changes in the surrounding milieu.

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

1. Macrophages polarised as pro-inflammatory (M1) exhibit stronger expression of the transcription factor NFAT5 in response to later Toll-like receptor (TLR) stimulation compared to non-polarised or M2-polarised macrophages.

2. Macrophages polarised as pro-inflammatory (M1) or anti-inflammatory (M2) are capable of inducing classical macrophage genes such as Nos2, Il12b and Il1b in response to TLR stimulation. This induction is dependent on NFAT5 in both types of polarised macrophages.

3. NFAT5 induces the expression of selected alternative polarisation genes in macrophages stimulated with IL-4; it is required for the expression of *Arg1* and *Relma,* which are involved in limiting Th2 responses, but it is dispensable for *Cd206*.

4. NFAT5-deficient macrophages have reduced capacity to phagocytose live bacteria and, in addition, reduced capacity to control intracellular bacteria burden.

5. NFAT5 in macrophages supports type 1 immune responses since it promotes pro-inflammatory Th1 cytokine expression in $CD4^+$ cells while restrains anti-inflammatory Th2 gene expression.

6. Defective pro-inflammatory activation of NFAT5-deficient macrophages makes then more susceptible to repolarisation towards anti-inflammatory cells when they are co-cultured with ID8 and LLC tumour cell lines.

7. *In vivo*, NFAT5-deficient macrophages are less efficient than wild-type macrophages in limiting tumour growth of intraperitoneal ID8 and subcutaneous LLC tumour models.

ANNEX

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