A study of the modulation of Toll-like receptor signalling in macrophages by Annexin-1

Polycarpou, Anastasia

For additional information about this publication click this link.
https://qmro.qmul.ac.uk/jspui/handle/123456789/672

A study of the modulation of Toll-like receptor signalling in macrophages by Annexin-1

Dr Anastasia Polycarpou

A thesis submitted to the Queen Mary, University of London
for the degree of Doctor of Philosophy
October 2010

Centre for Biochemical Pharmacology
William Harvey Research Institute
Barts and the London School of Medicine
Queen Mary University of London

I, Anastasia Polycarpou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated and appropriately referenced.

Signed

Date

ABSTRACT

Annexin-A1 (AnxA1) is an endogenous anti-inflammatory protein that has been shown to exert a protective role against the lethal effects induced by the Toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS). The aim of this PhD studentship was to expand these observations and investigate the possible cross-talk between AnxA1 and other TLR signalling pathways in macrophages. To this aim, we compared the response *in vitro* of AnxA1$^{-/-}$ to AnxA1$^{+/+}$ bone marrow-derived macrophages (BMDMs) after stimulation with TLR2, 3, 5, 7 and 9 ligands. AnxA1$^{-/-}$ BMDMs exhibited higher expression of the activation markers MHC II and co-stimulatory molecules CD40, CD80, CD86 at the basal level, but a similar upregulation after stimulation with different TLR agonists. Stimulation of AnxA1$^{-/-}$ BMDMs with MyD88-dependent TLR agonists caused an increased production of TNF-α and IL-6 compared to AnxA1$^{+/+}$. Conversely, stimulation with the TRIF-dependent ligand poly (I:C) caused a decreased production of IL-6, but not TNF-α, by these cells. Interestingly, comparison of MyD88 and TRIF-dependent downstream signalling pathways in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs showed different kinetics of NF-κB DNA-binding activity, IκB-α degradation and ERK1/2 phosphorylation. Consistent with this, measurement of MyD88-dependent or TRIF-regulated genes in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs indicated a different time course of expression following stimulation with TLR3 (TRIF-dependent), TLR9 (MyD88-dependent) and TLR4 (TRIF and MyD88-dependent) ligands. Finally, AnxA1$^{-/-}$ mice showed an increased survival after challenge with poly (I:C), in contrast to increased lethality after injection of LPS, compared to AnxA1$^{+/+}$ mice. These results suggest that endogenous AnxA1 influences mainly the MyD88-dependent pathway and to a lesser extent the TRIF-dependent pathway both *in vitro* and *in vivo*. In addition, this study provides future venues for the investigation of molecular mechanisms by which endogenous AnxA1 preferentially interferes with specific TLR signalling pathways.

'This work is dedicated with love to the memory of my grandmother Anastasia who will always be an inspiration for me'

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER 1 – INTRODUCTION

CHAPTER 2 – METHODS AND MATERIALS

CHAPTER 3 – RESULTS

CHAPTER 4 – DISCUSSION

CHAPTER 5 – BIBLIOGRAPHY

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Ac2-26	The Annexin-1 mimetic peptide Ac-2-26
Akt/PKB	Protein kinase B
ALXR/FPRL-1	Formyl-peptide receptor like 1
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AnxA1	Annexin-A1
AnxA2	Annexin-A2
AP-1	Activated protein 1
APC	Antigen-presenting cell
APS	Ammonium persulphate
Arg1	Arginase 1
BLP	Bacterial lipoprotein
BMDMs	Bone marrow-derived macrophages
BSA	Bovine serum albumin
C3H/HeJ	Mouse strain hyporesponsive to LPS
C57BL/6	Black 6 strain of mouse
C5L2	C5α-like receptor 2
CASP	Colon ascendens stent peritonitis
CD40L	CD40 ligand
cDNA	Complementary DNA
CLP	Caecal ligation and puncture
COX	Cyclo-oxygenase
CpG	Cytosine-phosphodiesterguanine DNA
cPLA$_2$	Cytosolic Phosholipase 2
C-terminus	Carboxyl-terminus
CTL-4	Cytotoxic-lymphocyte associated protein-3
DAMP	Danger associated molecular pattern
dH$_2$O	Distilled water
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDA	Extra domain A
EDTA	Ethylene diaminetetraacetic acid
EIE	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
ERK	Extra-cellular signal-regulated kinase
FACS	Flow activated cell sorter
FCS	Foetal calf serum
FPR	Formyl peptide receptor
FPRL-1/ALXR	Formyl peptide receptor like-1
FPRL-2	Formyl peptide receptor like-2
FSC	Forward scatter
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper
GPCR	G-protein-coupled receptor
h	Hours
HKLM	Heat-killed *listeria monocytogenes*
HMGB1	High mobility group box 1 protein
HNE	Neutrophil elastase
hr-AnxA1	Human recombinant AnxA1
HSP	Heat shock protein
HRP	Horseradish peroxidase
Hyppos	Hydrophobic portions
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
IB	Immunoblotting
IFN-β	Interferon β
IFN-γ	Interferon γ

Ig	Immunoglobin
IKK	IkB kinase
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17A	Interleukin-17A
IL-23	Interleukin-23
IP	Immunoprecipitation
IP-10/CXCL10	IFN-γ-inducible protein
IPAF	ICE-protease activating factor
IRAK	IL-1 receptor associated kinase
IRF	IFN response factor
ISRE	Interferon sensitive response element
ITAM	Tyrosine-based activation motif
JNK	Jun N-terminal kinase
kDa	Kilo Dalton
KO	Knock-out
LBP	LPS-binding protein
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MALP-2	Synthetic bacterial lipopeptide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MD-2	Myeloid differentiation protein 2
MEK	MAPK/ERK kinase
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MKK	MAP kinase kinase
MIF	Macrophage migration inhibitory factor
min	Minute(s)

mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response protein 88
NEMO	NF-κB essential modifier
NET	Neutrophil extracellular trap
NFAT	Nuclear factor for activated T-cells
NF-κB	Nuclear factor κ B
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric-oxide synthase
N-terminus	Amino-terminus
ODN	CpG-dinucleotide-containing sequence
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE$_2$	Prostaglandin E$_2$
PI3K	Phosphatidylinositol 3 kinase
PLA$_2$	Phospholipase A$_2$
PMN	Neutrophil, polymorphonuclear leukocyte
Poly (I:C)	Polyinosine-polycytidylic acid
PR3	Proteinase-3
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene fluoride
Pubmed	National Institute of Health Journal Database
RAGE	Advanced glycation end-products
Rel	Reticuloendotheliosis oncogene
RF	Rheumatoid factor
RH	Rel homology
RHIM	RIP homotypic interaction motif
RIP	Receptor interacting protein
RNA	Ribonucleic acid
r.p.m	Revolutions per minute
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus

RT	Room temperature
SARM	Sterile α- and armadillo-motif-containing protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SEM	Standard error mean
SIGIRR	Single immunoglobin IL-1 receptor-related molecule
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SNIP	Single-nucleotide polymorphism
SSC	Side scatter
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription protein
S.typhimurium	*Salmonella typhimurium*
TA	Transactivation
TAB	TAK binding protein
TAK	TGF-β-activated kinase
TANK	TRAF-family member-associated NF-κB activator
TBE	Tris-borate-EDTA
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylenediamine
TFPI	Tissue-factor pathway inhibitor
TGF-β	Transforming growth factor β
Th$_1$cell	T-helper 1 cell
Th$_2$ cell	T-helper 2 cell
TIR	Toll/IL-1 Receptor
TIRAP or MAL	TIR-domain-containing adapter protein
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TNT	Trinitrotoluene
TOLLIP	Toll-interacting protein
TRAF6	Tumor necrosis factor receptor-associated factor 6

TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAM	TRIF-related adapter molecule
TREM	Triggering receptor expressed on myeloid cells
TRIF	TIR-domain-containing adapter inducing IFN-β
TTBS	Tris-buffered saline solution containing tween-20
V	Volts
WT	Wild-type

CHAPTER 1:
INTRODUCTION

1.1. THE MACROPHAGE

The recognition of the role of macrophage as an immune cell has a history of more than 100 years, since Elie Metchnikoff first described phagocytosis, winning the Nobel Prize. Macrophages are present in all tissues. They arrive from circulating monocytes, which migrate into tissues and undergo differentiation. Therefore, they may be present as osteoclasts in the bone, microglial cells in the central nervous system, histiocytes in the connective tissue, Kupffer cells in the liver, alveolar and peritoneal macrophages, macrophages of the gastrointestinal tract, of the adipose tissue or other (Gordon and Taylor, 2005). In certain cases, such as the microglial cells of the central nervous system, mature macrophages may rise in the tissue from local proliferation of tissue-resident colony-forming cells and not from monocytes (Cuadros and Navascues, 1998, Naito et al., 1996).

Besides its function in the immune response, the macrophage has a very important homeostatic role. Indeed, macrophages are prodigious phagocytic cells that clear debris and cells that have undergone apoptosis or necrosis. They clear approximately $2x10^{11}$ erythrocytes each day contributing in the recycling of iron and haemoglobin (Beaumont and Delaby, 2009). Several receptors mediate this homeostatic clearance including scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors (Erwig and Henson, 2007).

While clearing the debris of necrotic cells, macrophages detect different 'danger' signals. The interaction of invading pathogens with macrophages can be either by

direct binding to surface receptors (nonopsonic) or mediated by opsonins coating the bacterial surface. Complement-opsonised bacteria bind to macrophages via the complement receptors but interaction of macrophages with non-opsonised bacteria is based on the recognition of evolutionary conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). Pathogens are able to exploit receptor interactions crucial to their survival or to use secretion systems to transport molecules into the cell altering the process of phagocytosis (Pluddemann et al., 2006).

Macrophages have been sub-divided as M1 macrophages for classically activated macrophages and M2 macrophages for alternatively activated macrophages. Another classification of the macrophages is based on the fundamental macrophage functions which are: host defence, wound healing, and immune regulation (Mosser and Edwards, 2008). One of the main characteristics of these cells is their plasticity that allows them to efficiently respond to environmental signals changing their phenotype (Stout and Suttles, 2004). These signals can arrive from both innate and adaptive immune response.

The classically activated macrophages are activated by a combination of tumor necrosis factor α (TNF-α) -produced after TLR stimulation- and interferon-γ (IFN-γ) (Edwards et al., 2006). Certain TLR agonists may induce both TNF-α and IFN-β, therefore activating macrophages without the requirement of additional IFN-γ. Classically activated macrophages are characterized by the enhanced killing of intracellular pathogens, and the increased secretion of cytokines. This production

of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and IL-23, although an important component of host defence, can cause extensive host-tissue damage. Classically activated macrophages also produce reactive oxygen radicals (ROS) and nitric oxide (NO), both of which have antimicrobial activity.

The alternatively activated macrophages arise through the presence of IL-4 or IL-13 (Gordon, 2003). These cells secrete components of the extracellular matrix and their main function is related to wound healing. They can also contribute to defence against helminths and nematodes (Mylonas et al., 2009) and they have also been associated with allergy (Kurowska-Stolarska et al., 2009).

Regulatory macrophages are the result of stress responses (Sternberg, 2006) or they can arise during the later stages of adaptive immune responses (Mosser, 2003). Their primary role is to dampen the immune response limiting inflammation. The production of the anti-inflammatory cytokine IL-10 is the most important and reliable characteristic of regulatory macrophages (Edwards et al., 2006). Many parasitic, bacterial and viral pathogens interfere with macrophage activation or induce the development of regulatory macrophages, therefore resulting defective pathogen killing (Mahalingam and Lidbury, 2002).

The phenotype of macrophages can change over time. Often, this phenotypic switch is associated with pathology. One characteristic example of switching from classically activated macrophages to regulatory macrophages is the case of cancer. The production of free radicals that the classically activated macrophages produce may lead to DNA damage and mutations that can predispose host cells to

neoplasia. As cancer progresses, these macrophages change their physiology towards a phenotype which resembles regulatory macrophages by producing high levels of IL-10 and de-activating neighbouring macrophages (Pollard, 2008). On the other hand, the switch from wound-healing macrophages to classically activated macrophages, which takes place in obesity may contribute to chronic inflammation in the adipose tissue, leading to insulin resistance and type 2 diabetes (Lumeng et al., 2007).

Another important function of macrophages is their role as an antigen-presenting cell (APC) (Gregg and Denis, 1991). As well as being resident in tissues, macrophages are also found in lymphoid organs. Resting macrophages have few or no major histocompatibility complex II (MHC II) molecules on their surface and do not express the B7 co-stimulatory molecules. When macrophages ingest microorganisms and recognise their PAMPs they become activated and the expression of MHC II and B7 molecules is induced (Nolan et al., 2009). Different macrophage populations express distinct levels of these molecules (Edwards et al., 2006) and, therefore, may have different potentials to present antigens to T-cells. Thus, classically activated macrophages express relatively high levels of MHC II and co-stimulatory molecules, alternatively activated macrophages only minimally up-regulate expression of these molecules, whereas regulatory macrophages often express high levels. Mature macrophages can divert the fate of naïve T cells via three signals. Signal 1 is the result of the interaction of the pathogen-presented peptide by MHC II molecules with the T-cell receptor (TCR). Signal 2 results from the binding of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) to the T-cell. Signal 3 is the result of the cytokine microenvironment.

However, there is very little evidence that macrophages initiate T-cell immunity (Lipscomb et al., 1986, Lyons et al., 1986), and the expression of the co-stimulatory molecules may just reflect an expansion of responses already initiated by dendritic cells (Ding et al., 1993).
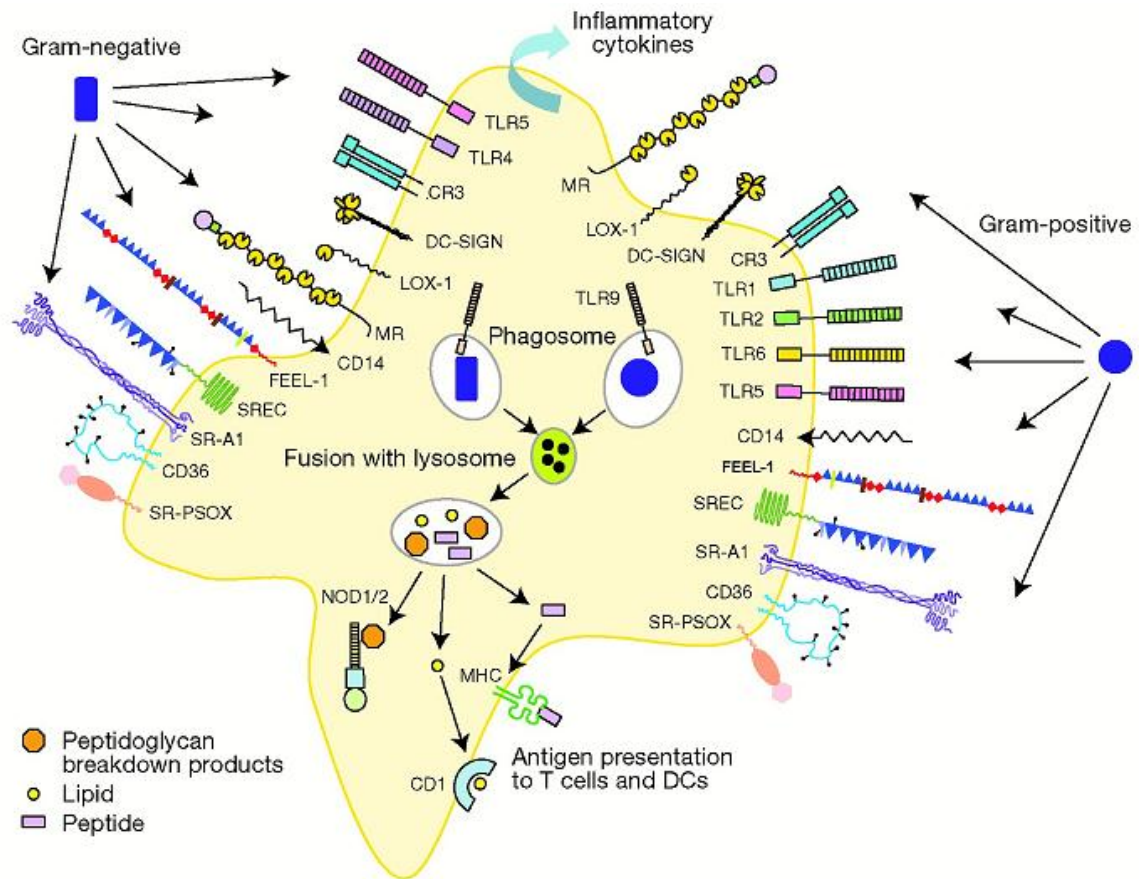
Figure 1.1: Macrophage receptors in bacterial recognition

Macrophages have multiple receptors both on the surface and in intracellular compartments that recognise Gram (+) and Gram (-) bacteria.

Abbreviations: CR3, complement receptor 3; DC-SIGN, dendritic-cell-specific intracellular-adhesion-molecule-3-grabbing non-integrin; FEEL-1, fasciclin, EGF-like, laminin-type EGF-like, and link-domain-containing scavenger receptor 1; LOX-1, lectin-like oxidised low-density lipoprotein receptor 1; MR, mannose receptor; NOD, nucleotide-binding oligomerisation domain; SR, scavenger receptor; SREC, scavenger receptor for endothelial cells; SR-PSOX, scavenger receptor for phosphatidylserine and oxidised low-density lipoprotein. Taken from (Pluddemann et al., 2006).
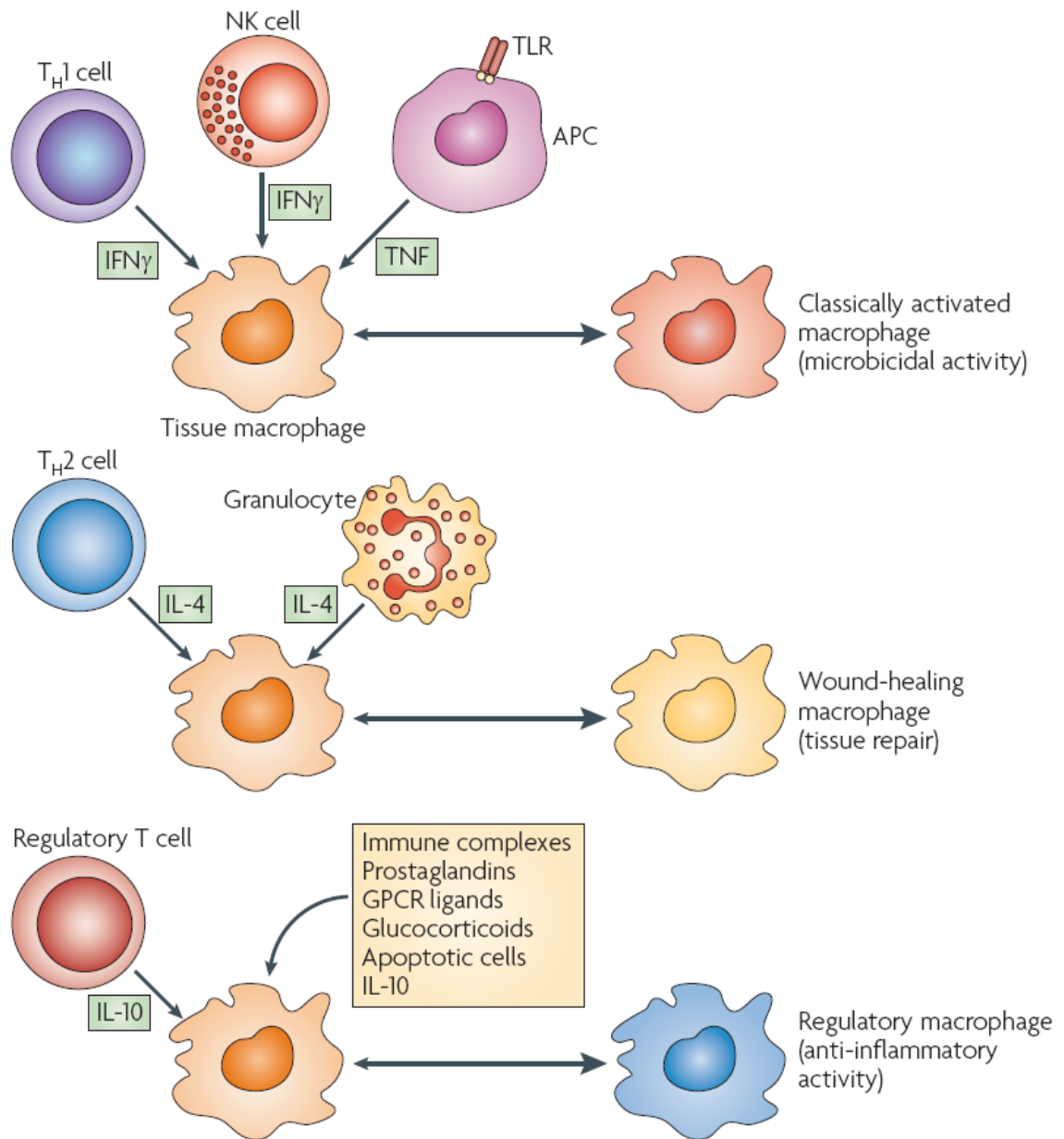
Figure 1.2: Cytokines produced by immune cells can give rise to macrophages with distinct phenotypes.

Classically activated macrophages arise in response to IFN-γ and TNF and have microbicidal activity. Wound-healing (alternatively activated) macrophages arise in response to IL-4 and have a role in tissue repair. Regulatory macrophages are generated in response to various stimuli, including immune complexes, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells or IL-10 and they suppress immune responses by producing high levels of IL-10. Taken from (Mosser and Edwards, 2008).

1.2. TLRs AND THE IMMUNE RESPONSE

1.2.1. Introduction

The immune response is initiated when the innate immune system is able to detect microbial intruders such as protozoa, bacteria, fungi, and viruses via PRRs. The Toll family of receptors have a central role as PRRs in the initiation of innate immune responses (Kaisho and Akira, 2006) and in the establishment of adaptive immunity. They recognise conserved microbial products known as PAMPs. These are motifs of microorganisms that are essential for their metabolism and survival. In addition, they recognise endogenous mediators that are released from dying cells termed "alarmins" or danger-associated molecular patterns (DAMPs), which further amplify the inflammatory response via TLRs.

TLRs are highly conserved through evolution. Toll was initially discovered in *Drosophila* as an essential receptor involved in embryogenesis and host defence against fungal infection (Lemaitre et al., 1996). This was followed by cloning and characterization of a human homologue, which has been shown to signal through the NF-κB pathway, inducing the expression of genes for inflammatory cytokines, as well as the expression of the co-stimulatory molecule B7.1, which is required for the activation of naive T cells by APCs (Medzhitov et al., 1997).

1.2.2. Mammalian TLRs and their localisation in cells and tissues

To date 11 TLRs have been identified in humans (TLR1-11) and 13 in mice (TLR1-13) (Akira and Hoshino, 2003). TLRs 1-9 are conserved between the human and the mouse.

Certain TLRs (TLR1, 2, 4, 5, 6, 10) are localised in the plasma membrane whereas others (TLR3, 7, 8, 9) are preferentially expressed in intracellular compartments such as endosomes (Takeda and Akira, 2005) (Fig 1.3). The subcellular localization is believed to be important for the discrimination of viral nucleic acids from self nucleic acids (Barton et al., 2006). For most TLRs it has been shown that endosomal maturation is a requirement for TLR-mediated recognition of their ligands (Hacker et al., 1998, Heil et al., 2004). Therefore, phagosomal/lysosomal or endosomal/lysosomal compartments could be the main sites for TLR recognition of microbial components (Takeda and Akira, 2005). Even TLR2 is recruited to macrophage phagosomes after exposure to zymosan, despite the fact that it is expressed on the cell surface (Underhill et al., 1999).

TLRs are expressed on different types of immune and non-immune cells. They are differentially expressed among immune cells (Muzio et al., 2000). Their surface expression appears very low and corresponds to a few thousand molecules per cell in monocytes and a few hundred in immature dendritic cells (Visintin et al., 2001). In addition to, TLR bearing cells may express different levels of TLRs according to their strategic position in the body. For example, peritoneal macrophages express significantly higher levels of TLR2 and TLR4 than splenic

macrophages (Liu et al., 2006). TLR expression is also modulated positively and negatively in response to a variety of stimuli. However, there is a complicated discrepancy between mRNA expression and responsiveness to TLR ligands and a lack of reliable antibodies to TLRs. Therefore, the expression of a TLR by a cell type does not necessarily mean that stimulation of this receptor will result cell activation. CpG, for example, does not stimulate neutrophils, despite the fact that they express TLR9 (Hornung et al., 2002).
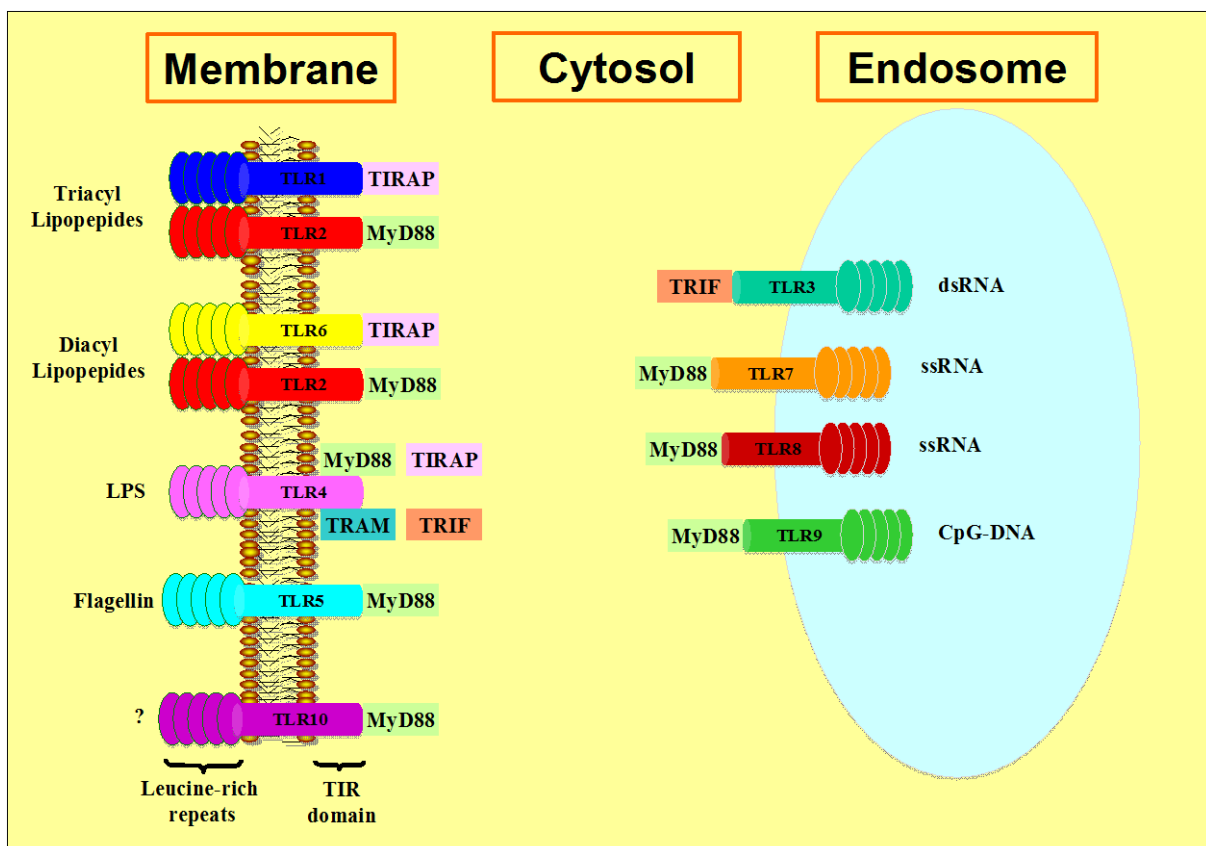
Figure 1.3: Human TLR localisation in the cell.
Cellular orientation of TLRs and examples of ligands showing the accessory factors associated with their signalling function.

1.2.3. Pathogen-associated Molecular Patterns

Representative ligands/PAMPs for TLRs include bacterial cell wall components such as lipopolysaccharide (LPS) of Gram (-) bacteria, which is recognised by TLR4 (Hoshino et al., 1999) or peptidoglycans and lipopeptides carried by Gram (+) bacteria recognised by TLR2 (Hirschfeld et al., 1999). Mycoplasma does not possess a cell wall but its plasma membrane contains lipopeptides (Razin et al., 1998). TLRs can also recognise proteins such as flagellin of flagellated bacteria recognised by TLR5 (Hayashi et al., 2001) and nucleic acids such as bacterial and viral DNA containing high frequency of CpG motives recognised by TLR9 (Hemmi et al., 2000) or viral single-stranded RNA (ssRNA) recognised by TLR7 and TLR8 (Lund et al., 2004), whereas TLR3 recognises viral double-stranded RNA (dsRNA) (Alexopoulou et al., 2001). TLR11 is involved in the recognition of uropathogenic bacteria (Zhang et al., 2004). TLR10 is believed to be an orphan member of the TLR family but has the ability to form homodimers and heterodimers with TLR1 and TLR2 (Hasan et al., 2005). Therefore, it is possible that TLR10 may recognise still unidentified ligands.

TLRs do not recognise only exogenous ligands but have the ability to recognise 'endogenous' ligands or 'alarmins' or DAMPS. Such ligands include heat-shock proteins (HSP; TLR2 or TLR4) (Ohashi et al., 2000, Vabulas et al., 2001), uric acid (TLR2) (Foell et al., 2007), hyaluronan (TLR2) (Scheibner et al., 2006), surfactant protein (TLR4) (Guillot et al., 2002), and fibronectin (TLR4) (Okamura et al., 2001).

One group of pathogens is not exclusively recognised by one TLR and one TLR can correspond to structurally unrelated ligands, often derived from different groups of pathogens (Janssens and Beyaert, 2003). There are, however, TLRs, which seem to be more ligand specific such as TLR3, TLR5, and TLR9.
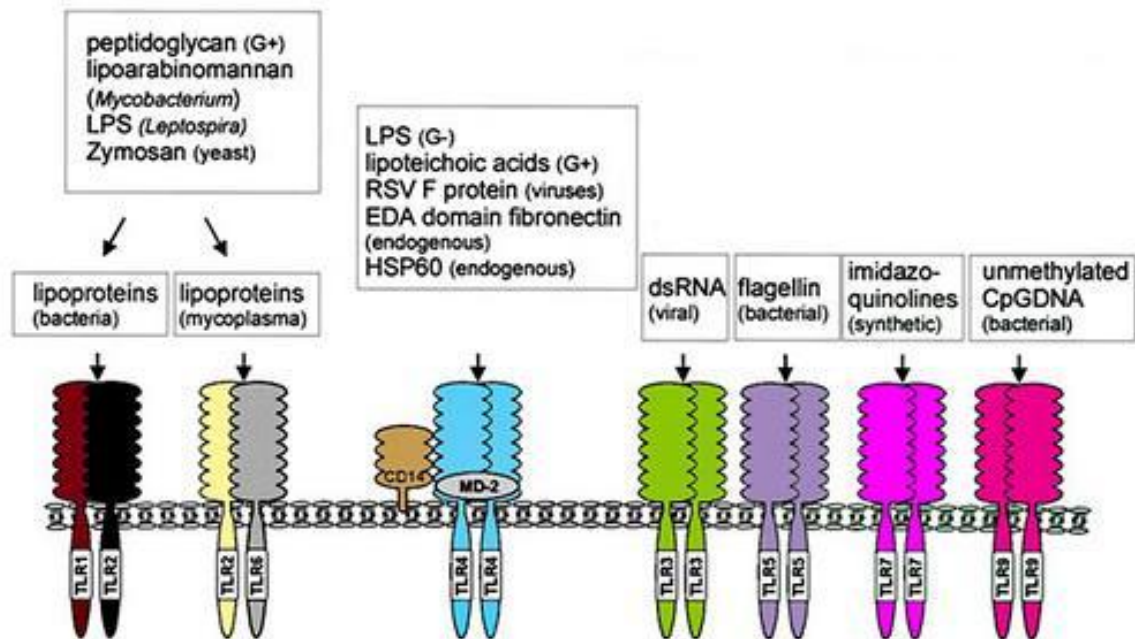
Figure 1.4: Mammalian TLRs and their ligands.
TLR1 and TLR6 do not signal as separate entities but act in cooperation with TLR2. TLR4 acts in a complex with CD14 and MD-2. RSV, respiratory syncytial virus; EDA, extra domain A. Taken from (Janssens and Beyaert, 2003).

1.2.3.1. LPS

Bacterial endotoxin (LPS) is a component of the outer membrane of Gram (-) bacteria and a causative agent of endotoxin shock. It is a glycolipid composed of a hydrophilic polysaccharide part and a hydrophobic domain known as lipid A, which is responsible for the molecule's biologic activity (Raetz, 1990). Lipid A, which is the only part of LPS recognised by TLR4, is conserved among species but is not a single molecule showing diversity among different bacteria (Miller et al., 2005). The receptor complex for LPS is composed of three proteins: TLR4, CD14 and myeloid differentiation protein-2 (MD-2) (Miller et al., 2005) (Fig 1.5). N-linked glycosylations of both MD-2 and TLR4 are important in maintaining the integrity of the LPS receptor complex (Ohnishi et al., 2001). In the bloodstream, enterobacterial LPS is carried by a serum LPS-binding protein (LBP), an acute-phase protein which binds to LPS and transfers an LPS monomer from the bacterial cell wall to CD14 (Schumann et al., 1990).

C3H/HeJ is a mutant mouse strain hyporesponsive to LPS. A loss-of –function mutation of these LPS-hyporesponsive mice lead to the discovery of the first mammalian Toll protein, which is known as TLR4 (Poltorak et al., 1998). Although TLR4 is the established receptor for LPS from enterobacteria, it has been observed that there are types of LPS such as those derived from *Porphyromonas gingivalis* (Asai et al., 2005), *Leptospira interrogans* (Werts et al., 2001), and *Legionella pneumophila* (Girard et al., 2003), which activate macrophages via TLR2. However, neither human nor murine TLR2 participates in LPS signalling

and it is believed that bacterial lipoproteins in LPS preparations are responsible for

the TLR2-mediated activity (Hirschfeld et al., 2000).
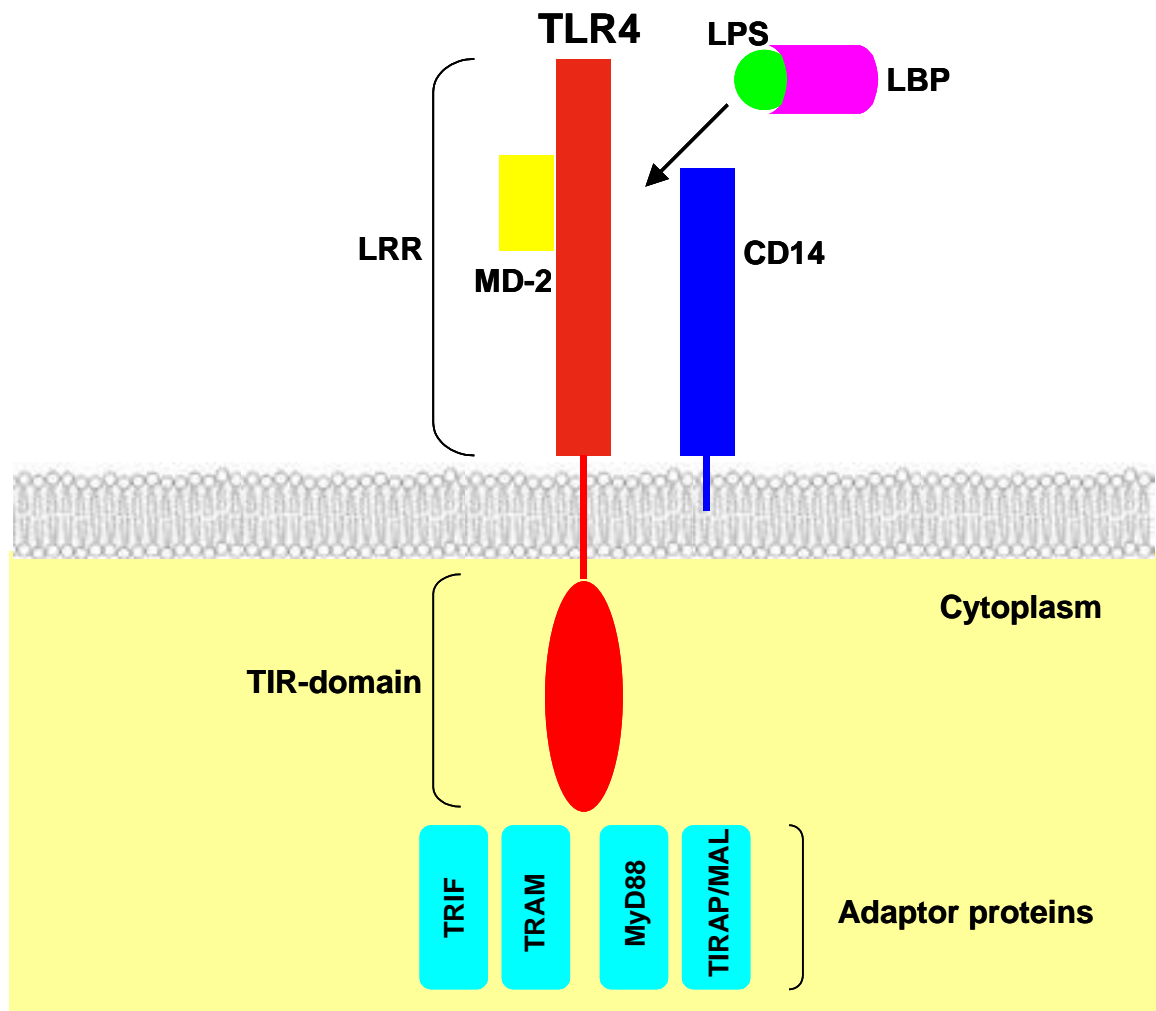
Figure 1.5: **The LPS receptor complex on macrophages.**
LPS is recognised by a complex of 3 proteins: CD14, TLR4, and MD-2. A serum LBP transfers LPS to CD14, which in turn concentrates LPS and presents it to TLR4-MD-2. MD-2 plays a role in LPS recognition and regulates the cellular distribution of TLR4. TLR4 acts as the signal-transducing receptor for LPS. All known TLR adaptor proteins are responsible for the TLR4-mediated signalling. LRR, Leucine-rich repeats; TIR, Toll-IL-1 receptor. Adapted from (Fujihara et al., 2003).

1.2.3.2. Lipoproteins

Lipoproteins and lipopeptides of the outer membrane of bacteria -abundant in Gram (+) microorganisms- are among the TLR2 ligands (Hirschfeld et al., 1999). TLR2 agonists are recognised by heterodimers between TLR2 and other TLR family members. For example, synthetic bacterial lipopeptide (MALP-2) is recognised between TLR2 and TLR6 (Takeuchi et al., 2001), whereas bacterial lipopeptide is recognised by TLR2 and TLR1 (Alexopoulou et al., 2002).

1.2.3.3. Double-stranded RNA

Viral replication within infected cells results in generation of dsRNA recognised by TLR3. TLR3-deficient mice showed reduced responses to a viral RNA mimic called polyinosine-polycytidylic acid (poly (I:C)) indicating the role of TLR3 in recognition of dsRNA (Alexopoulou et al., 2001).

1.2.3.4. Flagellin

Flagellin is a 55-kDa monomer obtained from bacterial flagella which helps the organism move through its aqueous environment. It is a conserved TLR ligand recognised by TLR5 (Hayashi et al., 2001) and forms an ideal PAMP since no host cells possess such a protein. Flagellated *Proteobacteria* such as *Helicobacter pylori* have evolved in such a way that they can avoid TLR5 by mutating the amino acid residues responsible for flagellin recognition by TLR5 (Andersen-Nissen et al., 2005).

1.2.3.5. Imidazoquinolines

Imidazoquinoline family contains members such as imiquimod and resiquimod, which have potent antiviral and antitumor properties (Miller et al., 1999) and are recognised by TLR7 (Hemmi et al., 2002).

1.2.3.6. Cytosine-phosphodiesterguanine (CpG) DNA

CpG DNA is DNA containing unmethylated CpG motifs, which is largely equivalent to bacterial DNA and is recognised by TLR9 (Hemmi et al., 2000). Unmethylated CpG-dinucleotide-containing sequences (CpG ODNs) are not frequent in vertebrate genomes, which are usually methylated therefore lack immunostimulatory activities (Hare and Taylor, 1985).

1.2.4. TLR structure

The cytoplasmic part of TLRs shows high similarity to that of the IL-1 receptor family, termed as Toll/IL-1 receptor (TIR) domain (Rock et al., 1998). The extracellular part is structurally unrelated to the IL-1 receptor and it bears leucine-rich repeats (LRRs) (Chiang and Beachy, 1994). How LRRs are involved in ligand recognition and signal transduction still remains unclear. It is still not understood how each TLR can recognise different ligands of such diversity.

The binding of a PAMP to a TLR causes dimerisation of the receptor. TLR2 forms a heterodimer with TLR1 or TLR6, but the other TLRs were believed to form homodimers (Saitoh et al., 2004). Recently however, the formation of a new heterodimer comprising TLR4 and TLR6 has been discovered, which recognises endogenous ligands such as low-density lipoprotein (LDL) and amyloid-β, promoting sterile inflammation (Stewart et al., 2010).
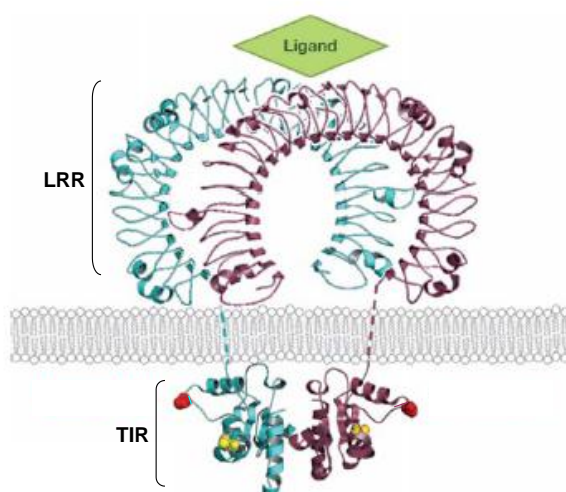
Figure 1.6: TLR structure

Schematic illustration of TLRs, based on the structure of the TLR3 ectodomain and the TLR2 TIR motif. TLR ectodomains are all likely to be dimerized, composed of numerous repeating LRRs. The cytoplasmic domains are compact, consisting mostly of a TIR motif. It is believed that ligands elicit a conformational change, allowing recruitment of specific adaptor proteins. Adapted from (Beutler et al., 2006).

1.2.5. TLR signalling pathways

1.2.5.1. Adaptor proteins

The intracellular signalling of TLR ligands is initiated by the TIR domain which contains adaptor molecules such as Myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adaptor inducing IFN-β (TRIF), TIR domain–containing adaptor protein (TIRAP) also known as MAL, and TRIF-related adapter molecule (TRAM) (Dunne and O'Neill, 2005). All TLRs use the adaptor protein MyD88 with the exception of TLR3, which exclusively uses TRIF (Yamamoto et al., 2002). Therefore, there are two distinct TLR signalling pathways: the MyD88-dependent and the MyD88-independent/TRIF dependent pathway. TLR4 is the only member of the TLR family that contains all adaptor proteins mentioned above and therefore it may signal through both TLR pathways. The major pathways activated by TLRs are passed through IkB kinase (IKK), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways.
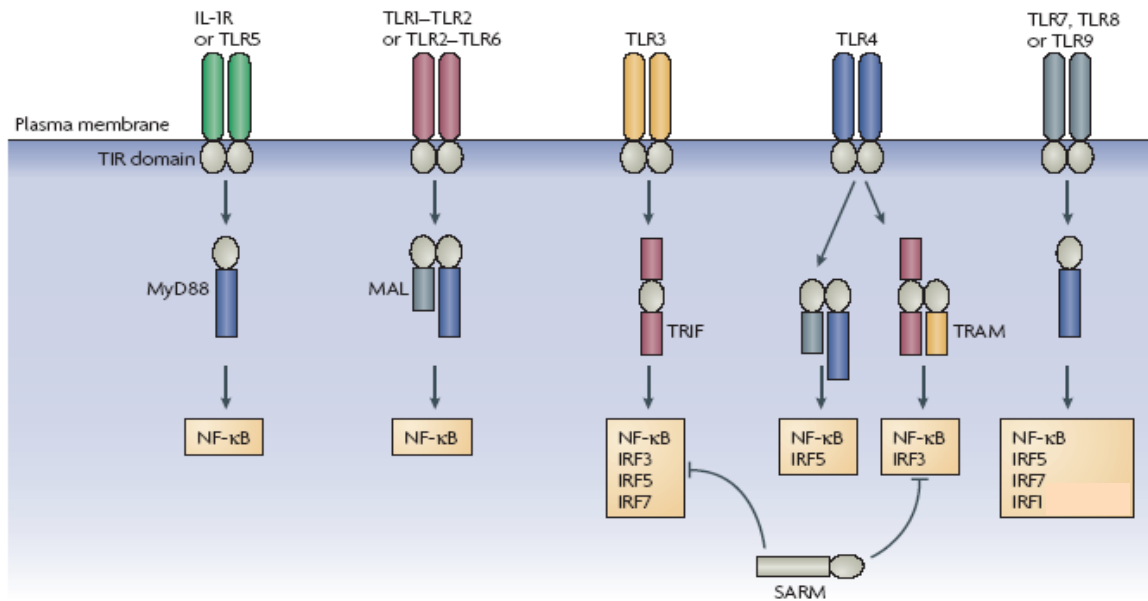
Figure 1.7: Adaptor proteins for the TLR/IL-1R superfamily.

Each receptor complex differentially uses each adaptor to positively regulate transcription factor activation. Sterile α- and armadillo-motif-containing protein (SARM) is the exception, which inhibits TRIF-mediated transcription factor activation. Adapted from (O'Neill and Bowie, 2007).

1.2.5.2. MyD88-dependent pathway

MyD88 has a TIR domain, which is involved in the interaction with TLRs, and a death domain (Watters et al., 2007), which is responsible for the recruitment of IL-1 receptor associated kinases (IRAKs) (Medzhitov et al., 1998). These are IRAK-1, IRAK-2, IRAK-4, and IRAK-M. IRAK-1 and IRAK-4 possess serine/threonine protein kinase activity (Wang et al., 2006) whereas IRAK-2 and IRAK-M negatively regulate TLR signalling (Hardy and O'Neill, 2004, Kobayashi et al., 2002). IRAKs are sequentially phosphorylated and dissociated from MyD88 and have binding motives for interaction with Tumor necrosis factor receptor-associated factor 6 (TRAF6) (Qian et al., 2001). TRAF6 belongs to an E3 ubiquitin ligase family and activates through ubiquitination TGF-β-activated kinase (TAK) 1 and the canonical IκB kinases IKKα and IKKβ that phosphorylate IκB and degrade it leading to the translocation of NF-κB to the nucleus (Deng et al., 2000). TAK1 phosphorylates two members of the MAP kinase kinase (MKK) family, MKK3 and MKK6, which activate Jun N-terminal kinase (JNK) and p38 (Irie et al., 2000). Extra-cellular signal related kinase (ERK) is activated through the activation of MAPK/ERK kinase 1 (MEK1) and MEK2 (Crews et al., 1992).

1.2.5.3. MyD88-independent/ TRIF-dependent pathway

Responses to poly (I:C) are relatively normal in MyD88-deficient mice indicating that TRIF is the sole adapter used by TLR3 (Yamamoto et al., 2003). TRIF may also lead to the activation of NF-κB and MAPKs (Yamamoto et al., 2003). It possesses 3 TRAF-6 binding domains that mediate interaction with TRAF6 (Sato et al., 2003). It also contains Rip homotypic interaction motif (RHIM), which mediates interaction with members of the receptor interacting protein (RIP) family (Meylan et al., 2004). Both TRIF-RIP1 and TRIF-TRAF6 interactions induce activation of NF-κB.

In addition, TRIF can activate the IFN-β promoter (Yamamoto et al., 2002). TRIF can interact with the canonical IκB kinases IKKϵ and TANK-binding kinase 1 (TBK1) (McWhirter et al., 2004). IFN response factor 3 (IRF-3) is then phosphorylated by these kinases and it translocates to the nucleus inducing several target genes.
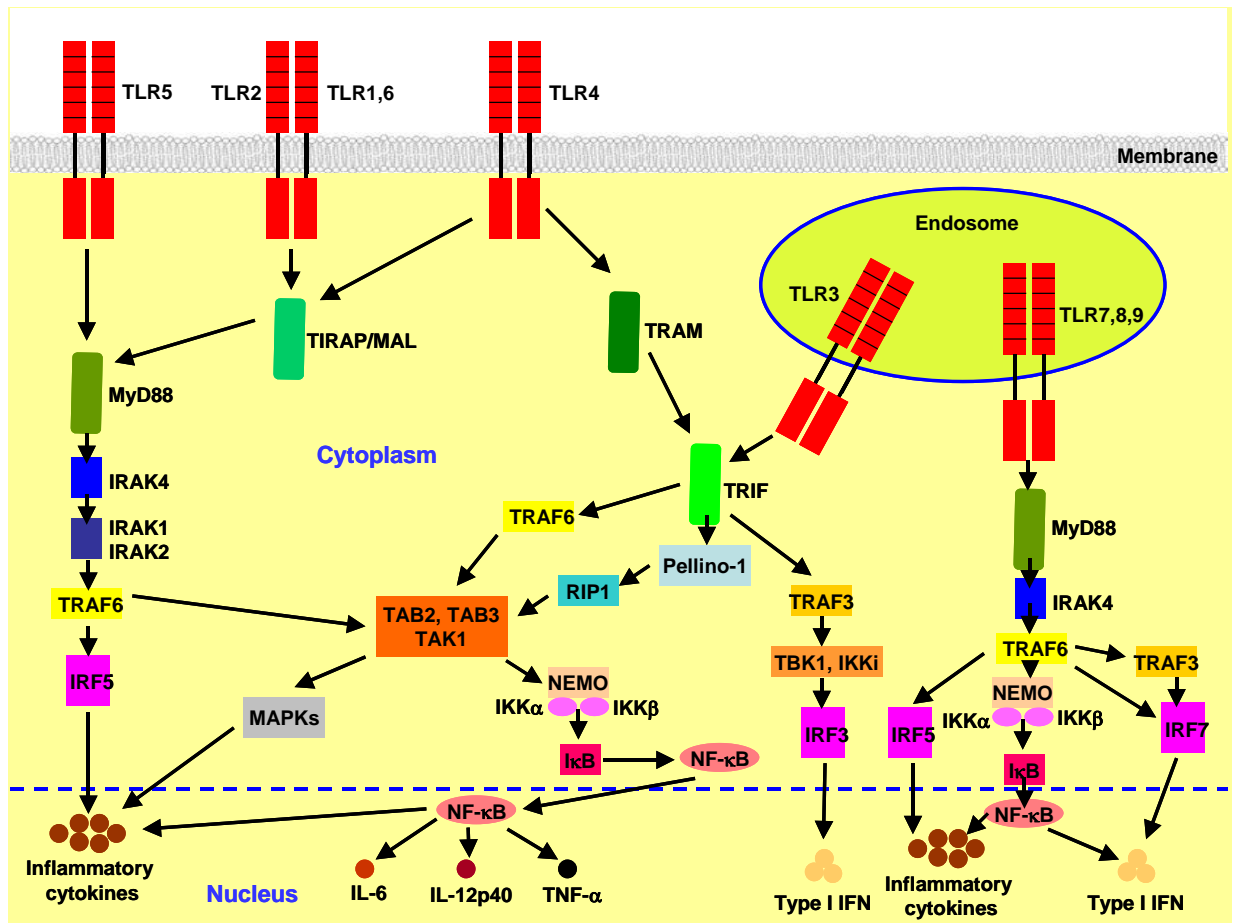
Figure 1.8: TLR signalling pathways.

Diagram showing the signalling complexity followed by each TLR. Both MyD88-dependent and TRIF-dependent pathway lead to the activation of NF-κB and the induction of inflammatory cytokines.

1.2.5.4. Transcription factors

TLR signalling can lead to activation of several transcription factors, including NF-κB (Doyle and O'Neill, 2006), Activating protein-1 (AP-1) (Jones et al., 2001a), and IRFs (Honda et al., 2005, Schoenemeyer et al., 2005, Yamamoto et al., 2003). As described previously, both signalling pathways activate NF-κB and the MAPKs (ERK1/2, JNK, p38) resulting in the expression of numerous genes encoding cytokines such as IL-6 and TNF-α and other inflammatory molecules such as platelet-activating factor, prostaglandins, enzymes, and free radicals, such as NO (Akira and Hoshino, 2003, Chi et al., 2006, Hu et al., 2008b, Jones et al., 2001a, Jones et al., 2001b, Yamamoto et al., 2002). In addition, the MyD88-independent signalling pathway activates IRF3, inducing the production of type I IFN (Toshchakov et al., 2002, Toshchakov et al., 2003). TLR7, TLR8 and TLR9 may also induce type I IFNs, including IFN-α and IFN-β, depending on MyD88 and using IRF7 as a transcription factor (Honda et al., 2005).

1.2.5.4.1. TLRs and NF-κB

NF-κB is not a single and unique molecule but a family of transcription factors that play an important role in the regulation of a variety of events such as inflammation, immunity, cell proliferation and apoptosis. It includes a collection of proteins containing a highly conserved DNA-binding and dimerisation region, the Rel homology (RH) domain (Hoffmann et al., 1999). This family includes two distinct groups of proteins. The first group includes p105, p100 and *Drosophila* Relish (Li and Stark, 2002). These proteins have long C-terminal domains containing

multiple copies of ankyrin repeats and can give rise to active shorter proteins such as p50, and p52 (Lin and Ghosh, 1996). Members of this group form dimers with members of the second group, which includes p65 (RelA), Rel (c-Rel), RelB, and *Drosophila* Rel proteins (Li and Stark, 2002). This second group of proteins possess one or more C-terminal transcriptional activation domains. Therefore, members of NF-κB proteins may form homodimers and heterodimers.

In unstimulated cells, NF-κB-family proteins exist as heterodimers or homodimers in the cytoplasm that are associated with a member of the IκB family of inhibitory proteins (Baeuerle and Baltimore, 1996). The IκB family includes IκBα, IκBβ, IκBε, and Bcl3. These proteins have an ankyrin repeat motif, important for the maintenance of NF-κB in the cytoplasm. Several extracellular signals such as TLR ligands activate signal transduction pathways leading to the activation of IκB kinase (IKK), which phosphorylates 2 serine residuals on IκB proteins (S32 and S36 of IκBα) (Ghosh et al., 1998). IKK is composed of 3 subunits: the catalytic subunits IKKα and IKKβ, and the regulatory subunit IKKγ, formerly known as NF-κB essential modifier (NEMO) (Karin, 1999). Other kinases involved in NF-κB activation are IKKε/IKKi and TBK/NAK/T2K (Fitzgerald et al., 2003a). Phospho-IκB is then ubiquitinated by ubiquitin ligase and degraded by the proteasome (Karin and Ben-Neriah, 2000). The dissociation of NF-κB from the IκB proteins unmasks the nuclear localisation signal of NF-κB, leading to its nuclear translocation and binding to the promoters of target genes. In addition to, there is also a second level of regulation of NF-κB, based on the phosphorylation of the second group of NF-κB proteins, resulting in the activation of transcriptional activity. Therefore, the

p65 subunit may be phosphorylated at serine residues within the two transactivation domains (TA1 and TA2) located in the C-terminus (Schmitz and Baeuerle, 1991).

Signalling through all TLRs leads to activation of NF-κB, which binds to a nucleotide sequence in genes producing proinflammatory cytokines such as IL-1, IL-6, and TNF-α. In fact, the first ever stimulus shown to activate NF-κB was the TLR4 ligand LPS (Sen and Baltimore, 1986).

In the MyD88-dependent pathway, the adaptor protein MyD88 associates with the TIR domain of the TLRs and recruits IRAK4 to the TLR receptor complex (Li et al., 2002). This facilitates IRAK4-mediated phosphorylation of IRAK1, which in turns dissociates from the receptor complex and associates with TRAF6 (Cao et al., 1996). Subsequently, TRAF6 interacts with a membrane bound pre-associated complex of TGF-β activated kinase (TAK1) and two TAK-1 binding proteins, termed TAB1 and TAB2 (Wang et al., 2001). A series of ubiquitinations occur on TRAF6 and TAK1. Phosphorylation activates TAK1, which in turn can phosphorylate the IKKs, subsequently leading to NF-κB activation (Karin and Ben-Neriah, 2000).

The MyD88-independent pathway signals through the adaptor protein TRIF. TLR3 exclusively uses TRIF (Yamamoto et al., 2002). On the other hand, TLR4 can also signal through the MyD88-independent pathway, but it requires both TRIF and TRAM (Fitzgerald et al., 2003a). TRIF binding to TLR3 recruits TRAF6 leading to TAK1 activation and subsequent NF-κB activation in an IRAK1 and IRAK4

independent manner (Sato et al., 2003). TRIF can also activate NF-κB in a TRAF6-independent way, through the association of a RHIM with RIP1 (Meylan et al., 2004).

The NF-κB activation associated with the MyD88-dependent pathway occurs approximately 30 min earlier than activation mediated by the MyD88-independent pathway (Covert et al., 2005). This is due the fact that the MyD88-independent pathway requires protein synthesis. In specific, the MyD88-independent pathway activates TNF-α production and secretion independent of NF-κB activation, mediated through the transcription factor IRF3. The secreted TNF-α subsequently binds to its receptors leading to activation of NF-κB (Covert et al., 2005).

The discovery of IκBζ revealed specificity in the regulation of different NF-κB subunits. In specific, IκBζ, induced by different TLR ligands, localises in the nucleus and it interacts with the p50 subunit. IκBζ overexpression results in increased IL-6 production in response to LPS, but it inhibits TNF-α production (Motoyama et al., 2005).

A breakthrough in the field of NF-κB has been the discovery that NF-κB activation follows an oscillatory pattern, which can be revealed using electrophoretic mobility shift assay (EMSA) (Hoffmann et al., 2002). This is based on the cycle of IκBα, which results in a strong negative feedback allowing turn-off of the NF-κB response. The expression of IκBα is linked to NF-κB since its activation induces the IκBα gene expression, which in turn binds to NF-κB, shutting down its

activation. On the contrary, the levels of IκBβ and IκBε remain low post-TLR stimulation, since their expression is not linked to NF-κB. Therefore, IκBβ and IκBε induce a monotonic increase in nuclear NF-κB and dampen the system's oscillatory potential, stabilizing NF-κB response during longer stimulations (Hoffmann et al., 2002). The number, period, and amplitude of oscillations determine the dynamics of gene expression (Nelson et al., 2004).

Although TNF-α and a wide variety of other stimuli induce oscillations in NF-κB activation dynamics, when cells were stimulated with LPS, a non-oscillatory pattern was observed (Covert et al., 2005). It was noticed that the NF-κB activation through TLR4 could be due to an interaction of the MyD88-dependent and TRIF-dependent pathway. Indeed, when LPS stimulated MyD88-deficient and TRIF-deficient fibroblasts an oscillatory pattern was revealed for both NF-κB and IκBα, whereas MyD88-TRIF-doubly deficient fibroblasts showed no NF-κB activation (Fig 1.9). In addition to, the model indicated that the MyD88-independent pathway requires a time delay of approximately 30min before NF-κB is activated, because it is dependent on the TNF-α synthesis through the activation of IRF3 (Covert et al., 2005).

Recently, however, another level of regulation of the inflammatory response has been discovered (Rao et al.). Although it has been known that the degradation of IκBs leads to the translocation of NF-κB to the nucleus and the initiation of the gene transcription, it has been reported that post-stimulation with LPS, IκBβ is re-synthesized in a hypophosphorylated form that can be detected in the nucleus (Rao et al.). This hypophosphorylated IκBβ can bind DNA with p65 and c-Rel,

forming complexes, which selectively bind to the TNF-α promoter, augmenting its transcription. Therefore, IκBβ leads to the prolonged expression of TNF-α. In concert with these results, IκBβ^{-/-} mice are resistant to LPS-induced septic shock (Rao et al.).
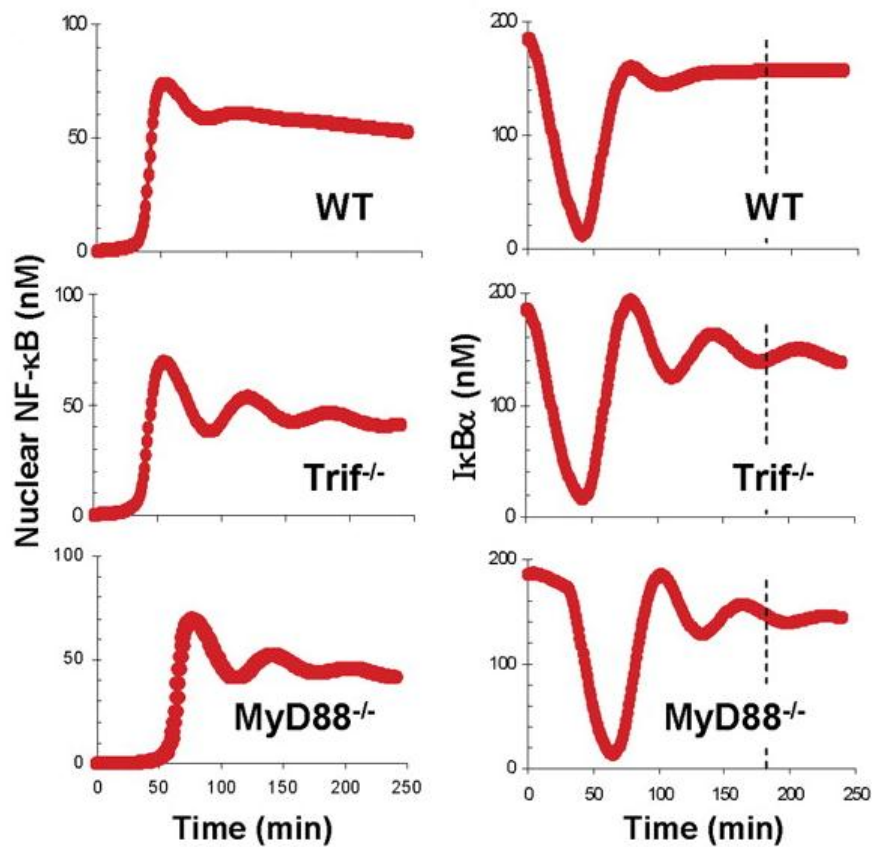
Figure 1.9: Modelling the activation of NF-κB.

The predicted time-courses of nuclear NF-κB activation and IκBα protein levels in LPS-stimulated WT, TRIF-deficient, and MyD88-deficient mouse embryo fibroblasts. Taken from (Covert et al., 2005).

1.2.5.5. Negative regulation of TLR signalling

When the inflammatory molecules induced by TLRs are produced in excess, they may cause serious systemic disorders. Therefore, mechanisms of control act to limit the exaggerated innate responses that may cause damage to the host.

Naturally expressed active soluble forms of TLRs capable of modulating cell activation have been discovered in human plasma, breast milk (LeBouder et al., 2003), and saliva (Zunt et al., 2009). These soluble TLRs provide the first line of regulation by functioning as decoy receptors (Liew et al., 2005).

Exposure of TLRs to a PAMP results in a severely reduced response to a subsequent challenge by the same PAMP. Therefore, when macrophages are treated with a sublethal dose of LPS, the cells become refractory to a subsequent exposure to LPS and this is a deactivation phenomenon, which in the case of LPS is known as endotoxin tolerance or LPS desensitization (Beeson, 1947b, Beeson, 1947a).

TLR signalling can be further controlled by intracellular regulators. Therefore, several molecules such as the short form of MyD88 (MyD88s), the kinase IRAK-M (Kobayashi et al., 2002), SOCS1 (Yasukawa et al., 2000), nucleotide-binding oligomerisation domain 2 (NOD2) (Pauleau and Murray, 2003), PI3K (Fukao and Koyasu, 2003), Toll-interacting protein (TOLLIP) (Didierlaurent et al., 2006), and A20 (Boone et al., 2004) negatively regulate TLR signalling. Transmembrane proteins such as single immunoglobin IL-1 receptor-related molecule (SIGIRR)

(Wald et al., 2003), T1/ST2 (Brint et al., 2004), and TRAILR (Diehl et al., 2004) can also downregulate TLR signalling.

Regulation may also occur through a reduction in the number of TLRs on the cell. This can take place by downregulation of the transcription and translation of TLR genes, or by degradation of TLR proteins (Liew et al., 2005). Such a mechanism is the ubiquitination–mediated degradation of TLRs (Chuang and Ulevitch, 2004). Certain anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) (McCartney-Francis et al., 2004) and IL-10 can also downregulate the function of TLRs (Muzio et al., 2000).

In addition, if all mechanism of negative regulation fail, apoptosis may occur ensuring that the hyper-responsive cells are eliminated (Aliprantis et al., 1999).

1.2.6. Cross-talk between TLRs

TLRs have a synergistic effect inducing their neighbour TLRs upon appropriate stimulation. Therefore, when macrophages were stimulated with TLR4 ligand LPS, their TLR9 expression increased and thus responded to CpG DNA more efficiently (An et al., 2002). Similarly, different combinations of TLR ligands have been used as stimulants in order to investigate the 'interaction' of TLRs in the induction of other inflammatory molecules. Thus, the TLR3 ligand poly (I:C) and the TLR9 ligand CpG-ODN demonstrated synergy in NO, IL-12, TNF-α, and IL-6 production after stimulation of murine macrophages (Whitmore et al., 2004). In addition, synergy between poly (I :C) and ODN was demonstrated *in vivo* for serum IL-6 and IL-12p40 levels (Whitmore et al., 2004). The differential effects of Gram (+) versus Gram (-) bacteria on the induction of nitric oxide synthase II (NOSII) and TNF-α were also investigated, proving a synergy between the two in NO production (Paul-Clark et al., 2006). A study performed by Bagchi et al using different TLR agonists to stimulate bone marrow-derived macrophages (BMDMs), showed that simultaneous activation of MyD88-dependent pathway and MyD88-independent pathway causes synergy whereas sequential activation causes priming (Bagchi et al., 2007). In addition, agonists that act through the same pathway induce tolerance (Bagchi et al., 2007). On top of this, MyD88-dependent and –independent agonists induced marked synergy in cytokine production *in vivo* (Bagchi et al., 2007).

1.2.7. TLR cooperation with other PRRs

Synergistic induction of cytokine production has also been observed in immune cells such as macrophages and dendritic cells when several TLR agonists were combined with ligands for other PRRs. Therefore, many TLR ligands synergise with NOD1 and NOD2 ligands for the induction of TNF and IL-12 p40 (Fritz et al., 2005, Tada et al., 2005, van Heel et al., 2005). In addition, several studies have reported a cooperation between TLRs and NALPs, NLR ICE-protease-activating factor (IPAF), receptors that recognise mannans and β-glycans such as dectin-1, and immunoreceptor tyrosine-based activation motif (ITAM) associated cell surface receptors such as TREM1 and TREM2 (Trinchieri and Sher, 2007). Moreover, a direct cooperation of TLR signalling and Notch signalling has been demonstrated, which results in different regulation of pro-inflammatory responses by macrophages (Palaga et al., 2008).

1.2.8. Toll like receptors and cell migration

The immune system relies on cell migration for the clearance of invading pathogens. There are two types of cell migration: inducible migration, which is the result of sensing of pathogens through PRRs such as TLRs, and homeostatic migration, which allows naive lymphocytes to circulate in lymphoid tissues.

Innate immunity relies on inducible migration. The recognition of PAMPs by TLRs initiates the immune response mediated by diffusible chemotactic factors and cell surface adhesion molecules, which recruits cells to infection sites. The activation of TLRs induces the expression of such molecules i.e. selectin, chemokines, and chemokine receptors. The procedure is initiated with the rolling of leukocytes on the vascular endothelial cells, a process mediated by selectins. Next, chemokines produced after the activation of TLRs bind to the luminal surface of the vascular epithelium inducing conformational changes to the integrins (Laudanna et al., 2002). This allows firm adhesion of the leukocytes to the vascular epithelium. Finally, the leukocytes migrate between the endothelial cells and extravasate into the tissue infection site.

TLR signaling may guide actin cytoskeleton rearrangements. It has been reported that LPS treatment may cause dendritic cells to strongly adhere, with some visible veils and actin cables, a process mediated by the scavenger receptor MARCO (Granucci et al., 2003). The polarization of dendritic cells after TLR activation may render the cells able to migrate. Their migration to lymph nodes and spleen is regulated by CCR7 expression, rendering the cells responsive to CCL19 and

CCL21 (Ohl et al., 2004). Another molecule that has been reported to regulate the dendritic cells migration has been the MHC II –associated invariant chain CD74 (Faure-Andre et al., 2008). TLR agonists may also regulate CCR expression in other cells types such as monocytic cells, changing the adhesive and migratory capacities of these cells (Nijhuis et al., 2007). In specific, stimulation of monocytic cells with TLR2 and TLR4 agonists resulted in a autocrine pathway of chemokine production and homologous down-regulation of the cognate receptors CCR1 and CCR2 (Parker et al., 2004). In neutrophils, stimulation with TLR agonists resulted in L-selectin shedding and chemokine expression, suggesting that TLR-stimulated neutrophils recruit, cells of the innate but not acquired immune system, to sites of inflammation (Hayashi et al., 2003). Therefore, after TLR stimulation, neutrophils expressed MIP-1α/CCL3, MIP-1β/CCL4 (active on monocytes/natural killer cells), IL-8/CXCL8, GRO-α/CXCL1 (active on neutrophils), and MIP-3α/CCL20 (active on immature dendritic cells) (Hayashi et al., 2003). In addition to, it has been reported that neutrophil migration may be regulated by the cross-talk of TLR2 with the cell surface protein CD47 (Chin et al., 2009). In macrophages, engagement of TLRs may increase cells motility via the iNOS/Src/FAK axis (Maa et al., 2011). The importance of CCR4 in leukocyte recruitment and lethality rate of mice after challenge with TLR ligands has also been addressed finding that there was a reduced lethality rate in response to TLR-induced endotoxaemia in the CCR4^{-/-} mice and this was associated with excessive early leukocyte migration (Ness et al., 2006).

1.2.9. TLRs and adaptive immunity

TLRs link innate and adaptive immunity (Iwasaki and Medzhitov, 2004). TLR-stimulated APCs activate T-cells together with antigen-presentation and promote the differentiation of naïve T-cells into T_H1 cells secreting IFN-γ, which mediates antiviral or antibacterial immunity or into T_H2 cells secreting IL-4 and IL-13, which are involved in allergic reactions and immunity against helminths (Abbas et al., 1996). Most TLR ligands support T_H1-skewed immune responses (Schnare et al., 2001) but this is not always the case (Eisenbarth et al., 2002).

Stimulation of TLRs on B cells can lead to polyclonal activation and production of low-affinity immunoglobin M (IgM) antibodies (Hayashi et al., 2005). B cell may respond to TLRs differently to ensure both self-tolerance and rapid response to reinfection (Iwasaki and Medzhitov, 2004).

Moreover, TLR3, TLR4, TLR7, and TLR9 may induce the production of type I IFN through different signalling pathways. Besides the role of interferons in antiviral activity, they have a critical involvement in controlling adaptive immune responses. Therefore, type I IFNs can promote memory T cell proliferation and prevent T cell apoptosis (Tough et al., 1999). In addition, type I IFN can induce IFN-γ secretion by $CD4^+$ T cells in humans (Sareneva et al., 1998). On top of this, IFN-α/β enables B cells to differentiate into plasma cells through the activation of dendritic cells (Le Bon et al., 2001).

1.2.10. TLRs and allergy

The hygiene hypothesis suggests that the increased incidence of allergy observed in western societies results from a decrease of infectious diseases in the early life of individuals (Strachan, 1989). According to this, the early exposure to bacterial or viral infections can lead to a T_H1 deviation of the immune status and a decrease of T_H2 cytokines associated with allergic conditions. Specifically, TLR9 signalling activated by CpG DNA is the strongest inducer of T_H1 differentiation (Kim et al., 1999). A number of studies have reported successful effects of CpG DNA to prevent murine and primate models of allergen-induced airway hyperresponsiveness (Broide et al., 1998, Fanucchi et al., 2004). However, contrary to the hygiene hypothesis, certain infections such as influenza virus infection can exacerbate allergic episodes (Dahl et al., 2004). Moreover, parasitic infections that provoke robust T_H2 cellular responses are associated with protection from the development of atopy (Lynch et al., 1993). On top of this, MyD88 null mice spontaneously developed higher levels of serum IgE than wild-type (WT) mice (Schnare et al., 2001).

1.2.11. TLRs and autoimmune disorders

TLR signalling appears to be critically involved the pathogenesis of autoimmune disorders when on a susceptible genetic background. As described previously, nucleic acid TLR ligands are recognised in different cellular departments from lipid and protein TLR ligands. This can protect the host from potential autoimmune reactions. However, in certain conditions such as deficient clearance of apoptotic cells, host-derived nucleic acids may trigger TLRs and lead to autoimmunity (LeBouder et al., 2003). Therefore, several pathogen-derived antigens, antigens from commensals, and endogenous self-antigens have the potential to induce TLR signalling (Ehlers and Ravetch, 2007).

The "Hydrophobicity Hypothesis' may represent a way of understanding the possible involvement of TLRs in the pathogenesis of autoimmunity (Seong and Matzinger, 2004). According to this, many of the immune system receptors have evolved to recognize and react to the hydrophobic portions (hyppos) of molecules when they become exposed. Therefore, a hyppos receptor normally binds to endogenous physiologically exposed hyppos but in certain cases can also bind to hyppos that are exposed because of injury, initiating inflammation.

Another form of self-reactivity is mimicry, where cells of the adaptive immune system cross-react with self-antigens (Fujinami and Oldstone, 1985). In this case, an acute or chronic infection might be associated directly with the onset of autoimmunity. During central B-cell and T-cell development in the bone marrow and thymus, respectively, a high percentage of self-reactive cells are generated, a

mechanism which is controlled by an initial checkpoint for tolerance. However, TLR signalling in different types of immune cells has been implicated in the breakdown of tolerance (Marshak-Rothstein, 2006).

In addition, nucleic acids TLR ligands, in contrast to protein or lipid TLR ligands, induce type I IFNs (Honda et al., 2005, Schoenemeyer et al., 2005, Yamamoto et al., 2003). LPS acting through TLR4 can induce IFN-β but not IFN-α (Toshchakov et al., 2002). TLR activation leading to type I IFNs may contribute to the pathogenesis of autoimmune disorders such as systemic lupus erythematosus (SLE) (Sibbitt et al., 1985). Other example is the activation of B cells to produce Rheumatoid factor (RF) by stimulation with CpG DNA (Viglianti et al., 2003).

1.3. PATHOPHYSIOLOGY OF SEPSIS

Although clinical manifestations of sepsis were known to Hippocrates and Avicenna, it was not until 1914 that Hugo Schottmuller realised that infection is a fundamental component of the disease. Sepsis can now be defined as a systemic inflammatory response syndrome (SIRS) that can occur during infection (Bone et al., 1992) and can be caused by Gram (-), Gram (+) bacteria, fungi and viruses. The incidence of sepsis is rising and the mortality reaches 25-30% in patients with severe sepsis and 50-60% in those who develop septic shock (Martin et al., 2003).

During sepsis, there is a release of high levels of PAMPs from invading pathogens and/or damaged host tissue. Therefore, the first stage of sepsis is a hyper-inflammatory state that is accompanied by an imbalanced cytokine response known as cytokine storm (Beutler et al., 1985, Fischer et al., 1992, Ohlsson et al., 1990, Tracey et al., 1987). For many years, it was believed that sepsis was the result of this overwhelming inflammatory reaction, which is characterised by cytokine-mediated pathology, coagulation and complement activation, which led to early mortality due to acute organ dysfunction. However, clinical trials with anti-inflammatory therapies failed to alter the outcome of patients with sepsis (Remick, 2003). Most current antisepsis therapies such as corticosteroids, drotrecogin-alfa activated, intensive insulin therapy, vasopressin, also have uncertain outcomes (Russell, 2006).

Although some patients die during the initial phase of hyper-inflammation, most succumb at later time points. It is now believed that sepsis involves both

exaggerated inflammation and immune suppression. During this immunosuppressive state neutrophils can undergo "immune paralysis" in which important intracellular pathways such as TLR signalling are shut-down (Marsik et al., 2003, Salomao et al., 2009, Salomao et al., 2008). In addition, there is a dysfunction of the adaptive immune system characterised by a diversion from an initial T_H1- to a T_H2-response (Song et al., 2000). Furthermore, increased apoptosis in lymphocytes and dendritic cells contributes to this immunosuppressive state (Lang and Matute-Bello, 2009).

Sepsis not only affects the immune system and the coagulation system but the autonomic nervous system (ANS) as well. More specifically, the initial inflammatory state activates afferent signals that are relayed to the *nucleus tractus solitarius* of the brain. This activates the vagus nerve and the cytokine release is inhibited via cholinergic receptors present on macrophages and other cells (the inflammatory reflex) (Borovikova et al., 2000, Tracey, 2002). It has been shown that electrical stimulation of the vagus nerve improves survival by decreasing the release of pro-inflammatory mediators in experimental sepsis (Huston et al., 2007).
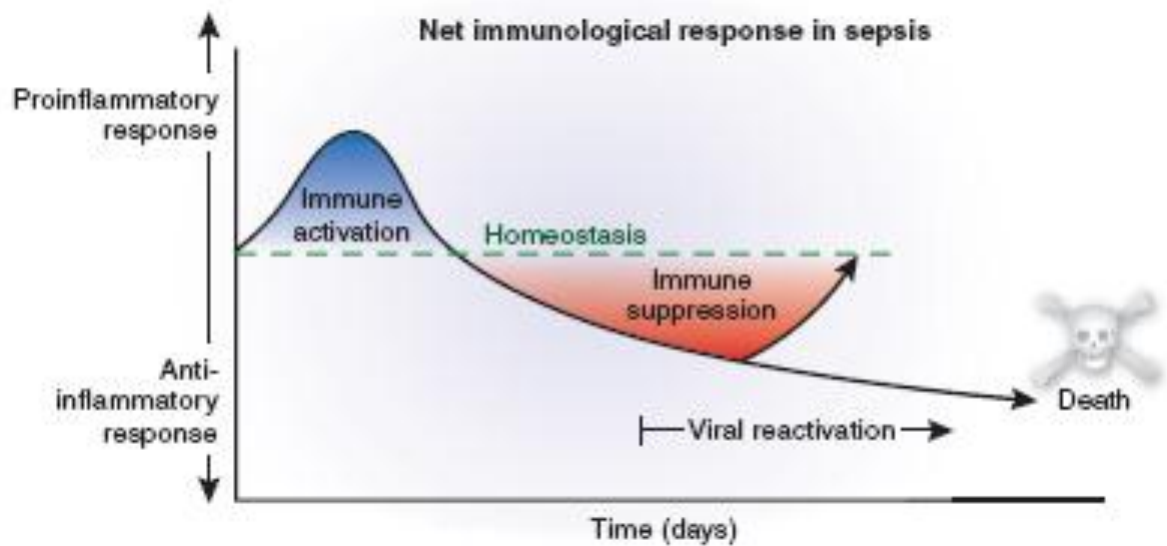
Figure 1.10: Net Immunological response in sepsis over time.

Early in sepsis the proinflammatory response predominates and deaths are due to cytokine storm-mediated events. As sepsis progresses, the anti-inflammatory response becomes predominant, secondary infections and viral reactivation may occur, and deaths are due to failure to control pathogens. Taken from (Hotchkiss et al., 2009).

1.3.1. Factors implicated in the pathogenesis of sepsis

1.3.1.1. Microbial pathogenesis

Gram (+) bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* are the most predominant microorganisms causing sepsis, whereas the most common Gram (-) bacteria are *Escherichia coli*, *Klebsiella spp*, and *Pseudomonas aeruginosa* (Opal et al., 2003). Several virulence characteristics contribute to the pathogenesis of sepsis, including virulence genes scattered across the bacterial genome (Merrell and Falkow, 2004), genomic islands (Hacker and Kaper, 2000), bacterial toxins (Schiavo and van der Goot, 2001), and quorum sensing (the ability of bacteria to assess their population density) (Bassler, 2002).

1.3.1.2. Host-pathogen interactions

1.3.1.2.1. The Coagulation system

The coagulation system is activated in patients with sepsis and it has been suggested that sepsis-related disseminated intravascular coagulation (DIC) is associated with organ failure and high mortality. In severe sepsis, activated monocytes, endothelial cells, and circulating microvesicles are sources of Tissue Factor- the primary initiator of coagulation in sepsis (Aras et al., 2004). Blood clotting is controlled by Tissue-factor-pathway inhibitor (TFPI), Antithrombin, Activated Protein C, and by the fibrinolytic system. During severe sepsis all these factors are impaired, resulting in a net procoagulant state. Although the administration of each of these factors was tested in clinical trials having as a

result the attenuation of coagulopathy (Abraham et al., 2005, Abraham et al., 2003, Warren et al., 2001), only Activated protein C was efficient in reducing mortality in patients with severe sepsis (Bernard et al., 2001).

1.3.1.2.2. The complement system

Complement may be activated by 3 pathways: classic, alternative, and lectin-binding pathway. Increased plasma concentrations of complement anaphylatoxins $C3\alpha$, $C4\alpha$, and $C5\alpha$ were found in clinical and experimental sepsis (Czermak et al., 1999, Ward, 2004). $C5\alpha$ is a central mediator at sepsis because it modulates other systems such as the coagulation cascade and TLR4-mediated responses such as the release of macrophage migration inhibitory factor (MIF) (Riedemann et al., 2004) and high mobility group box 1 protein (HMGB1) (Rittirsch et al., 2008). Anti-$C5\alpha$ antibodies reduced sepsis mortality caused by *E.Coli* (Stevens et al., 1986) and caecal ligation and puncture (CLP) (Czermak et al., 1999).

1.3.1.2.3. HMGB1

HMGB1 is a nuclear protein present in almost all eukaryotic cells, which is released by necrotic cells and from macrophages after activation by infectious agents (Lotze and Tracey, 2005), and its function is to stabilise nucleosome formation (Lichota and Grasser, 2003). HMGB1 is a late-acting proinflammatory cytokine in the pathogenesis of sepsis (Wang et al., 1999, Yang et al., 2004) and can be detected in high concentrations is sepsis patients (Sunden-Cullberg et al., 2005). Extracellular HMGB1 may interact with PRRs such as the receptor for

advanced glycation end-products (RAGE), TLR2 and TLR4 (Park et al., 2004). Interaction between C5α and its other receptor C5α-like receptor 2 (C5L2) triggers the release of HMGB1 in sepsis (Rittirsch et al., 2008), whereas activation of the cholinergic pathway suppresses its secretion by macrophages improving survival (Wang et al., 2004). Anti-HMGB1 antibodies improved survival in several experimental models of sepsis (Wang et al., 1999, Yang et al., 2004).

1.3.1.2.4. MIF

MIF is highly expressed by endocrine tissues and organs involved in stress such as hypothalamus, pituitary gland and adrenal glands. It is also produced by cells of the immune system such as macrophages, dendritic cells, neutrophils and T-cells (Calandra et al., 2003). Therefore, it is believed that MIF links the immune system with the endocrine system.

MIF is constitutively expressed by leukocytes and it is stored intracellularly (Calandra and Roger, 2003). When MIF is released, it acts as a classic pro-inflammatory cytokine by inducing phosphorylation and activation of ERK1/ERK2/MAPK pathway (Lue et al., 2006) and this is associated with increased PLA$_2$ activity (Sampey et al., 2001). MIF promotes the expression of cytokines such as TNF-α, IL-6 and molecules such as NO (Bernhagen et al., 1993) by increasing the expression of Cyclooxygegase-2 (COX-2) (Calandra and Roger, 2003, Sampey et al., 2001).

MIF secretion is induced by GCs and it acts as a physiological antagonist of GC activity (Calandra and Bucala, 1995). MIF interferes with GCs at a transcriptional and post-transcriptional level, overrides the immunosuppressive effects of GCs, and reverses GC-induced inhibition of TNF, IL-1, IL-6, IL-8, PLA_2 activity and arachidonic acid release and proliferation of T-cells (Mitchell et al., 1999). It is believed that MIF and GCs function together to modulate innate and acquired immune response.

Increased tissue and circulating levels of MIF have been observed in mice with sepsis (Calandra et al., 2000) whereas the administration of neutralising Abs for MIF reduced TNF-α and protected mice from lethal endotoxic shock or sepsis by *E.Coli* or CLP. Increased levels of MIF were detected in blood of patients with severe sepsis or sepsis shock, whereas neutralization of MIF improved survival (Calandra et al., 2000).

MIF is released by cells of the anterior pituitary gland after exposure to the endotoxin LPS (Bernhagen et al., 1993). MIF^{-/-} macrophages are hyporesponsive to LPS and Gram (-) bacteria and produce reduced levels of TNF-α and IL-6 (Roger et al., 2001), whereas MIF^{-/-} mice are resistant to lethal endotoxaemia (Roger et al., 2003). MIF upregulates TLR4 expression and therefore enables macrophages to respond rapidly to invasive bacteria. Therefore MIF^{-/-} macrophages have reduced levels of TLR4 mRNA and protein (Roger et al., 2001). MIF has also been implicated in the pathogenesis of Gram (+) sepsis and parasitic and viral infections (Assuncao-Miranda et al., 2010, Hou et al., 2009).

1.3.1.2.5. IL-17A

IL-17A is a proinflammatory cytokine mainly produced by T_H17 cells (Bettelli et al., 2006). It mediates the production of many other cytokines such as IL-1β, IL-6, and TNF-α. It has been shown that increased IL-17A levels are associated with adverse effects during experimental sepsis (Flierl et al., 2008) and that neutralization of IL-17A may improve survival (Flierl et al., 2008). In addition, the inflammatory response of macrophages to LPS was increased in the presence of recombinant IL-17A (Flierl et al., 2008).

1.3.2. Involvement of TLRs in sepsis pathogenesis

Epidemiological studies suggest a strong genetic component in the outcome of sepsis (Holmes et al., 2003). Several single-nucleotide polymorphisms (SNPs) in TLR genes have been associated with susceptibility to infectious diseases and the progression of sepsis in humans. The most extensively studied polymorphism is the Asp229Gly mutation of the TLR4 gene. Some groups have associated TLR4 polymorphisms with hyporesponsiveness to endotoxin (Arbour et al., 2000, Michel, 2003). Other studies, however, have contradictory results (Erridge et al., 2003, Imahara et al., 2005) and suggest that the relatively high frequency of this mutation in the Caucasian population may reflect modified response of carriers to alternative TLR4 agonists. The Asp229Gly polymorphism has been associated with lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules in humans (Kiechl et al., 2002). Although an association of this polymorphism with the risk of several bacterial infections especially Gram (-) has been shown (Agnese et al., 2002, Kiechl et al., 2002, Lorenz et al., 2002), no correlation has been demonstrated with the incidence or mortality of post-operative sepsis caused by polymicrobial infection (Feterowski et al., 2003). Rare TLR4 mutations have also been identified that have been shown to influence meningococcal infections (Smirnova et al., 2003). Other TLR polymorphisms that have been studied include the mutation Arg753Gln of TLR2 gene which has been associated with staphylococcal infections (Lorenz et al., 2000), tuberculosis (Ogus et al., 2004), Lyme disease (Schroder et al., 2005), and a common stop codon polymorphism of TLR5 gene which has been associated with susceptibility to pneumonia caused by Legionella (Hawn et al., 2003). In addition, a study

associated hypermorphic genetic variation in TLR1 with organ dysfunction, death, and Gram (+) infection in sepsis (Wurfel et al., 2008).

LPS from Gram (-) bacteria is believed to be an important trigger for the pathophysiology of sepsis. Several mediators of sepsis interact with TLR4. The complement anaphylatoxin C5a, which is produced in large amounts during the early stages of sepsis, negatively regulates TLR4-mediated responses (Hawlisch et al., 2005). Plasma levels of MIF, a cytokine that correlates with sepsis severity (Calandra et al., 2000), upregulate TLR4 by phagocytes (Calandra and Roger, 2003). HMGB1 is a late-acting proinflammatory cytokine in the pathogenesis of sepsis and it interacts with PRRs, including TLR2 and TLR4 (Park et al., 2004). The proinflammatory cytokine IL-17A has been correlated with adverse effects during experimental sepsis and the *in vitro* production of pro-inflammatory mediators in response to LPS was increased in the presence of recombinant IL-17A (Flierl et al., 2008). In addition, the activation of TLR4 in platelets initiates the formation of neutrophil extracellular traps (NETS) to esnare bacteria in the vasculature (Clark et al., 2007). Antibody-mediated blockade of TLR4 protected against polymicrobial sepsis (Daubeuf et al., 2007) and improved survival in experimental models of Gram (-) bacterial sepsis when administered both prophylactically and therapeutically (Roger et al., 2009). However, in human sepsis, clinical trials in which TLR4 was blocked did not show beneficial effects (van der Poll and Opal, 2008).

Besides the involvement of TLR4 in sepsis pathogenesis, it has been reported that the mRNA levels and surface expression of TLR2 in monocytes and neutrophils

were upregulated in septic patients when compared to healthy individuals (Armstrong et al., 2004, Harter et al., 2004, Schaaf et al., 2009, Tsujimoto et al., 2006). On the contrary, downregulation of TLR2 has been associated with death in septic patients (Schaaf et al., 2009), suggesting that TLR2 expression on monocytes could be a valuable sepsis marker (Viemann et al., 2005). Furthermore, the expression of TLR2 in macrophages has been upregulated in mice with experimental peritonitis induced by CLP (Tsujimoto et al., 2005, Williams et al., 2003), whereas TLR2^{-/-} mice displayed a survival advantage in Gram (-) sepsis caused by melioidosis (Wiersinga et al., 2007). Bacterial lipoprotein (BLP), which exerts its primary effects primarily through TLR2, delayed polymorphonuclear apoptosis arising during sepsis (Power et al., 2004). Moreover, a neutralizing anti-TLR2 antibody inhibited the release of TNFα and prevented lethal shock-like syndrome in mice challenged upon lipopeptide (Meng et al., 2004). However, the *in vitro* interaction of HMGB1 with TLR2 (Park et al., 2004) could not be confirmed *in vivo* (van Zoelen et al., 2009). In addition, in polymicrobial infection models such as the CASP model of septic peritonitis, it was shown that the survival rates of mice with single or combined deficiency of TLR2 and TLR4 was comparable to those of WT mice (Weighardt et al., 2002). On the contrary, MyD88^{-/-} mice exhibited improved survival suggesting that ablation of MyD88 may protect mice from the deleterious effects of polymicrobial sepsis (Weighardt et al., 2002). In addition, TLR3 has been suggested to be involved in viral sepsis. Indeed, studies have shown that TLR3^{-/-} mice are protected against the neurological implications of West Nile virus (Wang et al., 2004) and anti-TLR3 antibodies attenuate tissue necrosis and decrease sepsis-induced mortality (Cavassani et al., 2008).

Macrophages produce IL-10 in response to MyD88-dependent and TRIF-dependent TLR signals (Boonstra et al., 2006). When septic patients were challenged with TLR ligands such as LPS and Pam3CysK4, there was a reduction of TNF-α and an enhancement of IL-10 (Adib-Conquy et al., 2006). It has been recently proven that sepsis can be attenuated by bone marrow stromal cells via reprogramming of host macrophages to increase their IL-10 production (Nemeth et al., 2009), which inhibits macrophage microbicidal activity by blocking the endogenous production of TNF-α (Oswald et al., 1992).

Besides sepsis caused by infectious agents, injury may lead to the release of PAMPs into the circulation. In specific, trauma releases mitochondrial 'damage'-associated molecular patterns (DAMPs) including formyl peptides and mitochondrial DNA rich in CpG repeats. The latter is recognised by neutrophils through activation of TLR9, leading to inflammation and SIRS (Zhang et al.).

1.4. ANNEXINS

1.4.1. The Annexin superfamily

Annexins are a family of Ca^{2+} and phospholipid binding proteins which are evolutionary conserved and are expressed throughout the animal and plant kingdom (Gerke and Moss, 2002). More than 500 different gene products are present in most species (Morgan and Fernandez, 1997). In vertebrates 13 annexin subfamilies (A1-A13) have been identified (Rescher and Gerke, 2004). The name annexin is derived from the Greek word *annex* meaning hold together since nearly all annexins are characterised by the binding of membranes. By definition, a member of the annexin family must have the ability to bind in a Ca^{2+}-dependent manner to negatively charged phospholipids and to contain a structurally conserved segment called the annexin repeat.

Each annexin is composed of two domains: a divergent NH_2-terminal part and a conserved COOH-terminal protein core, which contains the Ca^{2+} and membrane binding sites (Raynal and Pollard, 1994). An annexin core generally comprises 4 segments of internal homology forming a highly α–helical disk (Raynal and Pollard, 1994). Ca^{2+} and possibly membrane binding can trigger exposure of the NH_2-terminal, which mediates interactions with protein ligands (Rosengarth and Luecke, 2003).

The binding of annexins to membranes is reversible, since removal of Ca^{2+} leads to the liberation of annexins from the phospholipids matrix (Raynal and Pollard,

1994). Therefore, certain annexins can mediate membrane vesicle aggregation (Raynal and Pollard, 1994), are implicated in exocytosis (Chasserot-Golaz et al., 2005, McArthur et al., 2009, Naidu et al., 2005) and endocytosis (Grewal et al., 2000, Jost et al., 1997, Morel and Gruenberg, 2009), and they can stabilise domains of the plasma membrane and organelle membranes (Hu et al., 2008a, Menke et al., 2005).

Besides lipid ligands, annexins have the ability to form complexes with EF hand-type Ca^{2+} binding proteins such as the S100 subfamily (Mailliard et al., 1996, Streicher et al., 2009), cytoskeletal proteins such as F-actin (Filipenko and Waisman, 2001, Locate et al., 2008) and profilin (Alvarez-Martinez et al., 1996, Alvarez-Martinez et al., 1997), macromolecules such as glycosaminoglycans (Ishitsuka et al., 1998) and a number of other ligands ranging from proteins to RNA (Filipenko et al., 2004, Hirata and Hirata, 1999).

Although annexins lack signal sequences guiding them to the canonical secretory pathway, they possess extracellular properties leading to responses such as the anti-inflammatory action of Annexin A1 (Perretti and Gavins, 2003) and the anti-thrombogenic action of Annexin A2 (Brownstein et al., 2004, Kwon et al., 2005, Ling et al., 2004, Zhu et al.). To date, no human diseases have been described which could be attributed solely to an annexin gene but still changes in expression, properties or localization of annexins may contribute to the pathophysiology of several diseases. The term 'annexinopathies' applies to such phenotypes, characterised by dysregulation of the normal antithrombotic properties of some extracellular annexins (Rand, 1999). Therefore, annexins have been associated

with diseases ranging from cardiovascular disorders (Cederholm and Frostegard, 2005, Gavins et al., 2006), cancer (Nair et al., Sharma et al., Song et al., 2009, Torosyan et al., 2009, Yan et al.) and diabetes (Lindgren et al., 2001). Although the likelihood of functional redundancy between annexins exists, it is a fact that changes in the expression of one annexin can affect the expression levels of another (Roviezzo et al., 2002).

1.4.2. The multifunctional role of Annexin A1

AnnexinA1 (AnxA1), originally known as lipocortin-1, is an endogenous 37kDa Ca^{2+} and phospholipid binding protein. The gene encoding the protein can be located on chromosome 19q24. AnxA1 contains a core with four 70-amino acid domains, where the binding sides for Ca^{2+} and phospholipids lie, and the 49 amino acid N-terminus of the protein, which contains canonical phosphorylation and proteolysis sites (Raynal and Pollard, 1994) and is believed to be responsible for its biological activity (Cirino et al., 1993). In the presence of $Ca^{2+} \geq 1$ mM the N-terminal domain which is normally buried in the core domain, is expelled revealing the active form of the protein (Rosengarth and Luecke, 2003).
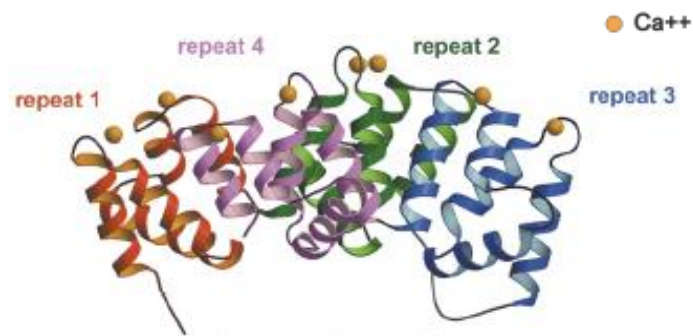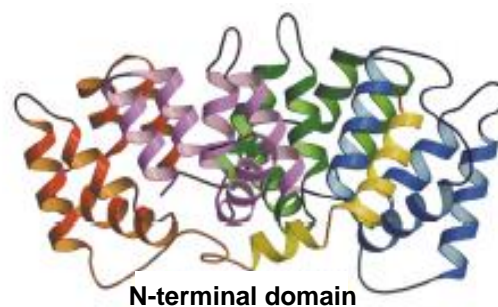
Figure 1.11: AnxA1 structure.
A. Three-dimensional structure of full-length AnxA1 in the presence of Ca^{2+} ions.
B. Full-length AnxA1 in the absence of Ca^{2+} ions. Adapted from (Rosengarth and Luecke, 2003).

Although AnxA1 is undetectable in plasma, it is found in many cells and tissues. AnxA1 is detected in all leukocyte subsets with the exception of B lymphocytes (Morand et al., 1995), whereas it is found in gelatinase granules in neutrophils (Perretti et al., 2000). Generally, differentiated cells tend to synthesise higher amounts of AnxA1, such as human alveolar macrophages when compared to their monocyte precursors (Ambrose et al., 1992).

After cell activation as GC treatment, AnxA1 is mobilised to the cell membrane, where it is secreted (Perretti et al., 1996b) and it binds to its receptor. The receptor for AnxA1 in humans is a G-protein-coupled receptor (GPCR) named formyl peptide receptor 2 (FPR2), formerly known as FPRL1, which is also the receptor for the anti-inflammatory molecule lipoxin A4 (Perretti et al., 2001). Peptides derived from the N-terminal domain of AnxA1 activate in vitro all receptors of the FPR family (FPR1, FPR2 and FPR3) (Dahlgren et al., 2000). Human FPRL1 has a structural homology with FPR2 in the mouse (Panaro et al., 2006, Selvatici et al., 2006) and therefore $Fpr2^{-/-}$ mice have been used to investigate the effect of AnxA1 in the absence of FPRs.

It has been reported that changes in the concentration of AnxA1 in human peripheral blood leukocytes can occur after exogenous administration of hydrocortisone (Goulding et al., 1990) and that there is a correlation between AnxA1 expression in these cells with the levels of serum cortisol (Mulla et al., 2005). Injection of hydrocortisone or dexamethasone causes an increase of AnxA1 in rat peritoneal leukocytes (Peers et al., 1993). In addition, treatment with dexamethasone may lead to an upregulation of the AnxA1 receptor in leukocytes

(Sawmynaden and Perretti, 2006). GCs promote the translocation of AnxA1 from intracellular to pericellular sites (Philip et al., 1997, Solito et al., 1994). Furthermore, AnxA1^{-/-} mice exhibit resistance to GCs (Hannon et al., 2003). It is now long established that AnxA1 is a downstream mediator of GCs, since the discovery of its involvement in the inhibition of the enzyme PLA_2 activity and subsequent inhibition of eicosanoid synthesis induced by GCs (Cirino et al., 1987); a mechanism through a 'specific interaction' with PLA_2 (Kim et al., 1994, Kim et al., 2001). However, the mechanism by which GCs regulate AnxA1 expression has not yet been fully elucidated since the AnxA1 promoter does not contain a complete consensus of glucocorticoid-response elements, suggesting that it is mediated through an indirect effect.

Besides the effect of AnxA1 on PLA_2 activity, administration of dexamethasone in LPS-treated rats reduced the expression of iNOS in the lung, an effect that was prevented by pretreatment with a neutralizing antiserum to AnxA1 (Wu et al., 1995). In the same study, an AnxA1 fragment blocked iNOS in macrophages stimulated with LPS, indicating that the extracellular release of AnxA1 mediates the effect of GCs on the expression of iNOS (Wu et al., 1995). A study using microglial cells demonstrated that the N-terminus peptide of AnxA1 Ac2-26 inhibits the LPS-induced expression of both iNOS and COX-2 (Minghetti et al., 1999). Another study confirmed the down-regulation of iNOS by Ac2-26 in a macrophage cell line and supported the notion that the anti-inflammatory effects of AnxA1 may be mediated by the release of the cytokine IL-10 (Ferlazzo et al., 2003). Indeed, IL-10 is an anti-inflammatory cytokine, which can downregulate the expression of both iNOS (Cunha et al., 1992) and COX-2 (Berg et al., 2001). Several TLR

ligands may upregulate IL-10 production in macrophages by activating the ERK pathway (Qian et al., 2006), which is also activated by endogenous AnxA1 (Alldridge et al., 1999).

It is now well established that AnxA1 has antimigratory effects on neutrophils and monocytes (Cirino et al., 1993, Lim et al., 1998, Perretti et al., 1996b, Perretti and Flower, 1993, Walther et al., 2000, Zouki et al., 2000) and anti-inflammatory effects when tested in several models of inflammation such as the air-pouch model (Cirino et al., 1993, Hayhoe et al., 2006, Perretti et al., 1996a, Perretti and Flower, 1993, Perretti and Flower, 1994) and the carrageenan paw oedema (Cirino et al., 1993, Cirino et al., 1989, Duncan et al., 1993).

AnxA1 has also been implicated in the adaptive immune response by acting as a molecular 'tuner' of TCR signalling. In specific, stimulation of naïve T-cells with hrAnxA1 and suboptimal doses of anti-CD3/CD28 increased cell activation and proliferation by affecting the transcription factors NF-κB, nuclear factor for activated T cells (NFAT) and AP-1 (D'Acquisto et al., 2007a). Stimulation of T cells via the TCR lead to a secretion of endogenous AnxA1 and externalisation of its receptor ALXR (D'Acquisto et al., 2007a). In addition, differentiation of naïve T cells in the presence of AnxA1 increased skewing to T_H1 cells (D'Acquisto et al., 2007a). Finally, patients with active rheumatoid arthritis, in which T cells are the dominant cell type in synovial infiltrate, had higher levels of AnxA1 in their blood CD4+ cells (D'Acquisto et al., 2007a).

Besides its role in inflammation, AnxA1 has been reported as a regulator of cell proliferation (Alldridge et al., 1999), and apoptosis (McKanna, 1995, Sakamoto et al., 1996). It has also been implicated in the apoptotic cell 'eat me' signal (Arur et al., 2003). Furthermore, *Mycobacterium tuberculosis* may block crosslinking of AnxA1 and therefore the apoptotic envelope formation on infected macrophages, leading to death by necrosis and thus contributing to virulence (Gan et al., 2008).
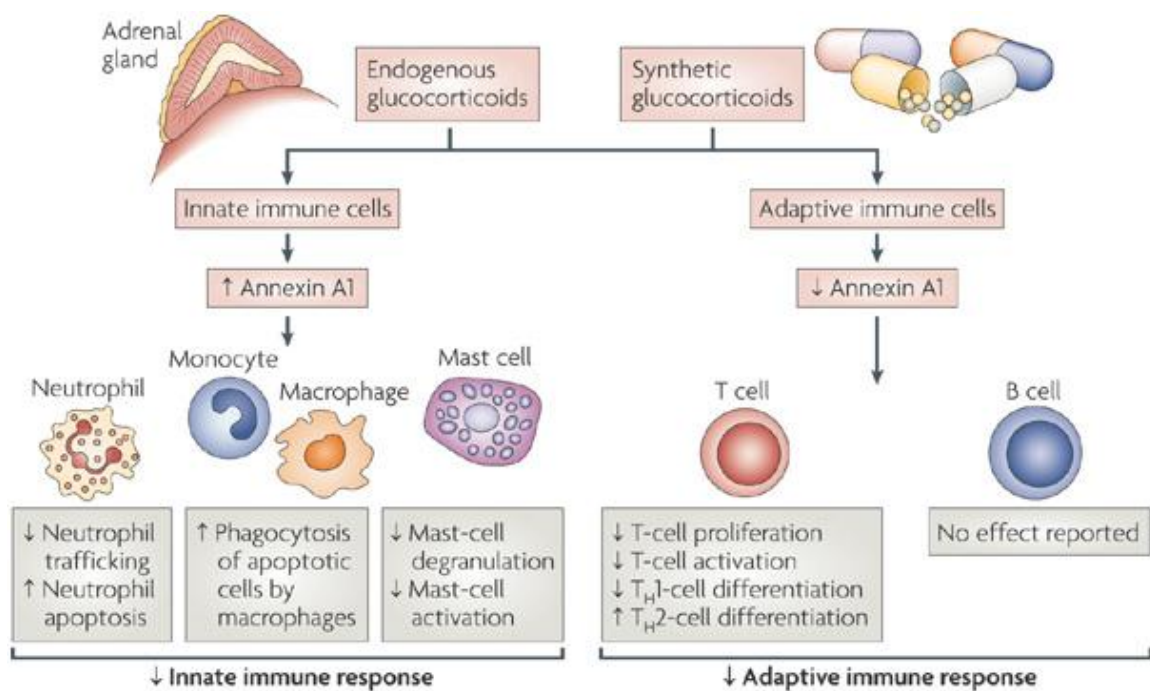
Figure 1.12: Model of glucocorticoid modulation of the AnxA1 pathway in immune regulation.
It has been proposed that endogenous and synthetic glucocorticoids can control innate and adaptive immune responses by modulating the expression and release of AnxA1. Exposure of innate immune cells, such as neutrophils, monocytes, macrophages and mast cells, to glucocorticoids induces the release of AnxA1. By contrast, exposure of T-cells to glucocorticoids leads to a reduction of AnxA1 expression. Taken from (Perretti and D'Acquisto, 2009).

1.4.3. AnxA1 and the macrophage

AnxA1 mRNA and protein are constitutively expressed in many different tissue specific macrophages i.e. peritoneal, alveolar, synovial, and microglial cells (Ambrose et al., 1992). The expression of the protein in macrophages increases following exposure to GCs (Ambrose et al., 1992, Cirino et al., 1993, De Caterina et al., 1993) depending upon the cell differentiation status (Ambrose et al., 1992). Cytokines such as IL-6 can increase cellular and tissue AnxA1 expression (Solito et al., 1998). In macrophages, the ATP-binding cassette (ABC) transporter system is responsible for the secretion of the protein (Wein et al., 2004). In these cells, AnxA1 inhibits cell trafficking and trans-endothelial migration (Perretti et al., 2002). Several inflammatory mediators produced by macrophages during the inflammatory response such as TNF-α (Sudlow et al., 1996), PGE$_2$ (Sudlow et al., 1996) and NO (Yang et al., 1998) can be inhibited by GCs in an AnxA1-dependent manner. It has been suggested that the inhibition of NO release and the expression of iNOS in the macrophages are associated with the increase of IL-10 and the decrease in IL-12mRNA (Ferlazzo et al., 2003). Moreover, an impaired phagocytic mechanism has been reported in AnxA1 null macrophages (Yona et al., 2006).

Interestingly, it has been shown that LPS increases FPR1 mRNA levels in macrophages by both enhanced transcription and stabilization of the FPR1 mRNA (Mandal and Hamilton, 2007, Mandal et al., 2005). This begs the question of why an agonist acting through a different receptor modulates the mRNA levels of a receptor belonging to the formyl receptor family. Furthermore, ligands that stimulate macrophages through TLR2 and TLR3 were also capable of inducing

FPR1 expression, implying that FPR1 modulation probably does not correlate with either the MyD88-dependent or MyD88-independent pathways (Mandal and Hamilton, 2007). Genes encoding formyl peptide receptors were also upregulated by LPS in murine microglial cells (Cui et al., 2002). In addition to, it has been reported that the mouse FPR2 is upregulated by TLR2 ligands in synergy with the intracellular receptor NOD2 (Chen et al., 2008) or by the TLR3 ligand poly (I:C) in synergy with the TLR7 ligand R837 (Chen et al., 2009b) in microglial cells.

1.4.4. AnxA1$^{-/-}$ mice present increased lethality to administration of LPS

A study performed by our group has demonstrated that administration of the TLR4 agonist LPS to AnxA1-deficient mice, produced a toxic response characterised by lethality not seen in WT mice, a phenotype which was partially rescued by exogenous administration of the human recombinant protein (Damazo et al., 2005). Which is the cause of death is still not understood. It was associated with an increase in the levels of IL-1, IL-6, and TNF-α in the plasma indicating that AnxA1 has a protective role in the endotoxemic murine microcirculation. In addition, LPS activated the AnxA1 gene in macrophages, a cell type with a critical role in the pathophysiology of sepsis since it initiates the immune response. Analysis of AnxA1-deficient macrophages has shown an increased response to LPS stimulation and an aberrant expression of TLR4. However, the mechanism by which AnxA1 exerts a protective role in endotoxemia and the role of AnxA1 in different types of infections (caused by Gram (-), Gram (+) bacteria and by viruses) remains unknown.
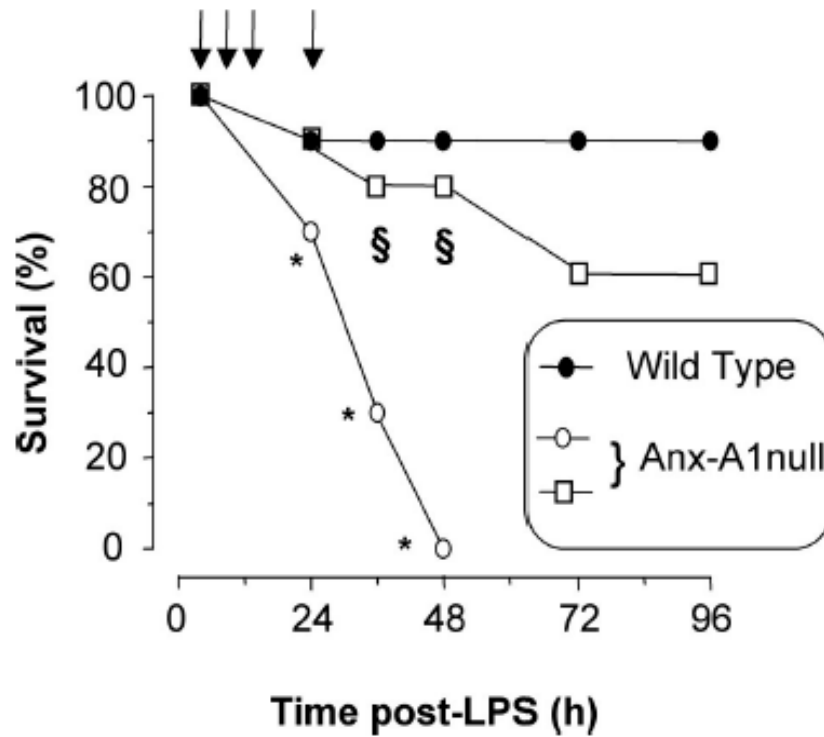
Figure 1.13: Modulation of mouse survival by AnxA1.

WT and AnxA1 null mice received 10mg/kg LPS intraperitoneally at time 0. A group of AnxA1 null mice was rescued by receiving 10ng of hr-AnxA1 at time 0, 4, 8, and 24h (arrows) after LPS administration. * P≤0.05 compare with WT mice. § P≤0.05 compared with untreated control mice. Taken from (Damazo et al., 2005).

1.5 HYPOTHESIS

AnxA1 is an endogenous homeostatic/protective mediator that is implicated in the resolution of several types of infection.

1.6 AIMS

- To compare the response of AnxA1$^{-/-}$ to AnxA1$^{+/+}$ macrophages after stimulation with different TLR ligands.

- To investigate whether stimulation of macrophages with different TLR ligands can modulate the AnxA1/FPR system.

- To analyse the signalling molecules in TLR pathways at which AnxA1 could exert an effect.

- To study the response of WT macrophages stimulated with different TLR ligands after treatment with hr-AnxA1.

- To test the effect *in vivo* of administrating different TLR ligands to AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice.

Previous research by our group clearly indicated that AnxA1 blocks the TLR4 signalling pathway with an as yet unidentified mechanism (Damazo et al., 2005). Since TLR4 signalling includes both the MyD88-dependent and the TRIF-dependent pathway (Fig 1.14), several approaches have been used to investigate the exact 'checkpoint(s)' on the TLR4 pathway at which AnxA1 could exert an effect (Fig 1.15). In addition, we extended our investigation in different types of inflammation caused by other TLR

ligands. In order to achieve these aims a panel of different TLR agonists has been used. Different adaptor protein(s) are recruited to each TLR after stimulation (Table 1.1).
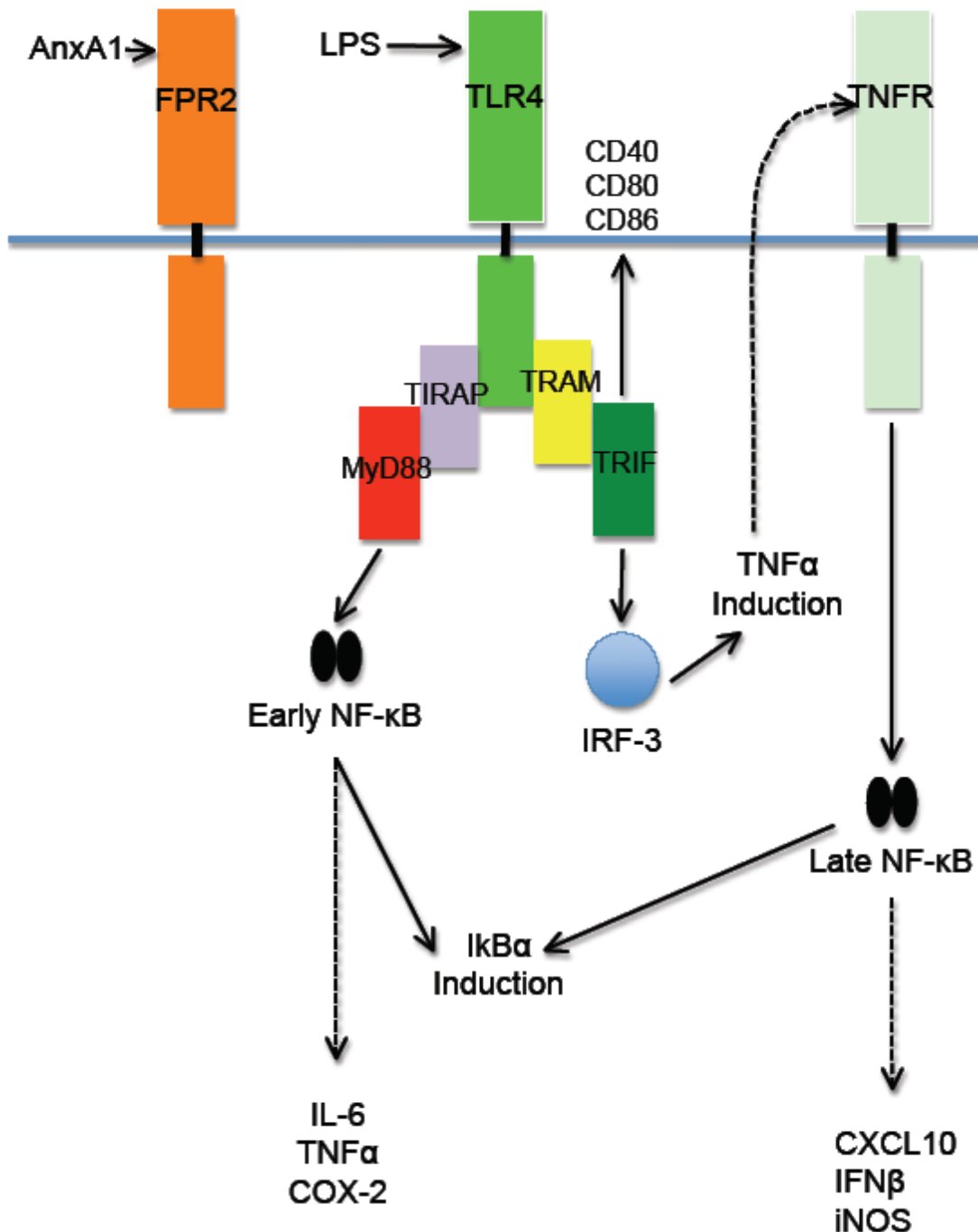
Figure 1.14: TLR4 signalling pathway

Both MyD88-dependent and TRIF-dependent pathways are part of the TLR4 signalling. These induce a number of transcription factors influencing the gene expression of several mediators of inflammation. Adapted from (Covert et al., 2005).
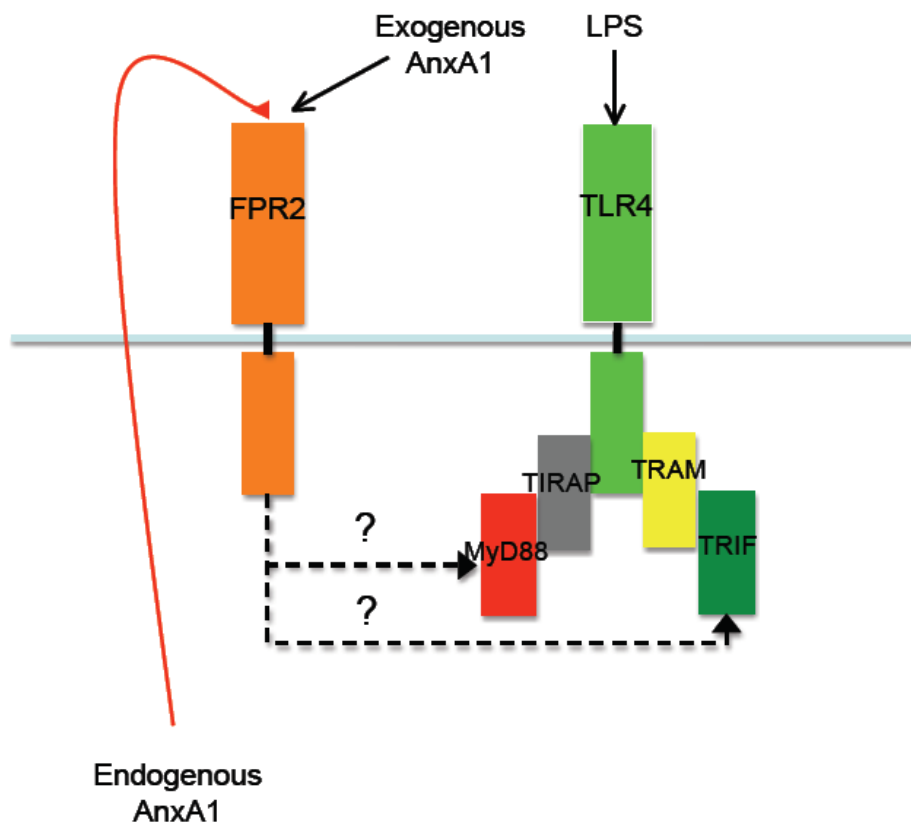
Figure 1.15: AnxA1 exerts an effect on TLR4 signalling pathway.

Exogenous AnxA1 acts on FPR2 but at the same time blocks the TLR4 signalling pathway with an as yet unidentified mechanism. Red line: release of AnxA1 upon TLR stimulation.

MyD88	TRIF	MyD88/TRIF
TLR2	TLR3	TLR4
TLR5		
TLR7		
TLR9		

Table 1.1: **Different adaptor molecules are recruited to the receptor after stimulation of different TLRs.**

CHAPTER 2:

MATERIALS AND METHODS

2.1. TLR ligands

The TLR agonists used for stimulation of BMDMs were: Heat-killed *Listeria monocytogenes* (HKLM - TLR2 agonist), poly (I:C) (synthetic analogue that resembles viral double-stranded RNA - TLR3 agonist), *Escherichia coli* LPS serotype 0111:B4 (principal cell wall component of Gram (-) bacteria - TLR4 agonist), flagellin from *S. thyphimurium* (protein component of bacterial flagellar filament - TLR5 agonist), loxoribine (synthetic guanosine analogue - TLR7 agonist), and ODN1826 (synthetic CpG oligonucleotide - TLR9 agonist) and were all purchased by InvivoGen (San Diego, U.S.A.).

2.2. Animals

Genetically modified AnxA1 KO mice on a C57BL/6 background and their WT littermates were purchased from B&K (UK) and were used throughout these experiments. Prior to sending to the commercial breeder, the colony was genotyped as previously described (Hannon et al., 2003). All mice were young adult males (6-8 weeks old) and had a body weight of 24-28g. Food and water were available *ad libitum*. Animals were kept under standard conditions and maintained in a 12-h light/dark cycle at $22 \pm 1\ ^{0}C$ in accordance with United Kingdom Home Office regulations (Guidance on the operation of Animals, Scientific Procedures Act 1986) and of the European Union directives.

2.3. Generation of conditioned medium

L929 fibroblasts were resurrected from aliquots stored in liquid nitrogen (-120 ^{0}C). L929 cells were gently warmed in a 37 ^{0}C water bath, plated in T175 cm^2 flasks containing DMEM (GIBCO, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS; GIBCO, Paisley, UK) and were maintained at 37 ^{0}C in a 5% CO_2 and 95% O_2 atmosphere. When the cells became confluent the medium was removed, they were washed with phosphate-buffered saline (PBS) and were plated with 100ml of fresh DMEM 10% FCS in T175 cm^2 flasks at 37 ^{0}C in a 5% CO_2 and 95% O_2 atmosphere. On day 3 of culture, the supernatant medium was collected, centrifuged and filtered through a 0.22 μm filter (Corning, Schiphol-Rijk, Netherlands). The aliquots were stored at -20 ^{o}C and were used as and when required to make conditioned medium containing Macrophage Colony Stimulating Factor (M-CSF) for the generation of BMDMs from bone marrow pre-cursor cells. Conditioned medium was considered DMEM medium containing 20% FCS, 30% L929 supernatant and 50μg/ml gentamicin.

2.4. Generation and culture of BMDMs

Young (6-8 weeks) AnxA1 KO mice and their WT littermates were sacrificed by cervical dislocation. The skin of the abdomen and legs were sterilised with 70% ethanol spray in a sterile flow cabinet. A transverse cut through the skin of the abdomen was performed in order to dissect away the skin and expose the hind limbs. In order to expose the femurs and tibia the muscles attaching the hind limbs to the pelvis were dissected. The femurs and tibia were washed and cleaned, to remove all attached flesh. Thereafter, they were washed for 1 min in 70% ethanol and then transferred to new plates containing tissue culture PBS. Each bone was held with forceps so that the tips of the bones (epiphyses) could be cut. The bone marrow cells were flushed out with a 10ml syringe containing PBS by placing the connected 25-gauge needle into the bone shaft. The procedure was repeated several times until the bones became white indicating that all the bone marrow was expelled. The marrow plugs were mechanically disrupted by the 25-gauge needle. The cells were resuspended into 50ml-falcon tubes and filtered through a 70μm strainer, then centrifuged for 5min at 1200 rpm and resuspended in conditioned medium. Viable cell counts using the Neubauer haemocytometer were performed. The total number of cells was calculated according to the following formula:

Total number of cells= number of cells counted in the grid x dilution factor x10^4 (factor of chamber) x volume of cell suspension.

Thereafter, cells were plated in a concentration of $2x10^6$/ml in 10 cm sterile Petri dishes (Corning, Shiphol-Rijk, Netherlands), and were incubated in a humidified atmosphere of 5% CO_2 at 37^oC for 7 days in conditioned medium to allow proliferation and differentiation. Fresh conditioned medium was added on day 4. Cultures were confluent by day 6 and the adherent cells were harvested gently by cell scrapers, counted, and plated in DMEM medium in order to be stimulated with different TLR agonists. The bone marrow which was flushed from the femurs and tibia of each mouse gave approximately $50\text{-}100 \times 10^6$ bone marrow cells and resulted in approximately $30\text{-}60 \times 10^6$ macrophages following 7 days of differentiation in conditioned medium. The differentiation of macrophages was confirmed by morphology using FACS at day 6 for the markers CD40, CD80, CD86 and MHC II.

2.5. Stimulation of BMDMs with TLR ligands

AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs were seeded at a density of 500.000/well in triplicate wells in order to be used for flow cytometry and cytokine assays, and of $2x10^6$/well for EMSAs and Western blots. Thereafter, the cells were incubated with medium alone or with one of the following TLR agonists: HKLM (final concentration $1x10^8$ freeze-dried cells/ml), poly (I:C) (25μg/ml), LPS (5μg/ml), flagellin (0.1μg/ml), loxoribine (1mM), and ODN (1μM). AnxA1$^{+/+}$ BMDMs were also stimulated with different concentrations of human recombinant AnxA1 (see below homology with the mouse protein). The macrophages were incubated at 37 ^oC under humidified 5% CO_2 for various time-points before use in assays. After incubation, supernatants were harvested and frozen at -20^oC prior to analysis for

cytokines by ELISA and for NO_2^- by the Griess reaction. RNA was extracted from cells using commercial column-based kit (Qiagen, West Sussex, UK) for RNA extraction or cells were removed by vigorous pipetting for analysis of markers of macrophage activation by flow cytometry or they were treated with cell lysis buffer to be used for Western blotting or were stored directly at −20°C for nuclear and cytoplasmic extracts.

```
☑ 34861   1    MAMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFNPSSDVAALHKAIMVKGVDEATIIDILTKRNNAQRQQI  80
☑ 34863   1    MAMVSEFLKQARFLENQEQEYVQAVKSYKGGPGSAVSPYPSFNVSSDVAALHKAIMVKGVDEATIIDILTKRTNAQRQQI  80

☑ 34861   81   KAAYLQETGKPLDETLKKALTGHLEEVVLALLKTPAQFDADELRAAMKGLGTDEDTLIEILASRTNKEIRDINRVYREEL  160
☑ 34863   81   KAAYLQENGKPLDEVLRKALTGHLEEVVLAMLKTPAQFDADELRGAMKGLGTDEDTLIEILTTRSNEQIREINRVYREEL  160

☑ 34861   161  KRDLAKDITSDTSGDFRNALLSLAKGDRSEDFGVNEDLADSDARALYEAGERRKGTDVNVFNTILTTRSYPQLRRVFQKY  240
☑ 34863   161  KRDLAKDITSDTSGDFRKALLALAKGDRCQDLSVNQDLADTDARALYEAGERRKGTDVNVFTTILTSRSFPHLRRVFQNY  240

☑ 34861   241  TKYSKHDMNKVLDLELKGDIEKCLTAIVKCATSKPAFFAEKLHQAMKGVGTRHKALIRIMVSRSEIDMNDIKAFYQKMYG  320
☑ 34863   241  GKYSQHDMNKALDLELKGDIEKCLTTIVKCATSTPAFFAEKLYEAMKGAGTRHKALIRIMVSRSEIDMNEIKVFYQKKYG  320

☑ 34861   321  ISLCQAILDETKGDYEKILVALCGGN  346
☑ 34863   321  ISLCQAILDETKGDYEKILVALCGGN  346
```

Query 34861: Human AnxA1
Query 34861: Murine AnxA1

Source: NCBI/ BLAST/ blastp suite-2sequences

2.6. Flow cytometry

Flow cytometry is a technique which can be used to determine the relative size (proportional to forward-scattered light- FCS) and granularity (proportional to side-scattered light- SSC) of a cell based on the scattering of light and the emission of fluorescence. It can also be used to identify specific surface markers on a cell.

Flow cytometry was used to investigate the phenotypic characteristics of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs and the cell surface expression of markers of macrophage activation. BMDMs were prepared as described in section 2.4 and were treated for 24 h with the different TLR ligands or medium alone. The plate was centrifuged at 2000 rpm for 5min and the supernatant was removed. Cells were detached from the plates by flashing with PBS and were collected in tubes, centrifuged at 8000 rpm for 5min and resuspended in FACS buffer (PBS containing 1%FCS and 0.02% NaN$_2$) containing CD16/CD32 FcγIIR blocking antibody (working dilution 1:1000; clone 93; eBioscience, Wembley, UK) for 30 min at 4°C in order to prevent unspecific binding of antibodies. Thereafter, cells were labelled for 1 h at 4°C with the following PE-conjugated antibodies (eBioscience, Wembley, UK): MHC II (1:1000; clone M5/114.15.2), CD40 (1:1000; clone 1C10), CD80 (1:1000; clone 16-10A1), and CD86 (1:1000; clone GL1), prior to analysis by FACScalibur using CellQuest software (Becton Dickinson, Fanklin Lakes, NJ). At least 10^4 cells were analysed per sample. If the FACS acquisition could not be performed the same day, the cells were fixed in 1% paraformaldehyde (PFA) at 4^{0}C for 1 hour. For isotype controls we used an IgG of the same subtype conjugated to the chromophore.

2.7. Analysis by ELISA

2.7.1. Mouse TNF-α and IL-6 ELISA

The collected supernatants of BMDMs stimulated with different TLR ligands were analysed for the levels of IL-6 and TNF-α by ELISA according to the manufacturer's instructions (eBioscience, Hatfield, UK). Briefly, 96-well high-binding plates were coated with the specific cytokine capture antibody and incubated overnight at 4°C. The standards were diluted as noted on the certificate of analysis and 2-fold serial dilutions were performed to make the standard curve. 100μl/well of standard and of each unknown sample was added to the appropriate wells. After a 2-h incubation at 37°C in order to allow the antigen present in the cell free supernatants to bind to the immobilised antibody already bound to the plate, the plates were washed extensively with the wash buffer (PBS 0.05% tween-20) in order to remove any sample that was unbound. Then, the samples bound to the antibody were detected by the addition of 100 μl of a detection antibody specific for the desired cytokine following incubation of 1-hour. Thereafter, the plates were incubated for 30min with the enzyme Avidin-Horseradish Peroxidase (HRP), and after an extensive wash to remove unbound antibody-enzyme reagent, 100 μl of substrate solution (tetramethylbenzidine TMB) was added to produce colour. The reaction was terminated by the addition of 50 μl of 1M H_3PO_4 turning the samples from blue to yellow and the absorbance was read at a wavelength of 450nm using a spectrophotometer (Labsystems Multiskan Bichromatic; Helsinki; Finland). The concentration was calculated from the standard curve using the software Graph Pad Prism^{TM}.
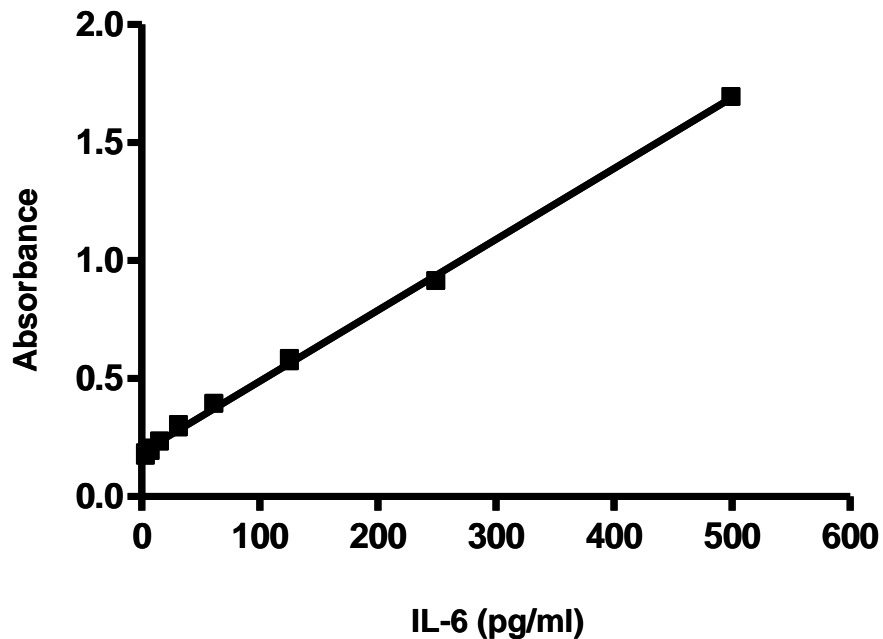
Figure 2.1: **Typical standard curve obtained during an IL-6 ELISA assay.**
Absorbance values were read at 450 nm and IL-6 concentration were determined from the standard curve; using the Graph Pad Prism^{TM}.

2.7.2. Mouse phospho-ERK1/2 ELISA

Cell lysates were prepared as described at section 2.9 and were analysed for the levels of phospho-ERK1/2 according to the manufacturer's instructions (R&D Systems; Abingdon; UK). Briefly, a 96-well plate was coated with the capture antibody and was incubated overnight at room temperature. The following day, the plate was washed with 0.05% tween-20 in PBS, was blocked by the addition of 300 µl of blocking buffer (1% BSA, 0.05% NaN_3 in PBS) per well and was incubated at room temperature for 2 h. A standard curve starting at the concentration of 20 ng/ml was prepared by serial dilution with IC Diluent #3 (1 mM

EDTA, 0.5% Triton X-100, 5 mM NaF, 1M urea in PBS). All the samples were diluted 1:1 with IC Diluent #Y (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 2M urea in PBS) in order to have a final concentration of urea 1M. 100 µl of standard or diluted sample was added to the appropriate wells and the plate was incubated for 2 h at room temperature. Thereafter, the detection antibody was added and the plate was incubated for another 2 h before the addition of streptavidin HRP and the incubation for 20 min. Finally, 100 µl of substrate solution (prior to use equal quantities of Color Reagent A- H_2O_2 and Color Reagent B- Tetramethylbenzidine were mixed) was added per well and the plate was incubated until the colour changed (approximately 1 hour). The reaction was terminated by the addition of 50 µl of 1 M H_3PO_4 per well and the optical density was determined at a wavelength of 450 nm using a spectrophotometer (Labsystems Multiskan Bichromatic). The software Graph Pad Prism[TM] was used for determination of the concentration of unknown samples from the standard curve.
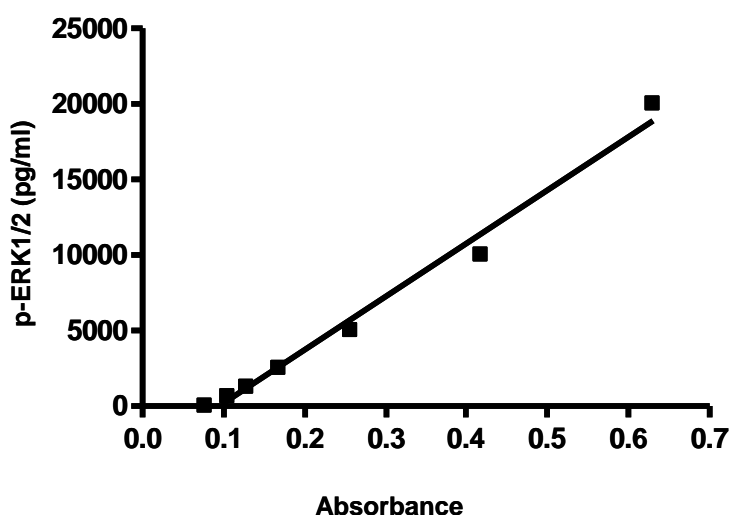
Figure 2.2: Typical standard curve obtained during a p-ERK1/2 ELISA assay.
Absorbance values were read at 450 nm and p-ERK1/2 concentration were determined from the standard curve; using the Graph Pad Prism[TM].

2.8. Bradford protein assay

200 μl per well of the Bradford reagent (1:5 of commercial stock solution in distilled water) was added in a 96-well flat bottom plate. 2 μl of each sample was added per well followed by incubation for 10min at room temperature. The absorbance was read at 595nm in a microplate reader (Labsystems, Waltham, MA). The protein concentration was calculated using the software Graph Pad Prism^{TM} knowing that the absorbance of 0.06 corresponds to 1 μg protein.

2.9. Preparation of whole cell lysates

The cells were lysed in ice-cold lysis buffer containing protease and phosphatase inhibitors for 5 min (1% Triton-X; 20mM Tris, pH 7.5; 150mM NaCl; 1mM MgCl$_2$; 1mM EGTA; 1mM DTT; 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1μM Aprotinin, 1μM Leupeptin; 1μM Pepstatin; 50mM NaF; 10mM Na$_4$P$_2$O$_7$; 1mM sodium orthovanadate (NaVO$_4$) and 1mM β- glycerophosphate). The lysates were centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were collected. The protein content was quantified by the Bradford method described at section 2.8.

2.10. Western blotting by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Western blotting is a technique, which allows the detection of proteins of interest and their separation according to their molecular weight. It firstly includes electrophoresis on a gel, secondly the transfer of separated proteins onto a membrane and thirdly incubation of the membrane with specific antibodies which may be detected using the ECL^{TM} system.

The samples were denatured with hot 6x Laemmli sample buffer (60% 4x Tris-HCl/SDS, pH 6.8; 36% glycerol; 0.1 g/ml SDS; 0.093 g/ml dithiothreitol (DTT); 0.12 mg/ml bromophenol blue). The SDS disrupts the hydrogen bonds and makes the proteins negatively charged. The reducing agent DTT cleaves disulphide bonds to completely unfold the protein structure, whereas bromophenol blue was added to identify the proteins as they migrate through the gel.

A discontinuous buffer system (Laemmli) was used for protein electrophoresis, constructed of a low percentage (4%) stacking and a higher percentage resolving gel, allowing proteins to concentrate prior to separation resulting in a far greater resolution. Throughout the experiments 8%, 10%, and 12% resolving gels were used according to the molecular weight of the protein of interest, the formulations of which are shown in Table 2.1. The resolving and stacking gels were prepared by combining all reagents except for the polymerization catalysts ammonium persulfate (APS) and N,N,N',N'-Tetramethylenediamine (TEMED), which were added immediately before pouring the gels.

Table 2.1: Formulations for SDS-PAGE resolving and stacking gels.

REAGENTS	RESOLVING			STACKING
	8%	10%	12%	4%
Polyacrylamide (37.5:1) (ml)	10.6	13.3	16	3.9
4xResolving buffer (ml)	10.4	10.4	10.4	-
dH$_2$O (ml)	18.6	15.8	13.2	18.3
Stacking buffer (ml)	-	-	-	7.5
10% APS (μl)	400	400	400	150
TEMED (μl)	40	40	40	30

Polyacrylamide: Proto Gel Acrylamide: 30% acrylamide and 0.8%bis-acrylamide (National Diagnostics, Atlanta, USA)
Resolving buffer: 1.5M Tris-HCl 0.4% SDS, pH 8.8 (National Diagnostics, Atlanta, USA)
Stacking buffer: 0.5M Tris-HCl 0.4% SDS, pH6.8 (National Diagnostics, Atlanta, USA)
APS: Ammonium Persulphate
TEMED: N,N,N',N'-Tetramethylenediamine

The gels were clipped onto the western blot apparatus. The bottom tray and the space behind each gel were filled with SDS running buffer (diluted from a stock solution of 5x concentrate: 0.12 M Tris-base, 0.96 M glycine, 0.017 M SDS). Thereafter, the gels were loaded with 5 μl of molecular marker (New England Biolabs, USA) in the first lane and the unknown denatured samples in the following lanes. The samples were then subjected to electrophoresis until the blue marker reached the bottom of the gel.

Once electrophoresis was complete, the gels were removed from the western blot apparatus, the stacking gel was discarded and the resolving gel was detached from the plate and placed into a bath of transfer buffer (0.02 M Tris-base, 0.14 M glycine, 20% ethanol). The gels were sandwiched firmly between Polyvinylidene Fluoride (PVDF; Millipore, Watford, UK) membranes and two pieces of blotting paper and foam inside a transfer cage. Protein was transferred onto PVDF membranes inside a transfer chamber (Hoefer) in the presence of transfer buffer.

Membranes were blocked for 1h at RT in 5% non-fat dry milk in Tris-buffered saline solution containing Tween-20 (TTBS: 130mM NaCl; 2.68mM KCl; 19mM Tris-HCl; 0.001% v/v Tween-20, pH 7.4)]. Membranes were then incubated with the primary antibody of interest diluted in 5% non-fat dry milk in TTBS at 4°C. Following 3 x 5 min washes with TTBS, the membranes were incubated for 1 h at RT with a horseradish peroxidase (HRP) conjugated IgG secondary antibody.

Membranes were washed 5 times for 5 min in TTBS prior to incubation with enhanced chemiluminescence (ECL) solution for protein detection. Briefly, an equal volume of solution ECL1 (2.5 mM luminol, 0.44% p-coumaric acid; 100 mM Tris-base; pH 8.5) was mixed with solution ECL2 (0.02% H_2O_2, 100 mM Tris-base; pH 8.5), 2.5ml was poured onto each membrane and incubated for 1 min at RT. Membranes were wrapped in Saran Wrap, placed in an X-ray film cassette, and transferred to the dark room. A sheet of X-ray film (Fujifilm, USA) was placed on top of the membrane, the cassette was closed and film was exposed for a minimum of 5 sec and up to 15 min in most instances.

The following primary Abs were used: pERK (Santa Cruz, Santa Cruz, USA; sc-7383; 1:500), ERK1/2 (Santa Cruz, Santa Cruz, USA; sc-94, sc-154; 1:5000), COX2 (Santa Cruz, Santa Cruz, USA; sc-1747; 1:1000), IκBα (Santa Cruz, Santa Cruz, USA; sc-371; 1:500), NOS2 (Santa Cruz, Santa Cruz, USA; sc-650; 1:1000), AnxA1 (ZYMED, San Francisco, USA; 71-3400; 1:5000) and AnxA2 (Santa Cruz, Santa Cruz, USA; sc-9061; 1:1000). The following secondary antibodies were used: polyclonal goat anti-rabbit/ HRP (Dako, Cambridgeshire, UK; P0448; 1:2000), polyclonal rabbit anti-goat/ HRP (Dako, Cambridgeshire, UK; P0449; 1:2000), polyclonal goat anti-mouse/ HRP (Dako, Cambridgeshire, UK; P0447; 1:5000).

2.11. Immunoprecipitation

The supernatant of BMDMs stimulated with the TLR ligands was collected in tubes. For each 500 μl of supernatant 2 μl of antibody (AnxA1 or AnxA2) and 35 μl of protein G coated sepharose beads were added. The beads were centrifuged 3 times in order to throw away the upper layer washing away the ethanol and were resuspended in TNT buffer before addition to the samples. The tip was cut for the sepharose beads. The tubes were agitated at 4 ^{o}C overnight. The following day, the samples were centrifuged at maximum velocity and the supernatant was discarded. The pellets were denatured with 6x sample buffer for 3-5 min. The usual procedure of the western blot was followed as described in section 2.10.

2.12. Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were harvested from BMDMs according to previously described protocols (Jorritsma et al., 2003). Briefly, the cells were centrifuged and resuspended in ice cold hypotonic NAR A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA in distilled H_2O) followed by the addition of NP-40 1%. They were vortexed and centrifuged giving a pellet of nuclei and a supernatant, which contained the cytoplasmic extracts. After washing again with ice cold NAR A, high salt NAR C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA in distilled H_2O) was added without resuspending. The samples were vortexed and centrifuged and the supernatant containing the nuclear extracts was collected and stored at $-80\,^{\circ}$C.

2.13. Elecrophoretic mobility shift assays

Nuclear extracts were harvested from 3 to 5 x 10^6 cells. Briefly, aliquots (3-5 μg) were incubated with 2μg poly (dI:dC) in 20μl of binding buffer with ^{32}P end-labelled, double-stranded oligonucleotide probes (5x10^5 cpm) and fractionated on a 4% polyacrylamide gel (37.5:1 cross-linking ratio) in 0.5% tris-borate-EDTA (TBE) for 2.5 h at 150 V. The NF-κB binding buffer (10x) was 100mM Tris-HCl (pH 7.5), 500mM NaCl, 10mM EDTA, 50% glycerol, 10 mg/ml albumin, 30mM GTP, and 10 mM dithiothreitol (DTT). The NF-κB double-stranded oligonucleotide probe was purchased from Santa Cruz, Santa Cruz, USA.

2.14. Real-Time Reverse Transcription PCR

2.14.1 Isolation of RNA

Total RNA was extracted from BMDMs using a commercial kit according to the manufacturer's protocol (Qiagen, West Sussex, UK). Briefly, cells were pelleted and lysed using RLT lysis buffer (contains guanidine isothiocyanate which inactivates RNases) with β-Mercaptoethanol. The lysates were homogenised by passing through a 20-gauge needle fitted to an RNAse free syringe for at least 5 times and resuspended in 70% ethanol. The samples were applied to an RNeasy mini column placed in a 2ml collection tube and were centrifuged for 15 sec at 10000 rpm to allow absorption of RNA onto the column membrane. Following several washes with wash buffers RW1 and RPE to remove contaminants, the RNA was eluted in RNAse free dH_2O.

2.14.2 Reverse transcription (cDNA synthesis)

cDNA was synthesized by reverse transcription using the AMV transcriptase kit (Promega, UK). Briefly, 5-10μg RNA samples were mixed with 0.5 μg oligo d(T) (Promega, UK) and RNase-free dH_2O and incubated at 70 ^{o}C for 10 min. Thereafter, the mix was transferred on ice and supplemented with 1μl RNase inhibitor (Promega, UK), 4μl AMV 5x first strand buffer (Promega, UK; contains DTT that forms precipitates and therefore it must be vortexed thoroughly), 1μl (10mM) dNTP's (Bioline, UK), and 1 μl dH_2O. After incubation at 4 ^{o}C for 5 min, 1μl AMV reverse transcriptase (Promega, UK) was added directly into each

sample and incubated to the PCR machine at 42 ^{0}C for 60 min followed by 70 ^{o}C for 10 min. The cDNA samples were stored at 4 ^{o}C until use. For long-term storage, cDNA was kept at –20 ^{o}C.

2.14.3 Real-Time Polymerase Chains Reaction (PCR)

Real-time PCR was performed using SYBR Green PCR Mix and fluorescent primers for the genes *mTNF-α*, *mIL-6, mCOX-2*, *mCXCL10*, *mIFN-β*, *mISG54*, *mNOSII*, and for each TLR gene specific primer (all from Qiagen; West Sussex, UK). Cycling conditions were set according to manufacturer's instructions. Sequence-specific fluorescent signal was detected by 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was determined relative to the abundance of the housekeeping gene *mGAPDH*. We used the comparative Ct method to measure the gene transcription in samples. The results are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of gene normalized to endogenous control (GAPDH) and to the sample with the lowest expression set as one.

2.15. Measurement of nitrites

To evaluate NO production, nitrite concentration in the supernatants of BMDMs was measured using the Griess reaction. Briefly, 100µl of the culture supernatant was mixed with an equal volume of Griess reagent, made after mixing immediately before use a 1:1 ratio of Griess A (1% sulphanilamide in 5% orthophosphoric acid) and Griess B (0.1% 2-(1-naphthylamino)ethylamine dihydrochloride)) in a flat bottom 96-well plate. The absorbance was read at 540nm using a spectrophotometer (Labsystems Multiskan Bichromatic; Helsinki; Finland), and the nitrite concentration was calculated using a standard curve of $NaNO_2$ starting at a concentration of 1 mM.

2.16. PGE_2 enzyme immunoassay

The method was conducted according to the manufacturer's instructions (Cayman Chemical Company, Michigan, USA). The method is based on the competition of PGE_2 and PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 specific monoclonal antibody. Samples were diluted 1:1 with PBS. A standard curve was prepared starting with a concentration of 1ng/ml by serial dilution with the culture medium. A microtitre plate pre-coated with the secondary antibody was used. Initially, 50 µl of enzyme immuno assay (EIA) buffer was added to the non-specific binding (NSB) wells and 50 µl of culture medium to the NSB wells and the maximum binding (Bo) wells. 50 µl of each standard and each unknown sample (B) was added to the appropriate wells. Thereafter, 50 µl of PGE_2 tracer was added to each well except the total activity (TA) wells, followed

by 50 μl of PGE$_2$ monoclonal antibody except the TA, NSB, and Blank wells. The plate was incubated for 18 h at 4 $^{\circ}$C. Thereafter, the plate was emptied and rinsed 5 times with wash buffer in order to remove any unbound ligand and 200 μl of Ellman's reagent was added per well. In the TA wells, 5 μl of tracer was also added. The plate was developed in the dark for 60-90min and was read at 405nm using a spectrophotometer (Labsystems Multiskan Bichromatic). The concentration of PGE$_2$ in the unknown samples was calculated using the software Graph Pad Prism^{TM} by plotting the standard curve of %B/Bo versus Log [PGE$_2$].
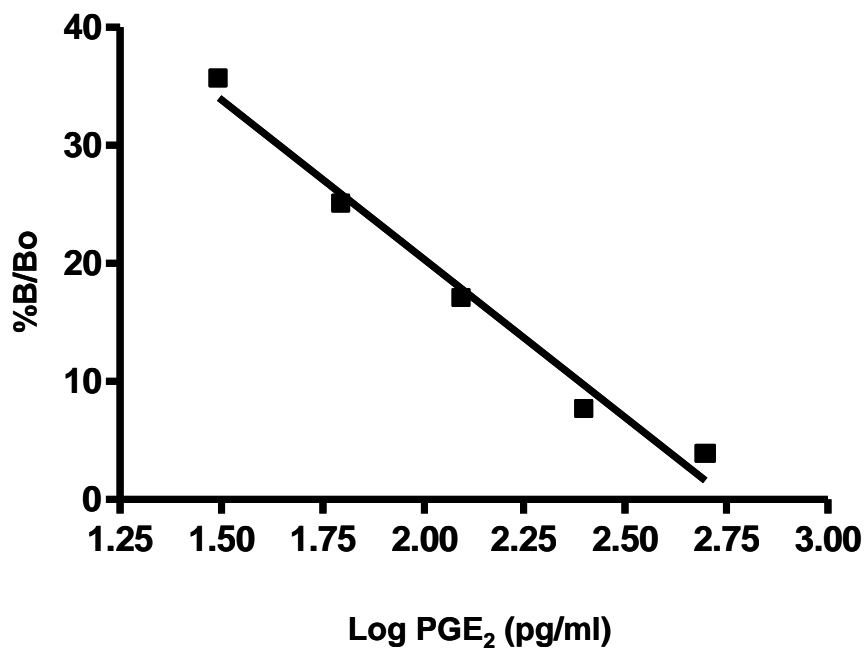
Figure 2.3: Typical standard curve obtained for the PGE$_2$ EIA system assay.

Absorbance values were read at 405 nm and PGE$_2$ concentration were determined from the standard curve; using the Graph Pad Prism^{TM}.

2.17. *In vivo* experiments

Male AnxA1-null mice on a C57BL/6 background and WT littermates were used throughout these experiments. In survival studies, peritoneal and systemic inflammation was produced by injection of 10µg/mouse or 20µg/mouse of poly (I:C) (InvivoGen, San Diego, U.S.A.). Control animals were injected intraperitoneally with an equal volume of PBS. Mice were monitored in 12h intervals for up to 96h.

2.18. Statistical analysis

Graph Pad Prism^{TM} software was used to create the graphs. All data were tested for normal distribution using the Shapiro-Wilk test. Normally distributed data was analysed by Student's t-test for two selected groups and two-way analysis of variance (ANOVA) using the Bonferroni post-tests correction. When data were not normally distributed, multiple sets were analysed using the Kruskal-Wallis test with Dunnet's post-test correction. When two data sets were compared, the Mann-Whitney test was applied. P-values lower than 0.05 (*P<0.05), 0.01 (**P<0.01) and 0.001 (***P<0.01) were sufficient to reject the null hypothesis and differences between groups were considered significant. Data are presented as means ± standard error of the means (S.E.M.) of n samples per group.

CHAPTER 3:

RESULTS

3.1. AnxA1 expression is modulated by TLR agonists

The activation of different cell types with inflammatory stimuli causes an increased expression and release of intracellular AnxA1 (D'Acquisto et al., 2007a, Damazo et al., 2005, Rescher et al., 2006). Using western blotting, we set to investigate if AnxA1 expression in BMDMs was modulated after stimulation with different TLR ligands.

As shown in Fig 3.1, AnxA1 is highly expressed in the cytosol of BMDMs. After 24 h stimulation with the TLR ligands at the concentrations seen in Table 3.1, it is constitutively released in to the culture supernatant. The peak and the time-course of this secretion differed between TLR agonists, and it was accompanied by depletion of the intracellular store, followed by resynthesis at later time-points. The peak of the intracellular AnxA1 depletion occurred approximately at 6 h after stimulation with HKLM and poly (I:C) (Fig 3.2A), followed by a return to the basal condition approximately at 24 h after stimulation. A similar depletion of intracellular AnxA1 was observed also for the TLR agonists LPS, flagellin, loxoribine, and ODN at later time-points (Fig 3.2B and Fig 3.2C).

Table 3.1: Toll-like receptor agonists used for stimulation of BMDMs

Toll-like Receptor	Ligand	Concentration
TLR 2	Heat Killed *Listeria Monocytogenes* (HKLM)	1×10^8/ml
TLR 3	Poly (I:C)	25μg/ml
TLR 4	Lipopolysaccharide (LPS)	5μg/ml
TLR 5	Flagellin	0.1μg/ml
TLR 7	Loxoribine	1mM
TLR 9	ODN1826	1μM
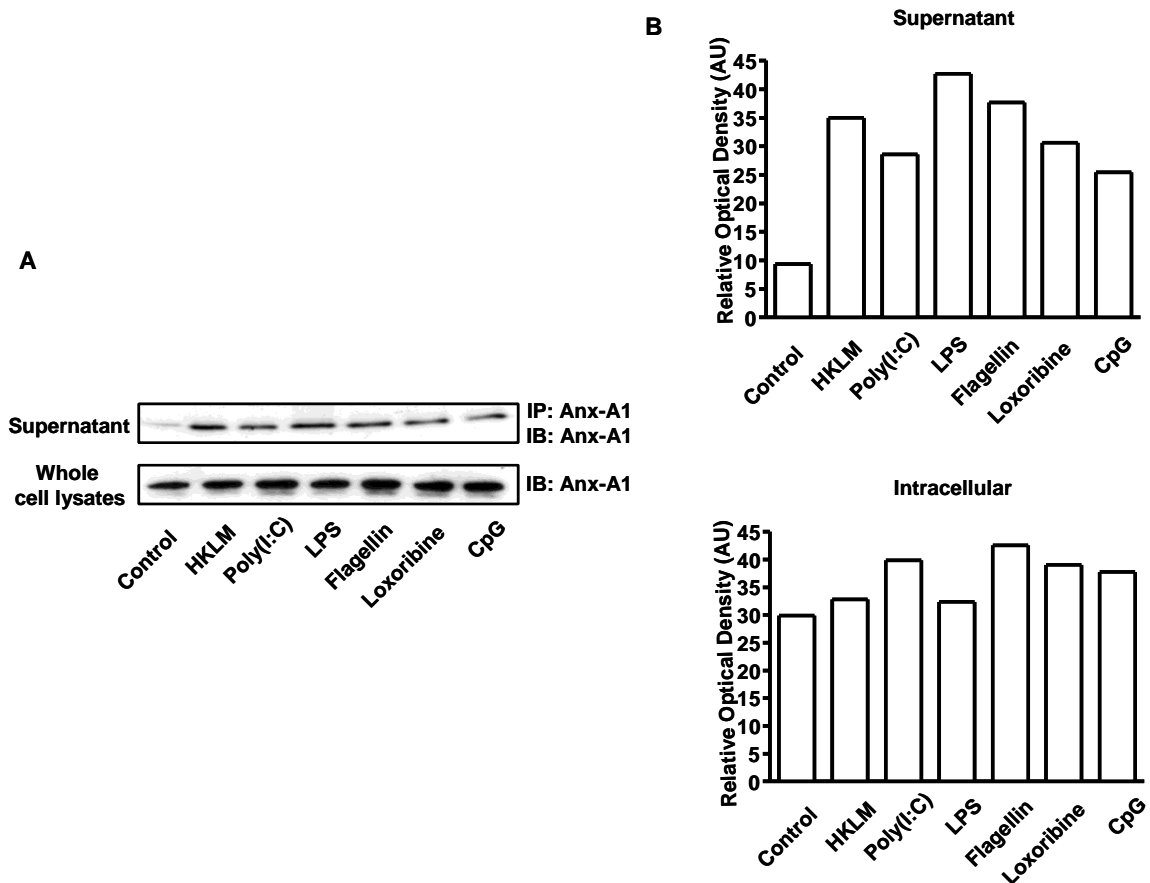
Figure 3.1: AnxA1 is secreted by bone marrow-derived macrophages after TLR stimulation.

(A) Western blotting analysis of AnxA1 expression in culture supernatants and cell lysates of bone marrow-derived macrophages stimulated with the TLR agonists for 24 h. To assess AnxA1 levels in culture supernatants, samples were immunoprecipitated with an anti-AnxA1 antibody and then immunoblotted. Data shown are from a single experiment.

(B) Bar graphs in the figure indicate the densitometric analysis of AnxA1 expression of the corresponding blots.

IP: Immunoprecipitation; IB: Immunoblotting
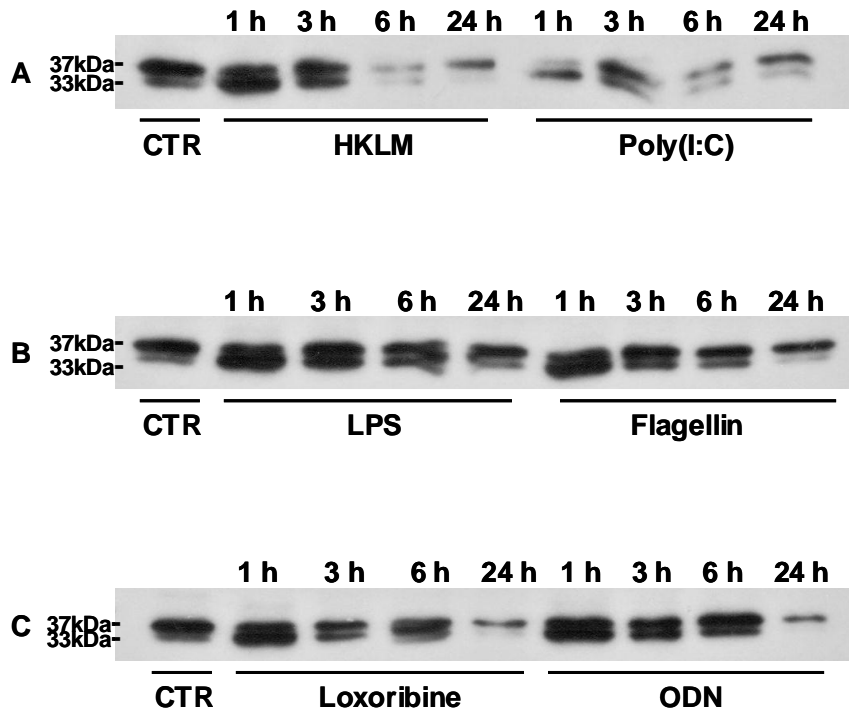
Figure 3.2: AnxA1 is modulated by TLR ligands.

Western blotting analysis of AnxA1 protein expression in whole cell lysates of BMDMs stimulated with the TLR agonists HKLM, poly (I:C) (A), LPS, flagellin (B), loxoribine, ODN (C) for the indicated times. Data shown are from a single experiment and are representative of n=2 experiments.

37kDa: Molecular weight of full-length AnxA1

33kDa: Molecular weight of cleaved form of AnxA1 (N-terminus absent)

SUMMARY

In this section the production and release of AnxA1 by BMDMs was investigated

- HKLM, poly (I:C), LPS, flagellin, loxoribine and CpG increased the intracellular content of AnxA1 as well as its release into the supernatant

3.2 Treatment of macrophages with human recombinant AnxA1

In order to investigate whether AnxA1 exerts an anti-inflammatory effect on macrophages, we used two different types of cells: a. RAW 264.7 cells- a macrophage cell line and b. BMDMs isolated from wild-type C57/BL6 mice. In both cases, the plated macrophages were incubated with one TLR agonist alone or in combination with hr-AnxA1 (at 3 different concentrations). The culture supernatant was collected at different time-points of incubation. Following this, the IL-6 and TNF-α content was measured by ELISA.

3.2.1 RAW macrophages

RAW macrophages were treated with one of the different TLR agonists at the concentrations seen in Table 3.2 alone or in combination with hr-AnxA1 (0.1, 0.01 or 0.001ng/ml). After 3 and 24h of incubation the supernatant was collected in order to perform ELISA for IL-6 and TNF-α.

The IL-6 release after 3h of incubation with different TLR agonists was undetectable and therefore is not reported. At 24h incubation with all the tested TLR ligands, the expected increase in the supernatant IL-6 content occurred (Fig 3.3). When the cells were treated in combination with hr-AnxA1, we observed a different response to each TLR agonist. Indeed, hr-AnxA1 lowered the content of IL-6 after treatment with the ligands poly(I:C), flagellin, loxoribine and CpG. This did not seem to be concentration-dependent since all 3 different concentrations of hr-AnxA1 had a similar effect, significantly decreasing IL-6 to a similar extent. The

results were different, however, for the TLR4 ligand LPS, where only the lowest concentration of hr-AnxA1 had as an effect reducing IL-6. On the contrary, after stimulation with the TLR2 ligand HKLM, hr-AnxA1 had no effect on IL-6 release at any concentration tested.

The production of TNF-α was recorded at both time-points for all the tested TLR ligands (Fig 3.4). At 3h of incubation, hr-AnxA1 effectively decreased the TNF-α release after treatment with the TLR5 ligand flagellin at the two higher concentrations, whereas for the other ligands no effect was recorded. However, when the 24h release was measured, hr-AnxA1 appeared more effective. Similarly to IL-6, hr-AnxA1 decreased TNF-α when combined with the ligands poly (I:C), flagellin, loxoribine and CpG. This phenomenon appeared concentration-dependent. For certain ligands (poly (I:C), flagellin) the highest concentration used was the most effective, whereas for others (loxoribine, CpG) the lowest. Again for the TLR4 ligand LPS, only the lowest concentration of hr-AnxA1 significantly decreased TNF-α, whereas after stimulation with HKLM, hr-AnxA1 had no effect at any concentration.

Table 3.2: **Toll-like receptor agonists used for stimulation of RAW macrophages**

Toll-like Receptor	Ligand	Concentration
TLR 2	Heat Killed *Listeria Monocytogenes* (HKLM)	1×10^7/ml
TLR 3	Poly (I:C)	$25 \mu g$/ml
TLR 4	Lipopolysaccharide (LPS)	500ng/ml
TLR 5	Flagellin	10ng/ml
TLR 7	Loxoribine	1mM
TLR 9	ODN1826	100nM
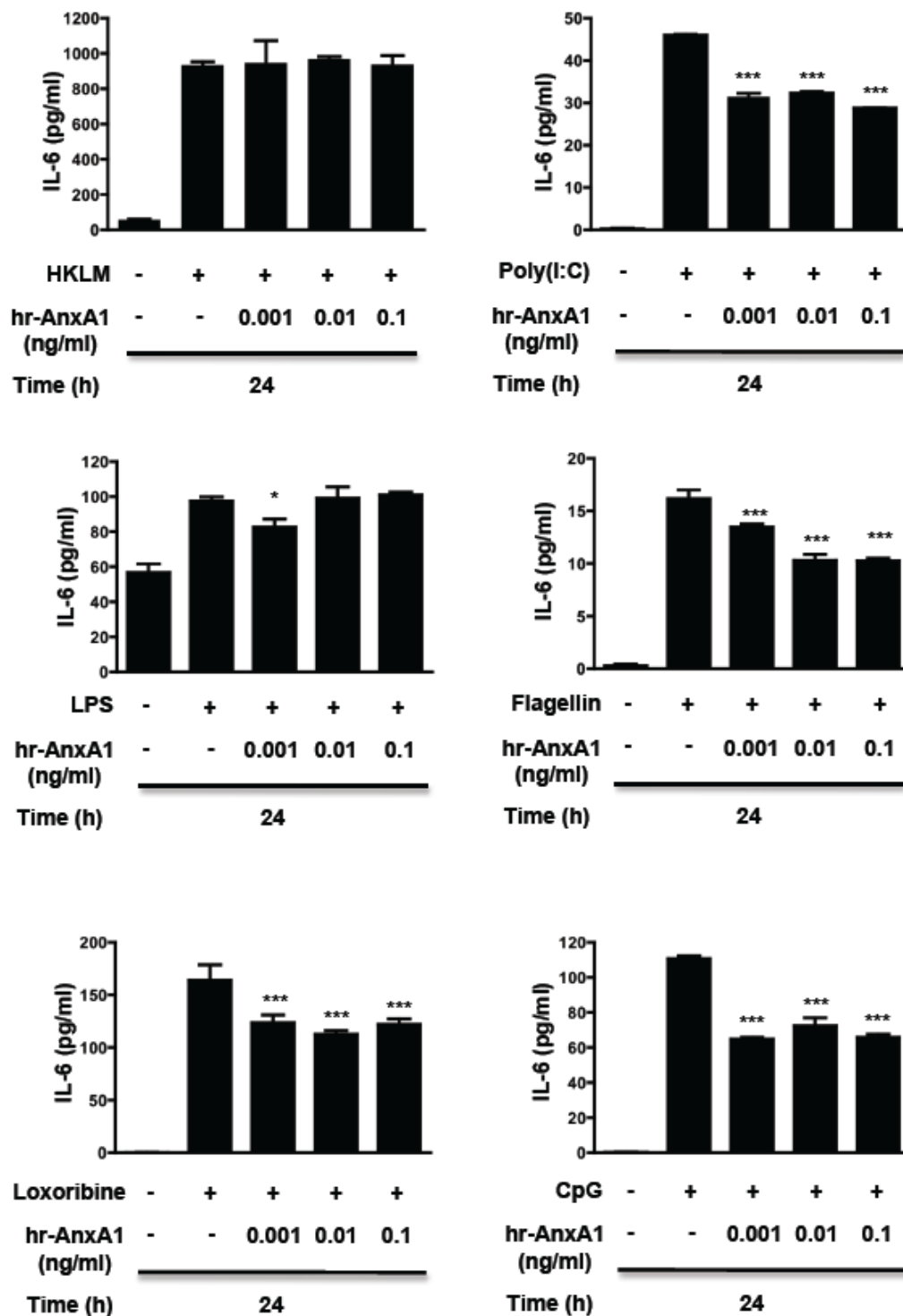
Figure 3.3: AnxA1 decreases IL-6 in RAW cells after stimulation with TLR ligands

RAW macrophages were incubated with different TLR ligands alone or in combination with 3 different concentrations of hr-AnxA1 for 24h. The supernatants were collected and analyzed for the content of IL-6 by ELISA. Bar graphs show the values from a single experiment representative of n=4 experiments with similar results. * $p<0.05$; ** $p<0.01$; *** $p<0.001$
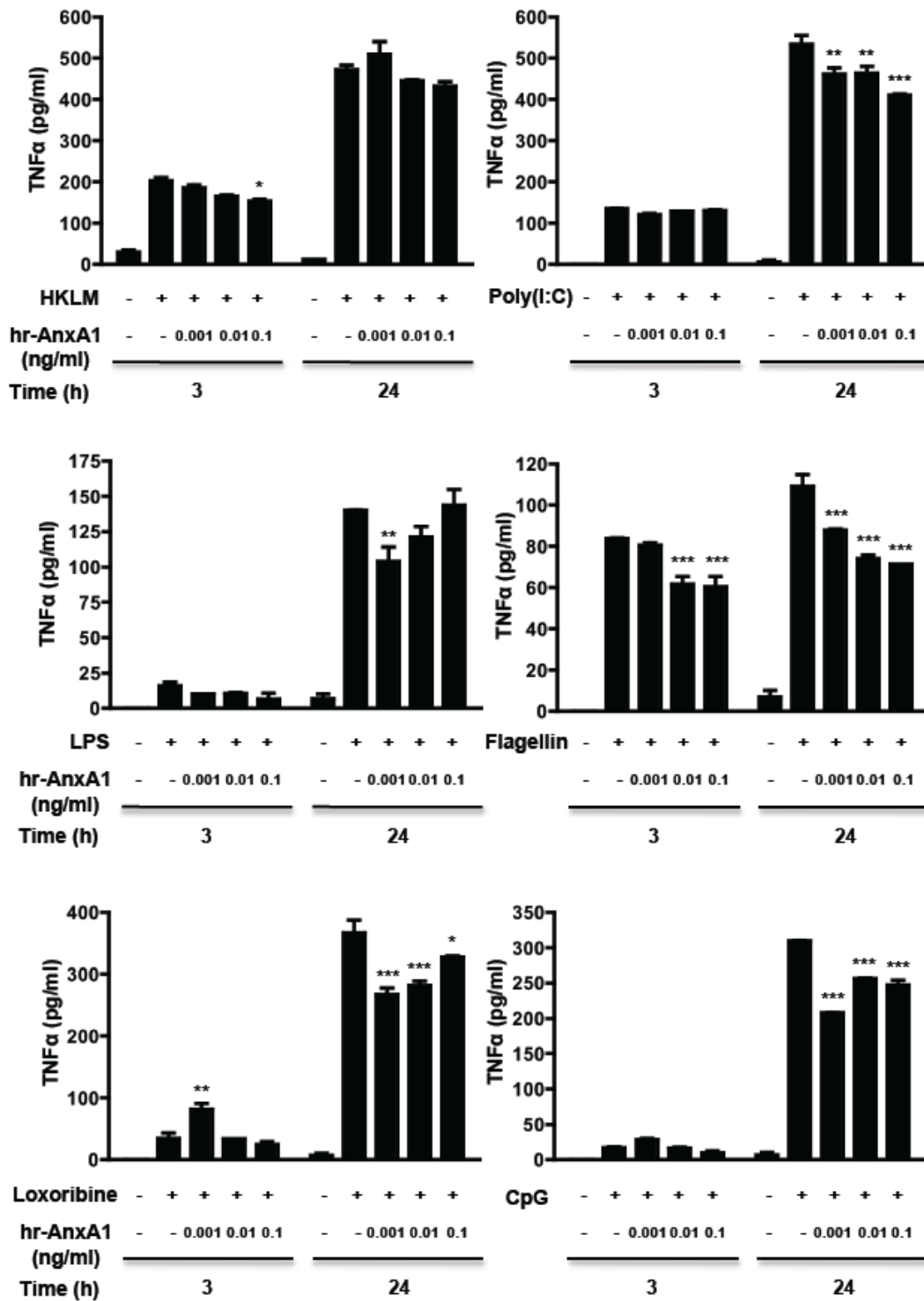
Figure 3.4: AnxA1 decreases TNF-α in RAW cells after stimulation with TLR ligands. RAW macrophages were incubated with different TLR ligands alone or in combination with 3 different concentrations of hr-AnxA1. The supernatants were analysed for TNF-α by ELISA. Bar graphs show the values from a single experiment representative of n=4 experiments with similar results.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.2.2 Bone marrow-derived macrophages

After the results using the RAW macrophage cell line, we repeated the experiment using BMDMs. In this case different concentrations (Table 3.1) of TLR ligands were used to stimulate the cells to produce pro-inflammatory cytokines.

At 3h of culture, hr-AnxA1 did not lower significantly the IL-6 release after treatment with any TLR ligand (Fig 3.5). When IL-6 was measured at 24h the results were ligand-specific (Fig 3.5). For HKLM and loxoribine hr-AnxA1 did not have any effect. On the other hand, hr-AnxA1 at the lowest concentration (0.001ng/ml) was effective in significantly reducing the IL-6 release post-stimulation with poly(I:C), LPS, flagellin, and CpG. Surprisingly, however, the highest concentration hr-AnxA1 (0.1ng/ml) was effective in reducing IL-6 release only after stimulation with flagellin.

When the TNF-α release was measured at 3h, no significant differences were observed after treatment with hr-AnxA1 (Fig 3.6). On the contrary, at 24h post-stimulation the hr-AnxA1 was effective in reducing TNF-α release at the lowest concentration (0.001ng/ml) post-treatment with HKLM, poly(I:C), loxoribine and CpG, whereas after stimulation with flagellin, only the highest concentration of 0.1ng/ml was the effective (Fig 3.6).

SUMMARY
In this section the effect of exogenous AnxA1 in the
release of IL-6 and TNF-α from BMDMs was investigated.

- hr-AnxA1 seems to have an anti-inflammatory effect
  in respect to IL-6 and TNF-α in a TLR-ligand
  specific and concentration-specific manner. Lower
  concentrations were the most effective.
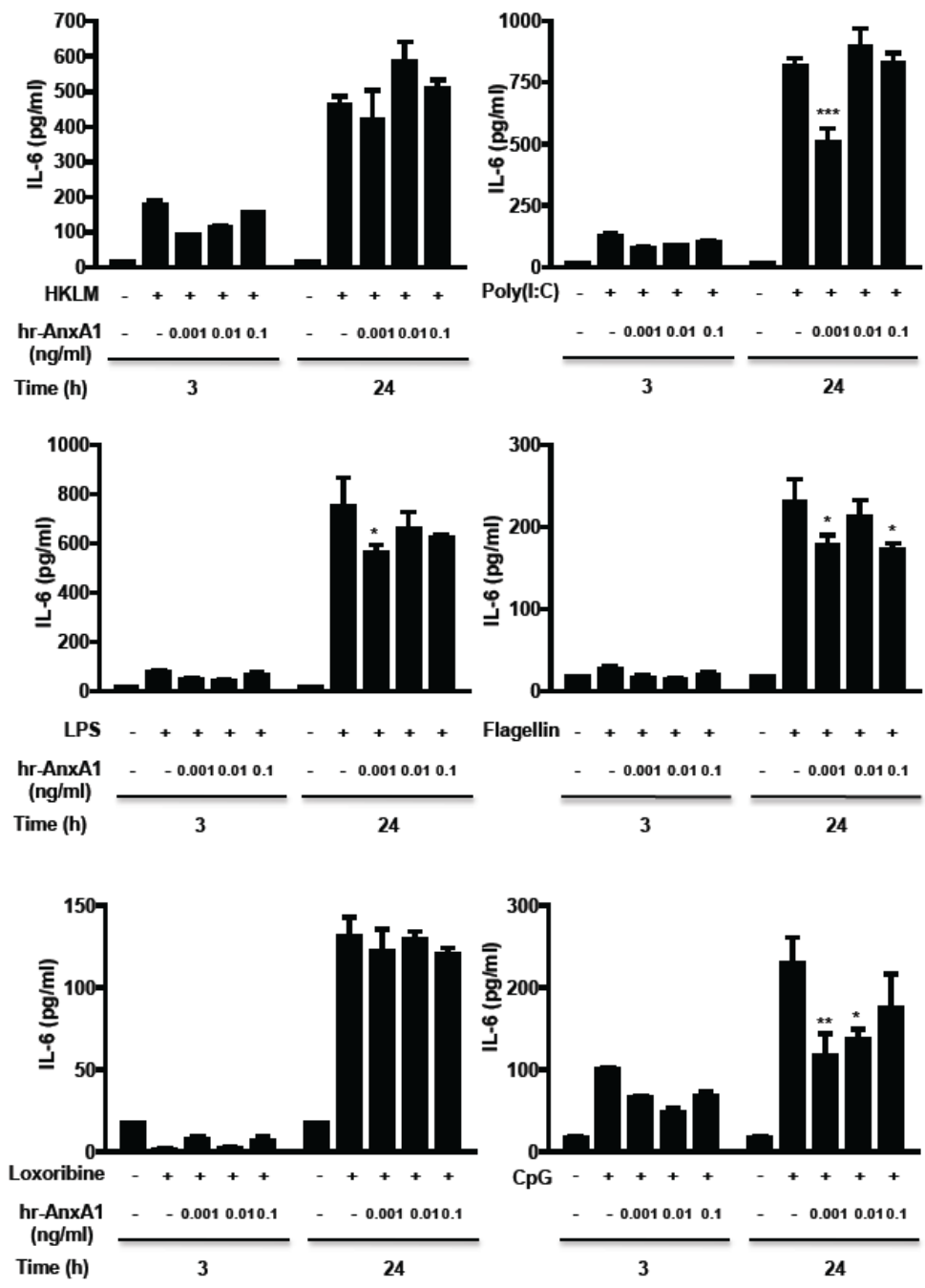
Figure 3.5: AnxA1 decreases IL-6 release from BMDMs after stimulation with TLR ligands

BMDMs were incubated with different TLR ligands alone or in combination with 3 different concentrations of hr-AnxA1 for the indicated times. The supernatants were collected and analyzed for the content of IL-6 by ELISA. Bar graphs show the values from a single experiment representative of n=2 experiments with similar results.   * p<0.05; ** p<0.01; *** p<0.001
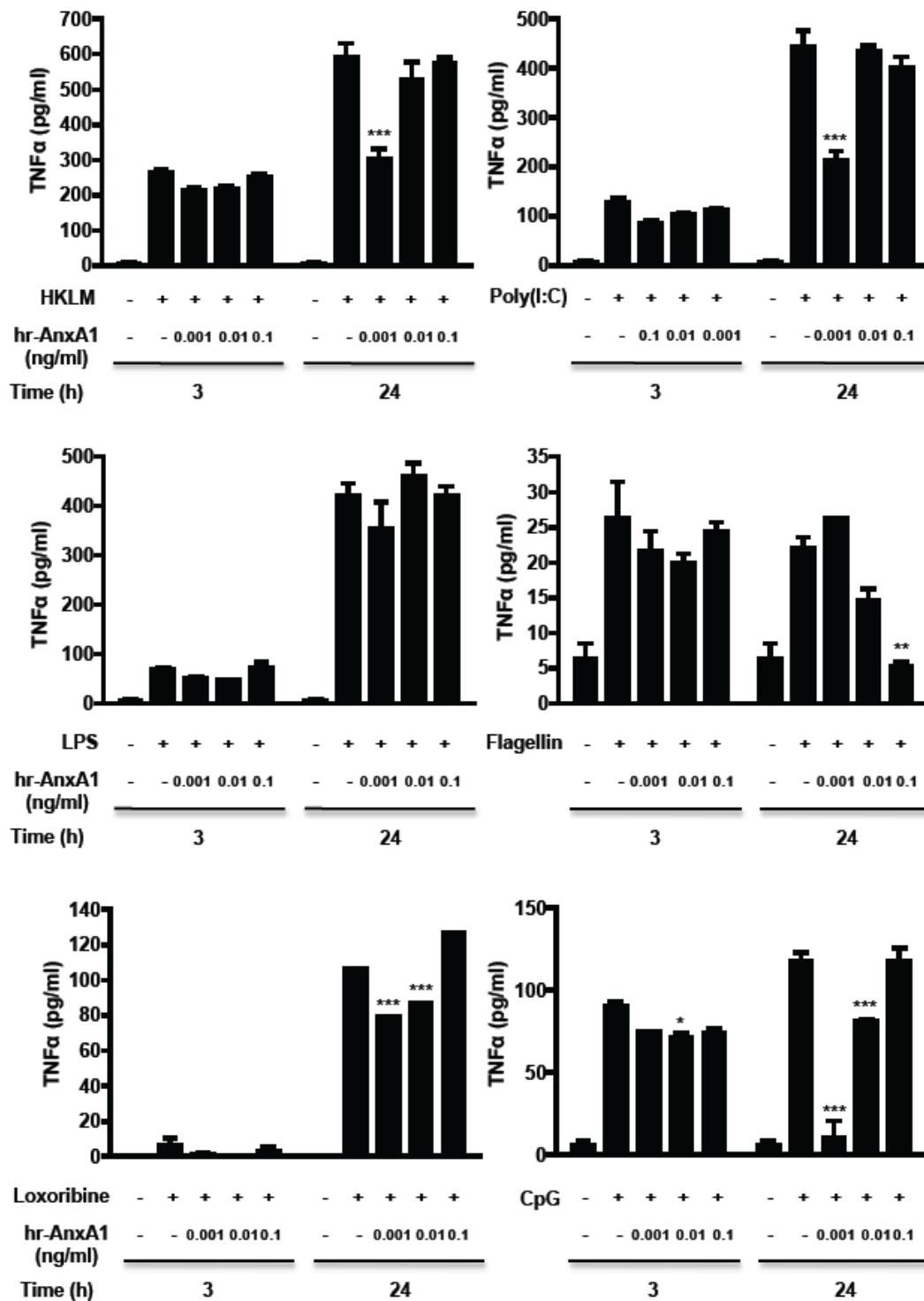
Figure 3.6: AnxA1 decreases TNF-α release from BMDMs after stimulation with TLR ligands

BMDMs were incubated with different TLR ligands alone or in combination with 3 different concentrations of hr-AnxA1. The supernatants were analysed for TNF-α by ELISA. Bar graphs show the values from a single experiment representative of n=2 experiments with similar results.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.3 Comparison of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs

Since the TRIF-dependent TLR ligand poly (I:C) and the MyD88-dependent TLR ligands and LPS which uses both pathways, do not behave in a consistent way in RAW cells and in BMDMs and since endogenous AnxA1 is secreted extracellularly probably acting at a surface receptor, we decided to further investigate the effect of complete deletion of AnxA1. Therefore, all the following experiments used BMDMs isolated from knockout mice (AnxA1$^{-/-}$) and compared them to BMDMs from wild-type (AnxA1$^{+/+}$) mice. Several different parameters of their function were assessed to establish the possible involvement of AnxA1 in different types of inflammation.

3.3.1 AnxA1^{-/-} BMDMs exhibit a different phenotype for activation markers

To compare the phenotypic characteristics of AnxA1^{+/+} and AnxA1^{-/-} macrophages, flow cytometric analysis was used to assess expression levels of several molecules related to macrophage activation such as MHC II and the co-stimulatory molecules CD40, CD80, and CD86.

The results revealed an increased expression of all activation markers in AnxA1^{-/-} BMDMs compared to AnxA1^{+/+} BMDMs at 24 h of cell culture (Fig 3.7 and Fig 3.8). Four independent experiments were performed and the Median Fluorescence Intensity (MFI) readings at the basal condition revealed a consistent 20% difference of all markers in the AnxA1^{-/-} cells compared to the AnxA1^{+/+} cells. This indicates that the AnxA1^{-/-} BMDMs have a different phenotype even before the addition of the TLR ligands.

Subsequently, we stimulated the BMDMs with the 6 different TLR ligands for 24 h at the concentrations shown in Table 3.1. The cells were collected and we performed the same analysis as above. The results showed that almost all TLR agonists upregulated the expression of MHC II, CD40, CD80, and CD86 in both AnxA1^{+/+} and AnxA1^{-/-} macrophages (Fig 3.7 and Fig 3.8). Moreover, different TLR ligands upregulated these markers differentially. This is in agreement with several other studies, which describe upregulation of MHC II (Lee et al., 2003, Liu et al., 2006) and co-stimulatory molecules (Edwards et al., 2006, Hoebe et al., 2003, Lee et al., 2003, Liu et al., 2006, Shen et al., 2008) after stimulation of WT macrophages with TLR agonists.

Although all TLR ligands upregulated MHC II in both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs, variations were observed between the different TLR ligands (Fig 3.7A). When the AnxA1$^{-/-}$ BMDMS were stimulated with TLR ligands acting exclusively through the MyD88-pathway (HKLM, flagellin, loxoribine, CpG), the percentage increase over basal MFI MHCII was similar to the AnxA1$^{+/+}$ BMDMs. In addition, when the percentage ratio of MFI of AnxA1$^{-/-}$ to AnxA1$^{+/+}$ cells was calculated for these ligands, the difference was approximately 20%, simply reflecting the higher basal MFI of AnxA1$^{-/-}$ cells. On the other hand, stimulation of both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ cells through the TRIF-pathway (poly(I:C), LPS), resulted a much greater upregulation of MHC II compared to the MyD88-dependent pathway ligands. However, this upregulation after stimulation with TRIF-mediated TLR ligands was impaired in the AnxA1$^{-/-}$ compared to the AnxA1$^{+/+}$ cells, resulting overall a lower MFI in these cells.

The MFI of the co-stimulatory molecules CD40, CD80, and CD86 after stimulation of the BMDMs with different TLR ligands, was higher in the AnxA1$^{-/-}$ BMDMs, reflecting the increased basal MFI in these cells (Fig 3.7B and Fig 3.8). Indeed, when the MFI of AnxA1$^{-/-}$ compared to AnxA1$^{+/+}$ cells was calculated, a difference of approximately 20% was revealed for both MyD88-dependent and TRIF-dependent ligands. However, although the percentage increase over basal MFI capacity i.e. the capacity of TRIF-mediated TLR ligands to further upregulate these molecules, was overall much higher compared to the MyD88-mediated ligands, it was similar when AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs were compared.

In conclusion, AnxA1 deletion for BMDMs appears to promote a more mature basal phenotype. When stimulated with the different TLR ligands, the expected cellular upregulation of these markers occurs, giving an overall higher MFI compared to their WT littermates. However, the capacity of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ macrophages to upregulate these co-stimulatory molecules, appears similar despite the much greater upregulation that occurs after stimulation with the TRIF-mediated TLR ligands poly(I:C) and LPS. On the other hand, these TRIF-mediated TLR ligands also lead to an upregulation of MHC II, yet this MHC II upregulation is impaired by the AnxA1$^{-/-}$ cells.
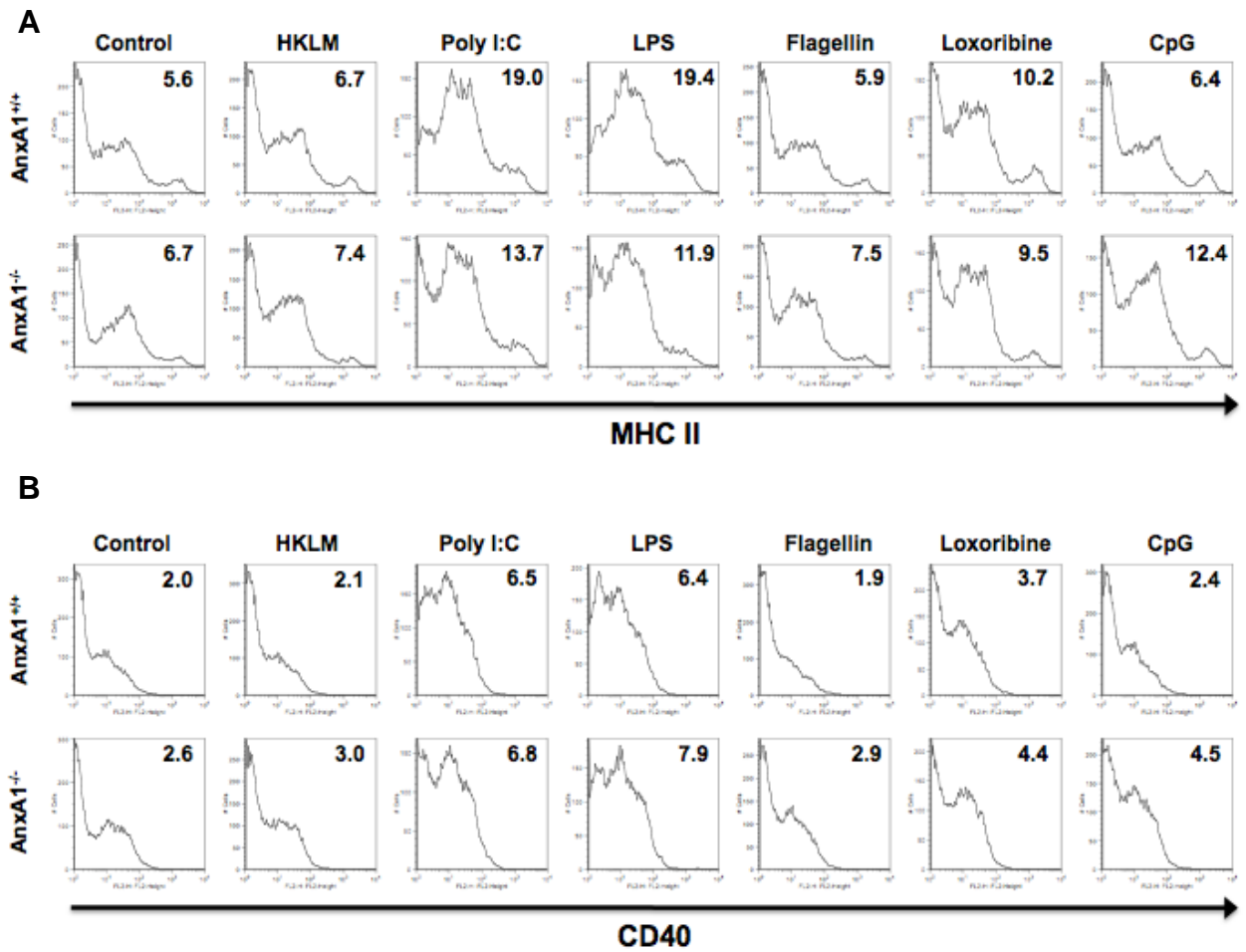
Figure 3.7: Phenotypic comparison of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs after stimulation with different TLR agonists.

Flow cytometric analysis of (A) MHC II, and (B) CD40 in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs after stimulation with TLR ligands for 24 h. Numbers in histograms show median fluorescence intensity from a single experiment and are representative of n=4 experiments with similar results.
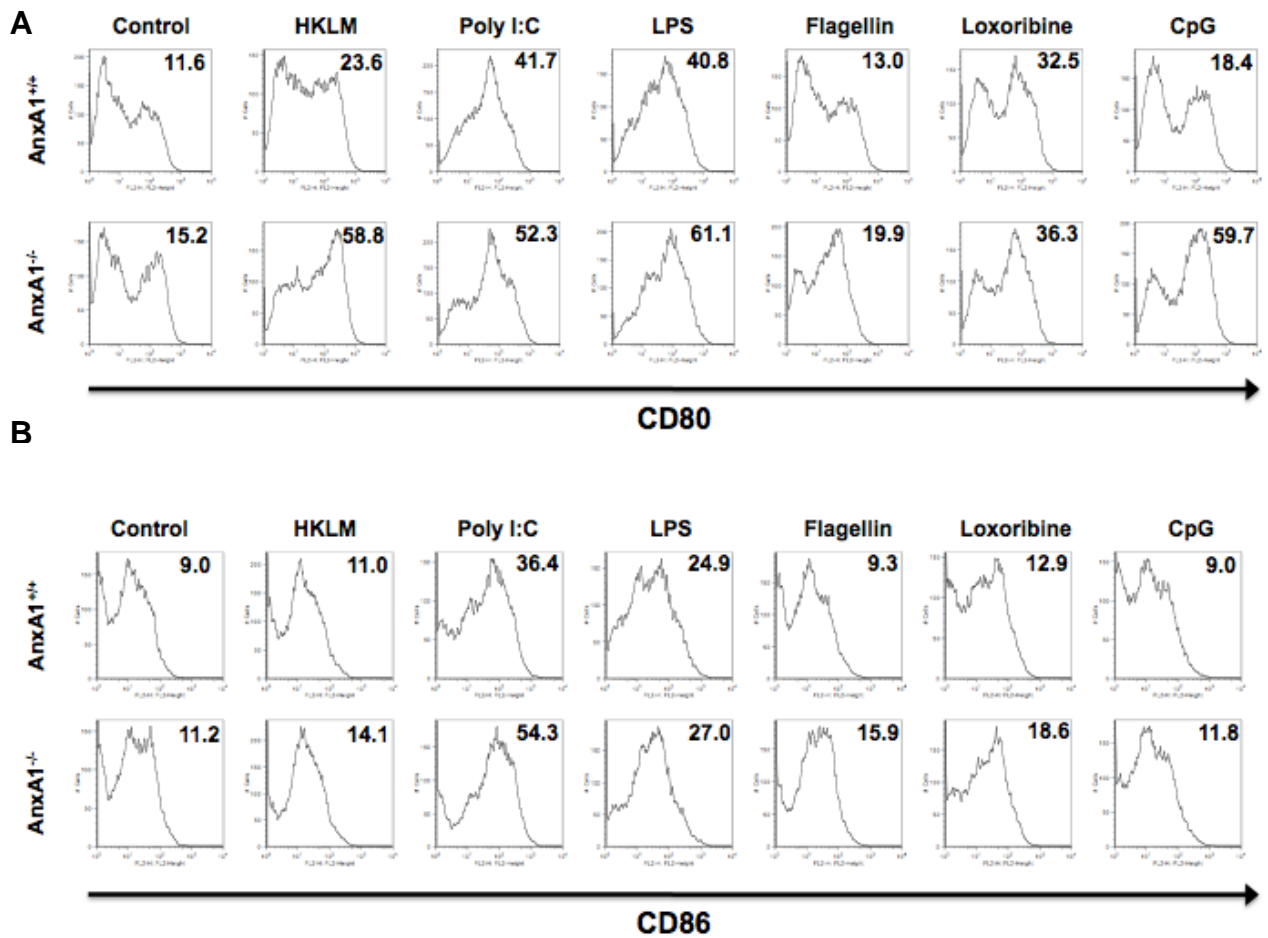
Figure 3.8: Phenotypic comparison of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs after stimulation with different TLR agonists.

Flow cytometric analysis of (A) CD80, and (B) CD86 in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs after stimulation with TLR ligands for 24 h. Numbers in histograms show median fluorescence intensity from a single experiment and are representative of n=4 experiments with similar results.

3.3.2 Exaggerated cytokine production by AnxA1$^{-/-}$ BMDMs

Stimulation of BMDMs with TLR ligands induces the production of proinflammatory cytokines such as IL-6 and TNF-α (Akira and Hoshino, 2003, Alexopoulou et al., 2001, Bagchi et al., 2007, Fang et al., 2004, Heil et al., 2003, Hemmi et al., 2000, Hoshino et al., 1999, Rowlett et al., 2008). To investigate whether there was a difference in this production in the absence of AnxA1, we stimulated AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs with 6 different TLR agonists as shown in Table 3.1 for 3, 6 or 24 h, and then measured IL-6 and TNF-α production by ELISA.

Firstly, the levels of IL-6 and TNF-α at the basal level were measured in unstimulated AnxA1$^{+/+}$ and AnxA1$^{-/-}$ cells. As seen in Fig 3.9, the basal release of TNF-α was similar at the two types of cells. However, the IL-6 release by the AnxA1$^{-/-}$ macrophages was significantly reduced compared to their WT littermates.

As expected (Hoshino et al., 1999, Fang et al., 2004, Bagchi et al., 2007, Rowlett et al., 2008, Alexopoulou et al., 2001, Heil et al., 2003, Hemmi et al., 2000), all TLR agonists tested stimulated the BMDMs to produce IL-6 (Fig 3.10) and TNF-α (Fig 3.12) in a time-dependent manner. The peak release of TNF-α was seen approximately 6 h after which a plateau was reached in the case of LPS, loxoribine, and CpG. On the contrary, HKLM, poly (I:C) and flagellin induced maximal cytokine production at later time-points. The extent of cytokine production differed between TLR ligands. In WT macrophages, certain ligands such as LPS strongly induced the production of IL-6 and TNF-α whereas others, such as flagellin, induced only low levels of these cytokines.

The activation of AnxA1$^{-/-}$ BMDMs with all the TLR ligands tested also resulted in the production of IL-6. However, the IL-6 production by the AnxA1$^{-/-}$ BMDMs was enhanced compared to WT cells after stimulation with the ligands LPS (Fig 3.10C), loxoribine (Fig 3.10E), and CpG (Fig 3.10F). On the other hand, in the first 6 h post-stimulation with flagellin, similar IL-6 levels were induced in AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs, whereas 24 h post-stimulation there was an impaired production of the cytokine by the AnxA1$^{-/-}$ cells (Fig 3.10D). A different pattern was also revealed after stimulation with the TLR3 ligand poly (I:C), where the production of IL-6 was markedly reduced by about 50% in the AnxA1$^{-/-}$ compared to the AnxA1$^{+/+}$ BMDMs at all time points (Fig 3.10B). More specifically, the cumulative results of 6 independent experiments revealed that the ratio of IL-6 production between AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs after stimulation with the ligands LPS, loxoribine and CpG was greater than 1, indicating the increased production of this cytokine by the AnxA1$^{-/-}$ cells. After stimulation with poly (I:C) the ratio was approximately 0.5, mirroring the decreased production of the cytokine in the AnxA1$^{-/-}$ BMDMs.

The production of TNF-α was enhanced in the AnxA1$^{-/-}$ BMDMs compared to AnxA1$^{+/+}$ BMDMs after stimulation with all tested TLR agonists with the exception of the TRIF-dependent ligand poly (I:C) (Fig 3.12). The ratio of TNF-α production in AnxA1$^{-/-}$ cells to AnxA1$^{+/+}$ cells after stimulation with all the MyD88-dependent TLR ligands was greater than 1 at all time-points, revealing the increased production of this cytokine by AnxA1$^{-/-}$ BMDMs.

A IL-6 (pg/ml) — Basal

B TNF-α (pg/ml) — Basal
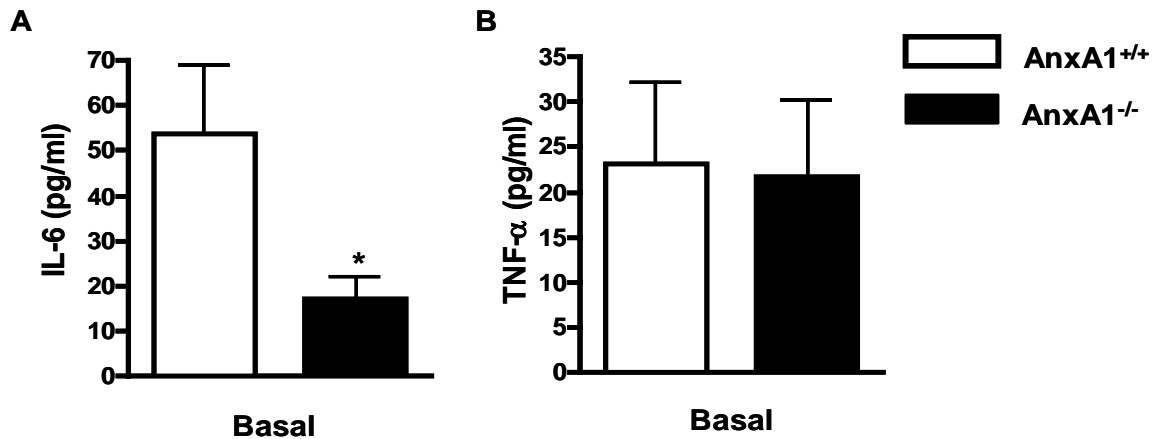
AnxA1$^{+/+}$
AnxA1$^{-/-}$

<u>**Figure 3.9**</u>: **Impaired basal IL-6 but not TNF-α production by AnxA1$^{-/-}$ BMDMs.**

Bar graphs showing the IL-6 (A) and TNF-α (B) secreted into the culture supernatant by AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs after incubation for 24 h with medium alone. Values are mean±S.E.M of 4 experiments with similar results.

* $p < 0.05$

SUMMARY

In this section the effect of endogenous AnxA1 in the release of IL-6 and TNF-α from BMDMs stimulated with TLR agonists was investigated

- AnxA1$^{-/-}$ BMDMs expressed higher levels of IL-6 after stimulation with the ligands LPS, flagellin and CpG

- AnxA1$^{-/-}$ BMDMs expressed reduced levels of IL-6 after stimulation with poly (I:C)

- AnxA1$^{-/-}$ BMDMs expressed higher levels of TNF-α after stimulation with HKLM, LPS, flagellin, loxoribine and CpG
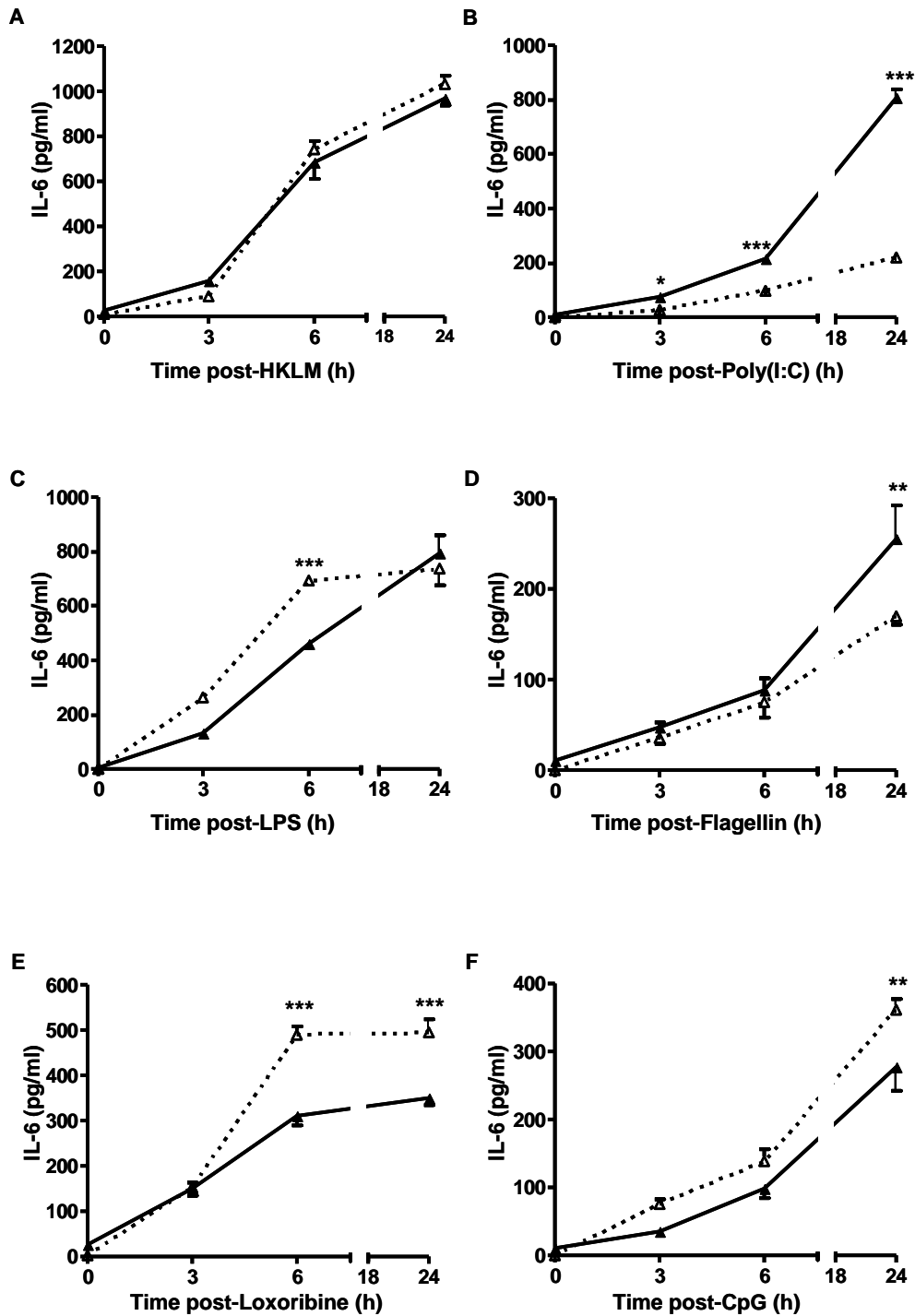
Figure 3.10: Production of IL-6 by AnxA1$^{-/-}$ BMDMs stimulated with TLR agonists. Bone marrow-derived AnxA1$^{+/+}$ and AnxA1$^{-/-}$ macrophages were incubated with different TLR agonists for the indicated times, and supernatants were collected and analyzed for IL-6 content. Values are means±S.E.M of a single experiment in triplicate wells. Data a're representative of n=4 different experiments. Continuous line: AnxA1$^{+/+}$ BMDMs; Dashed line: AnxA1$^{-/-}$ BMDMs

* p<0.05; ** p<0.01; *** p<0.001
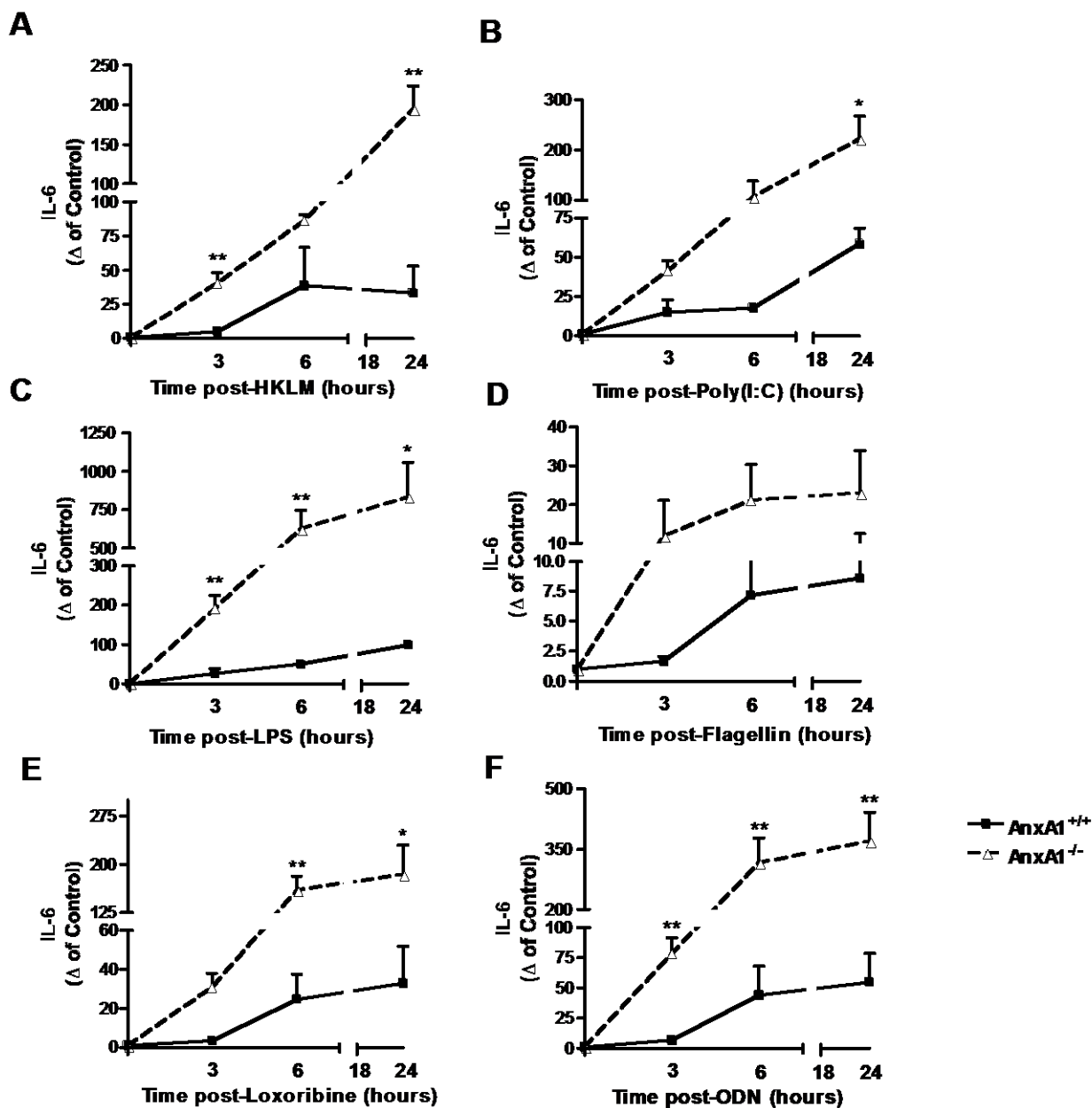
Figure 3.11: Production of IL-6 by AnxA1$^{-/-}$ BMDMs in response to different TLR ligands. Bone marrow-derived AnxA1$^{-/-}$ and AnxA1$^{+/+}$ macrophages were incubated with the different TLR agonists for the indicated times, and supernatants were collected and analyzed for the content of IL-6. Values are expressed as cumulative means±S.E.M. of the Δ of control for IL-6 release. Data are from n=4 different experiments.
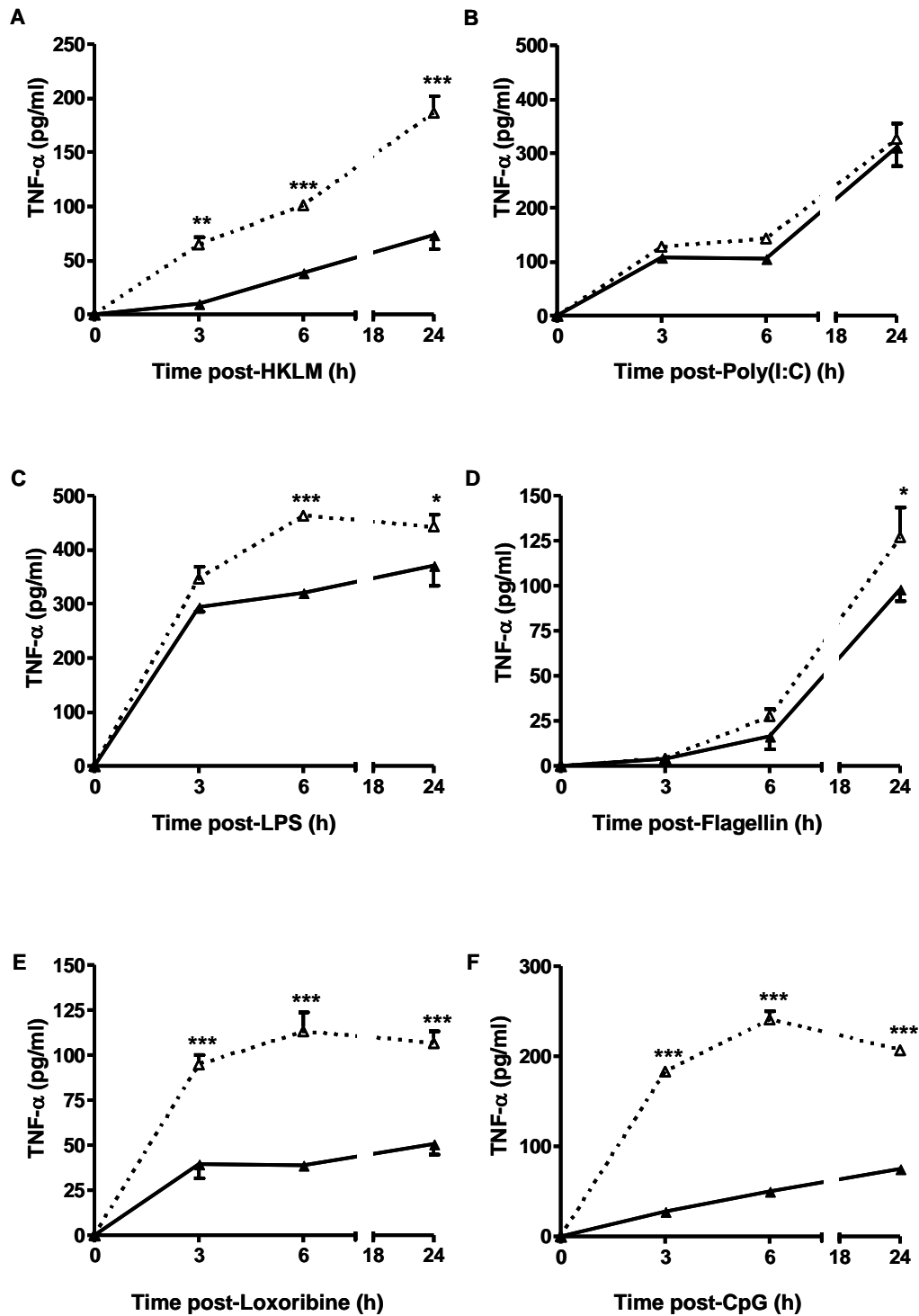
Figure 3.12: Exaggerated production of TNF-α by AnxA1$^{-/-}$ BMDMs after stimulation with MyD88-dependent TLR ligands. Bone marrow-derived AnxA1$^{+/+}$ and AnxA1$^{-/-}$ macrophages were incubated with different TLR agonists for the indicated times, and supernatants were collected and analyzed for content of TNF-α. Values are mean±S.E.M of a single experiment in triplicate wells. Data are representative of n=4 different experiments. Continuous line: AnxA1$^{+/+}$ BMDMs; Dashed line: AnxA1$^{-/-}$ BMDMs

p<0.05; ** p<0.01; *** p<0.001
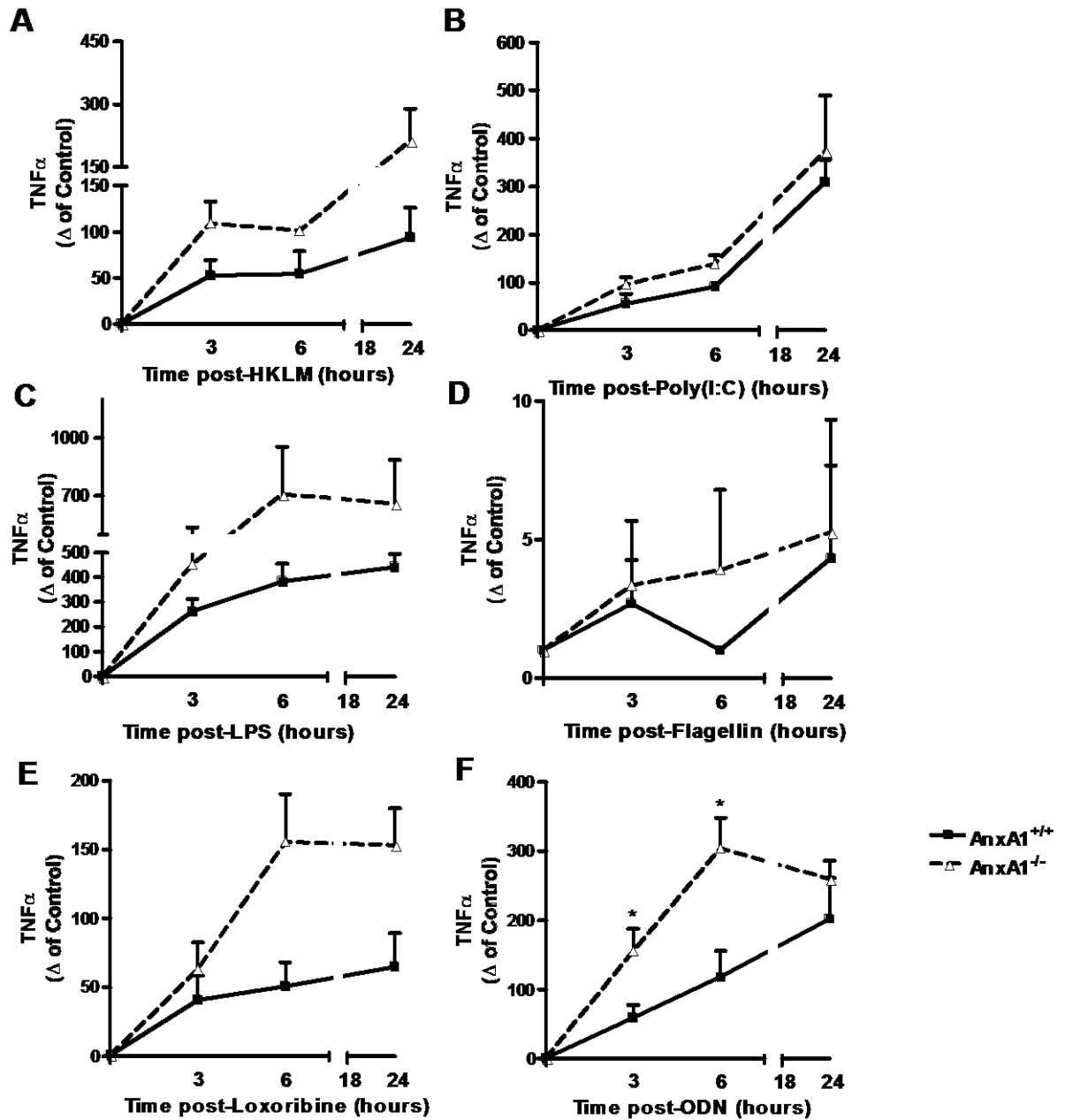
Figure 3.13: **Exaggerated production of TNF-α by AnxA1$^{-/-}$ BMDMs relative to AnxA1$^{+/+}$ BMDMs after stimulation with MyD88-dependent TLR ligands.**

Bone marrow-derived AnxA1$^{-/-}$ and AnxA1$^{+/+}$ macrophages were incubated with the different TLR agonists for the indicated times, and supernatants were collected and analyzed for TNF-α content. Values are expressed as cumulative means±S.E.M. of the Δ of control for TNF-α content. Data are from n=4 different experiments.

3.3.3 Impaired NO production of AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C)

The production of NO was measured by the Griess reaction in the culture supernatants of BMDMs after stimulation for 3, 6, and 24 h with the different TLR agonists. The results of our experiments showed that there was no production of nitrite at 3 and 6 h post-stimulation with any TLR ligand (data not shown), but a significant production at 24 h after stimulation with the ligands poly (I:C) (Fig 3.14 B), LPS (Fig 3.14C), and CpG (Fig 3.14F) in both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs.

The production of nitrite in the AnxA1$^{-/-}$ BMDMs was compared to the AnxA1$^{+/+}$ BMDMs. When the results of 4 independent experiments were combined, we observed a similar secretion of nitrite in the supernatant of the two types of cells after stimulation with LPS (Fig 3.14C) and CpG (Fig 3.14F). However, the production of NO after stimulation with the TLR3 ligand poly (I:C) was significantly impaired in the AnxA1$^{-/-}$ BMDMs (Fig 3.14B).
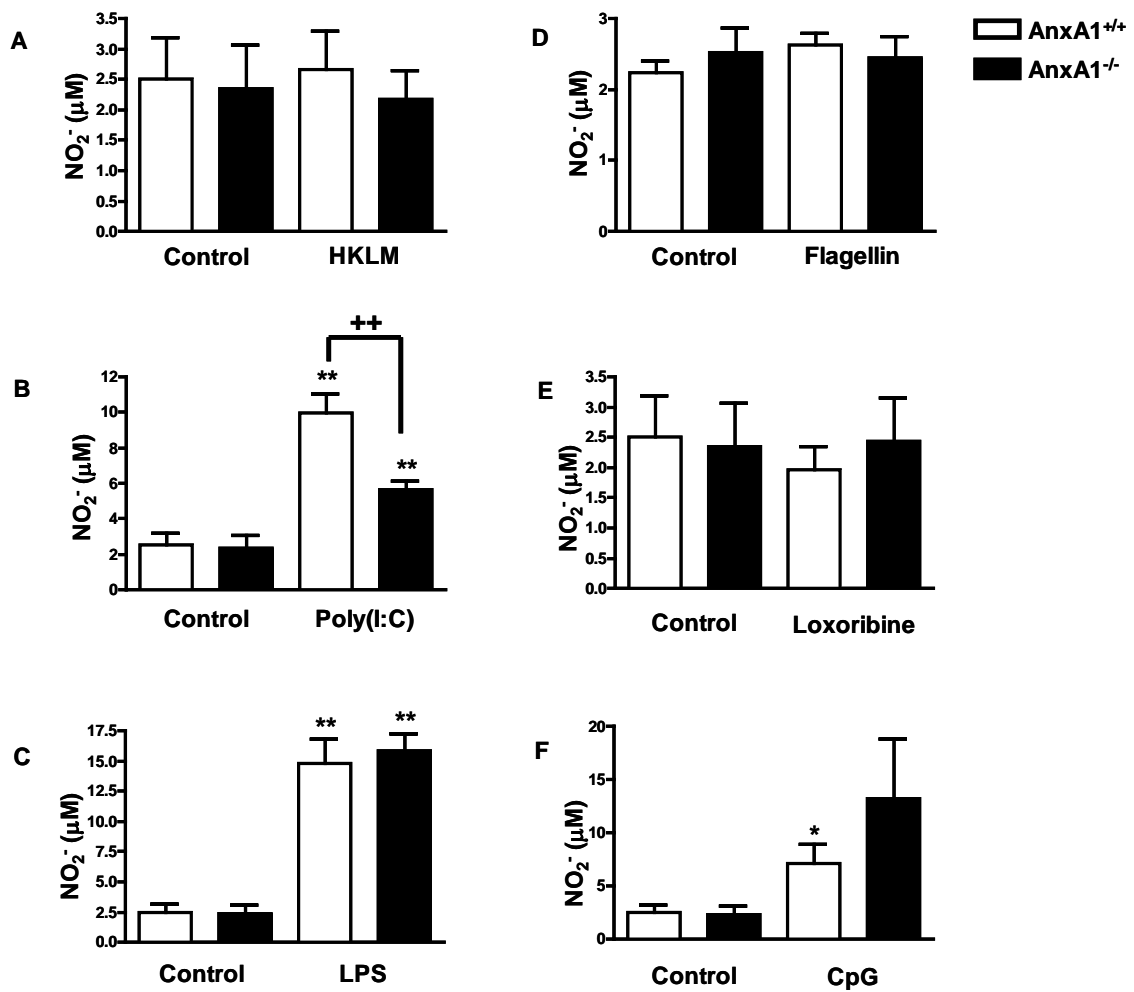
Figure 3.14: Impaired production of NO in AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C).

Bar graphs indicate the content of NO$_2^-$ present in the cell supernatant revealed by the Griess reaction. Values are means±S.E.M. of n=3 experiments.

* p<0.05; ** p<0.01 compared to control; ++ p<0.01 AnxA1$^{-/-}$ compared to AnxA1$^{+/+}$ BMDMs.

3.3.4 Enhanced PGE$_2$ secretion of AnxA1$^{-/-}$ BMDMs after stimulation with CpG

Since PGE$_2$ is secreted by BMDMs after stimulation with certain TLR ligands (Buczynski et al., 2007, Chen et al., 2004, Chen et al., 2009a, Pindado et al., 2007, Steer et al., 2006, Uematsu et al., 2002, Yeo et al., 2003), PGE$_2$ enzyme immunoassay was performed in the culture supernatant in order to investigate whether the PGE$_2$ production in the AnxA1$^{-/-}$ cells was different to the expected. Indeed, our results were consistent with the literature since the ligands HKLM, poly (I:C), LPS and CpG induced the secretion of PGE$_2$ of both the AnxA1$^{+/+}$ and AnxA1$^{-/-}$ macrophages after a 24 h incubation (Fig 3.15 A, B, C, F). On the other hand, the ligands flagellin and loxoribine did not induce the PGE$_2$ production in the AnxA1$^{+/+}$ or in the AnxA1$^{-/-}$ BMDMs (Fig 3.15 D, F). Interestingly, although the concentration of PGE$_2$ detected in the AnxA1$^{-/-}$ cells was similar to that seen in the AnxA1$^{+/+}$ cells after stimulation with HKLM, poly (I:C), and LPS, the PGE$_2$ detected was significantly increased in the AnxA1$^{-/-}$ cells after stimulation with the TLR9 ligand CpG (Fig 3.15 F).
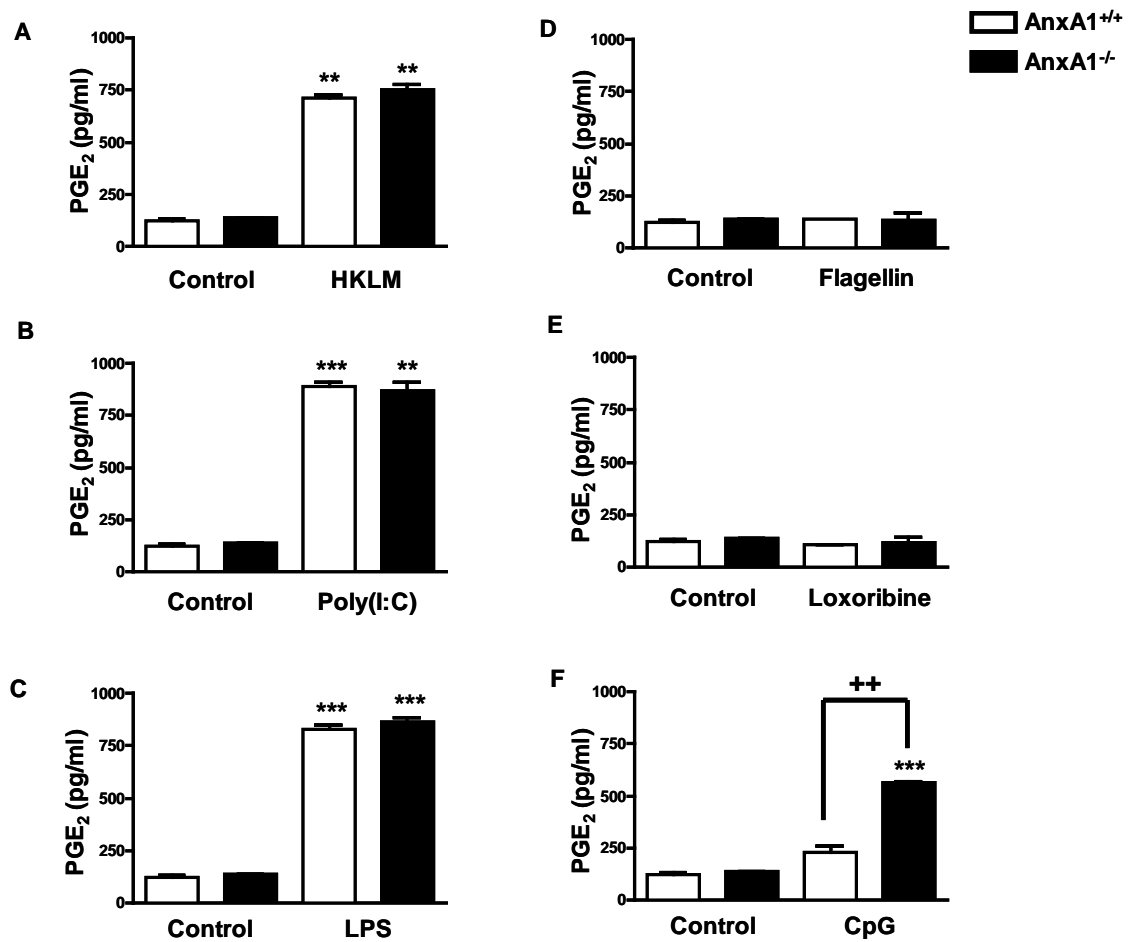
Figure 3.15: Increased PGE$_2$ production by AnxA1$^{-/-}$ BMDMs triggered by CpG.

Bar graphs indicate the content of PGE$_2$ present in the cell supernatants revealed by ELISA. Data are from a single experiment.

** $p<0.01$; *** $p<0.001$ compared to corresponding control;

++$p<0.01$ AnxA1$^{-/-}$ compared to AnxA1$^{+/+}$ BMDMs.

3.3.5 AnxA1^{-/-} BMDMs express higher levels of TLRs

Variable TLR expression in BMDMs could provide an explanation for the differences regarding cytokine production observed between AnxA1^{-/-} and AnxA1^{+/+} BMDMs. A higher receptor expression could trigger different cell response and enhanced production of pro-inflammatory cytokines. Therefore, we performed real-time PCR in order to investigate the relative TLR mRNA expression in unstimulated AnxA1^{+/+} and AnxA1^{-/-} BMDMs. Indeed, as shown in Fig 3.16, the expression of all the TLRs that are involved in the ligation of our tested agonists (TLR2, TLR3, TLR4, TLR5, TLR7, TLR9) was markedly increased in the AnxA1^{-/-} BMDMs.
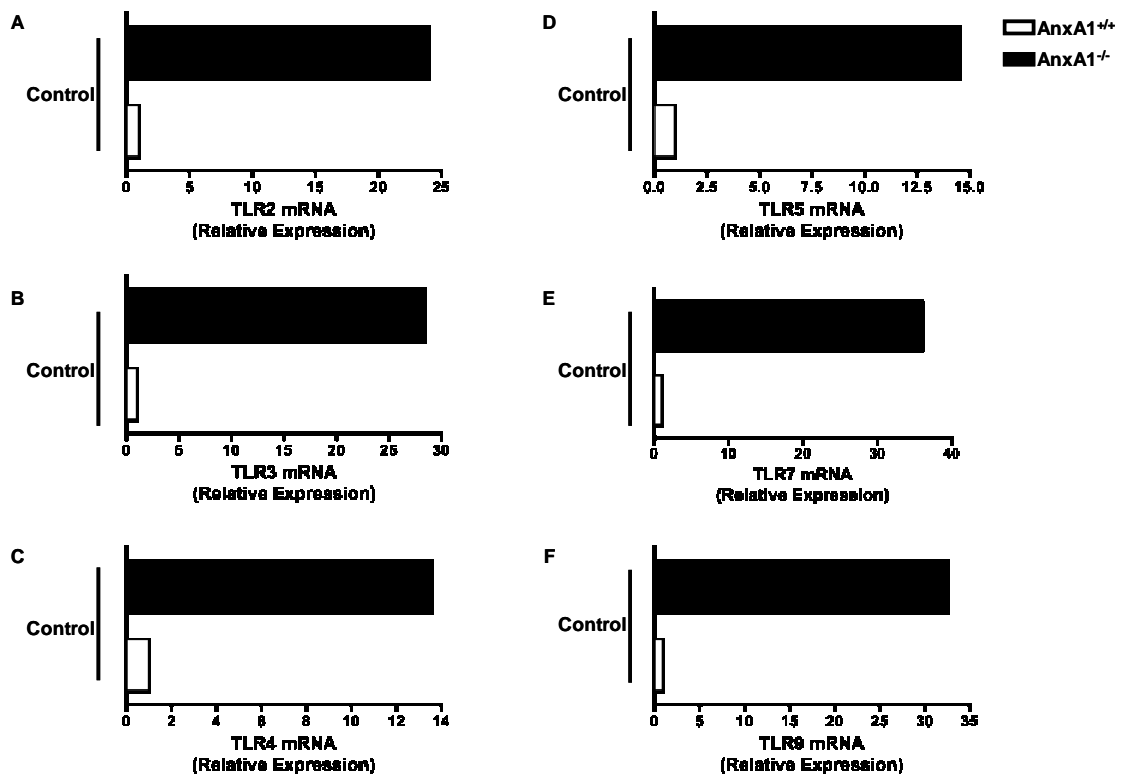
Figure 3.16: Increased TLR expression of AnxA1^{-/-} BMDMs.
Analysis of TLR expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs by real time PCR. Values are from a single experiment.

3.3.6 Enhanced NF-κB DNA-binding in AnxA1^{-/-} BMDMs

Both MyD88-dependent and MyD88-independent/TRIF-dependent pathways lead to the activation of NF-κB (Kawai and Akira, 2007). More specifically, both pathways are characterised by proteosomal degradation of the IκBα complex and translocation of NF-κB to the nucleus where it acts as the major transcription factor of the signalling pathway and leads to the expression of numerous proinflammatory genes such as IL-6 and TNF-α. One gene activated by NF-κB is the gene responsible for its own inhibitor IκBα. After the synthesis of IκBα, it is translocated to the cytoplasm to bind to NF-κB, preventing it from entering the nucleus and binding to DNA. Therefore, IκBα is responsible for a strong negative feedback in the NF-κB cycle. Oscillations in the NF-κB activity by electrophoretic mobility shift assay (EMSA) have been described, controlled by the three IκB isoforms (IκBα, IκBβ, and IκBε) (Hoffmann et al., 2002). The number, period, and amplitude of these oscillations may control the dynamics of gene expression (Nelson et al., 2004), in a similar way that the quality of sound is altered by pressing the keys and pedals of a piano. Since we observed important differences in IL-6 and TNF-α between the two genotypes, we set out to investigate whether there was also a difference in the NF-κB DNA binding that could explain our results. Therefore, we performed EMSA for NF-κB DNA binding and western blotting for IκBα expression. Initially pilot studies were performed using 5µg of nuclear extract, which was changed to 10µg of nuclear extract to increase the quality of the signal.

As seen in Fig 3.17, even under basal conditions, before stimulation with any TLR ligand, there were differences between the two genotypes. The NF-κB DNA binding in AnxA1$^{-/-}$ was decreased compared to AnxA1$^{+/+}$ BMDMs. At the same time, the IκBα protein expression by AnxA1$^{-/-}$ BMDMs was increased indicating less degradation of the complex and reduced translocation of NF-κB to the nucleus (Fig 3.17).

After addition of the different TLR agonists to the WT cells, the normal activation of NF-κB occurred (Fig 3.18), as described elsewhere (Alexopoulou et al., 2001, Bagchi et al., 2007, Hemmi et al., 2000, Muller et al., 2001, Yamamoto et al., 2003), followed by parallel degradation of IκBα (Fig 3.19). Densitometric analysis of both NF-κB EMSA and IκBα western blotting revealed a different kinetic of NF-κB activation/ IκBα degradation for each TLR ligand and important differences between AnxA1$^{+/+}$ and AnxA1$^{-/-}$ cells (Fig 3.20, 3.21, and 3.22).

An oscillatory pattern for NF-κB activation and IκBα degradation was seen after stimulation with all the MyD88-dependent TLR ligands and the TRIF-dependent ligand poly (I:C) (Fig 3.20, 3.21, 3.22). On the other hand, the NF-κB activation after stimulation with LPS followed a linear pattern (Fig 3.22), as described by Covert *et al* (Covert et al., 2005). Indeed, when both MyD88 and TRIF-pathways were stimulated, as in the case of TLR4 stimulation, NF-κB followed a non-oscillatory behaviour, possibly due to the interaction of the two pathways (Covert et al., 2005).

Stimulation with all TLR ligands provoked a different kinetic of NF-κB activation/ IκBα degradation by AnxA1$^{-/-}$ BMDMs. Surprisingly, AnxA1$^{-/-}$ cells exhibited NF-κB oscillations of higher amplitude after stimulation with all TLR ligands (Fig 3.20, 3.21 and 3.22). This was accompanied by an analogous profile of IκBα degradation (Fig 3.20, 3.21 and 3.22). Enhanced NF-κB DNA-binding occurred by AnxA1$^{-/-}$ BMDMs at 30min post-stimulation with all the strictly MyD88-dependent TLR ligands. On the contrary, the peak of NF-κB activation was delayed after stimulation with poly (I:C) or LPS (Fig 3.22). Therefore, higher NF-κB DNA-binding occurred by AnxA1$^{-/-}$ cells at 60min after TRIF stimulation.

.

SUMMARY

In this section the differences in NF-κB DNA binding and IκBα expression between AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs after stimulation with TLR ligands were investigated.

- AnxA1$^{-/-}$ BMDMs exhibit NF-κB activation oscillations of higher amplitude after stimulation with all the TLR ligands.

- Enhanced NF-κB DNA-binding by AnxA1$^{-/-}$ BMDMs occurred within 30min of stimulation with MyD88-dependent TLR ligands but required 60min to trigger TRIF
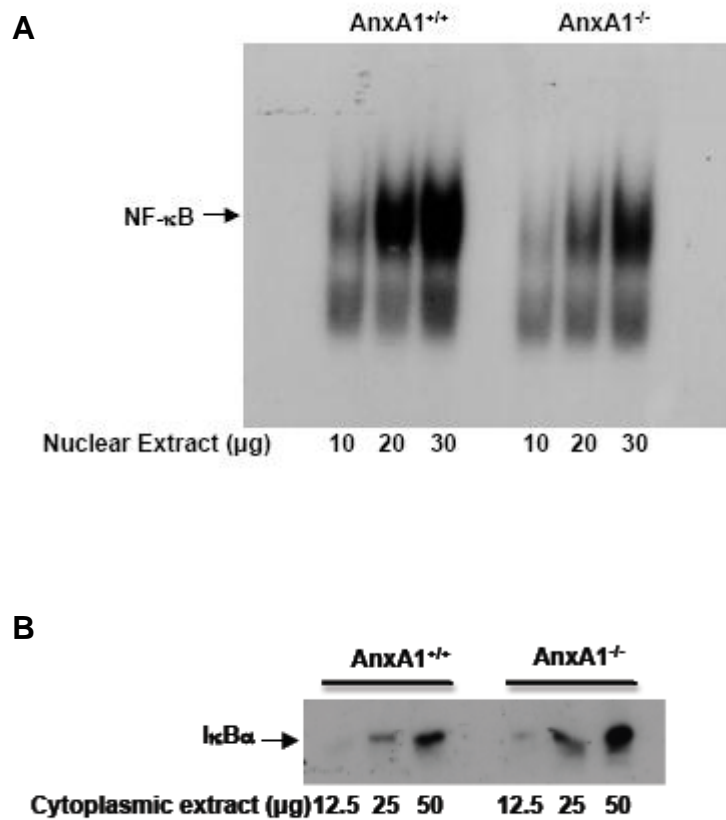
Figure 3.17: Impaired NF-κB DNA binding in AnxA1^{-/-} BMDMs under basal conditions.

A) Electrophoretic mobility shift assay showing NF-κB/DNA-binding activity in increasing concentrations of nuclear extracts of AnxA1^{+/+} and AnxA1^{-/-} BMDMs.

B) Western blot showing IκBα expression in increasing concentration of cytoplasmic extracts of AnxA1^{+/+} and AnxA1^{-/-} BMDMs. Data are from a single experiment representative of n=4 experiments.
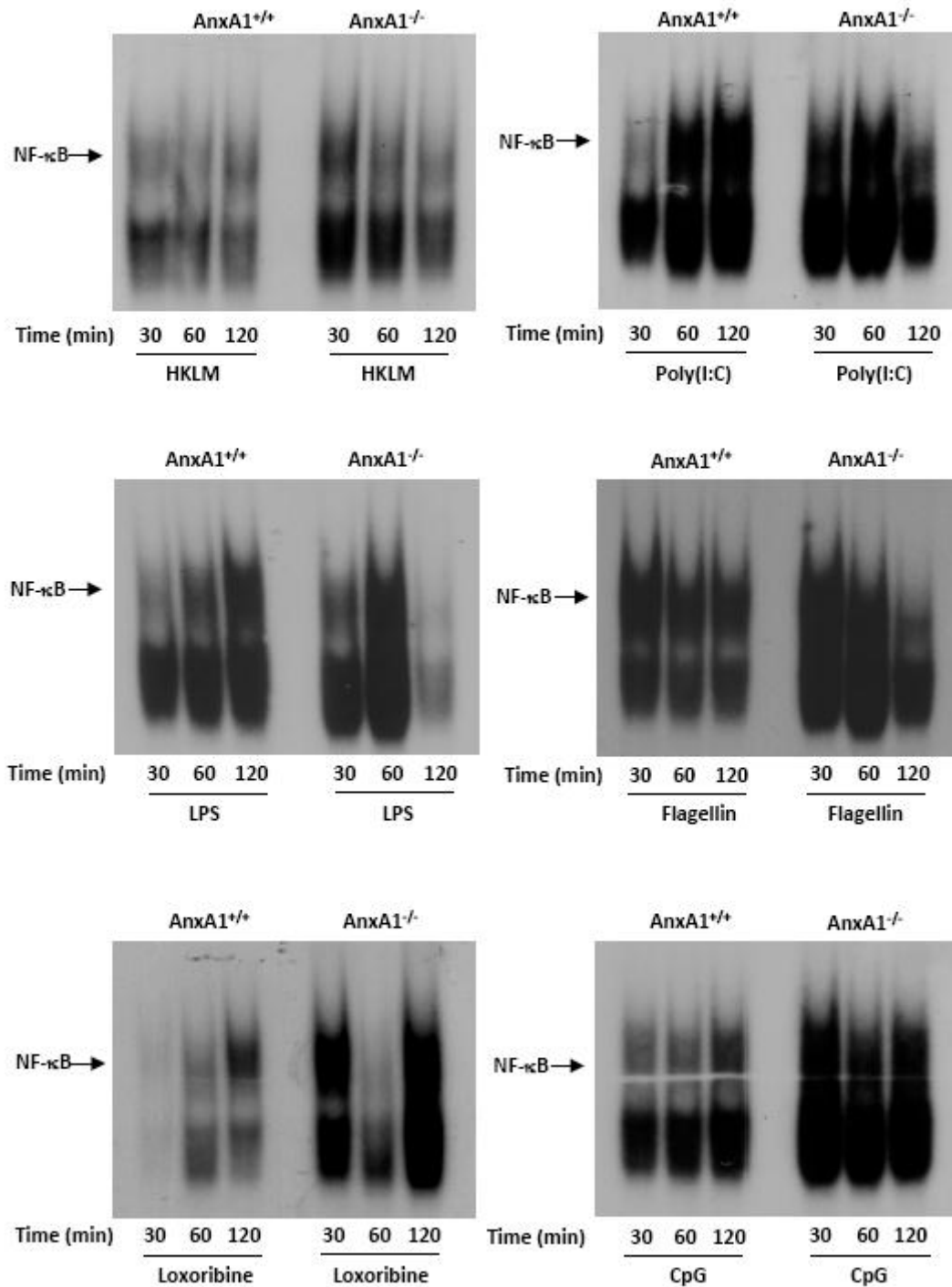
Figure 3.18: Increased NF-κB DNA binding in AnxA1^{-/-} BMDMs after stimulation with different TLR agonists.

Electrophoretic mobility shift assay showing NF-κB/DNA-binding activity in nuclear extracts of AnxA1^{+/+} and AnxA1^{-/-} BMDMs stimulated with the TLR ligands for the indicated times. Data are from a single experiment representative of n=4 experiments.
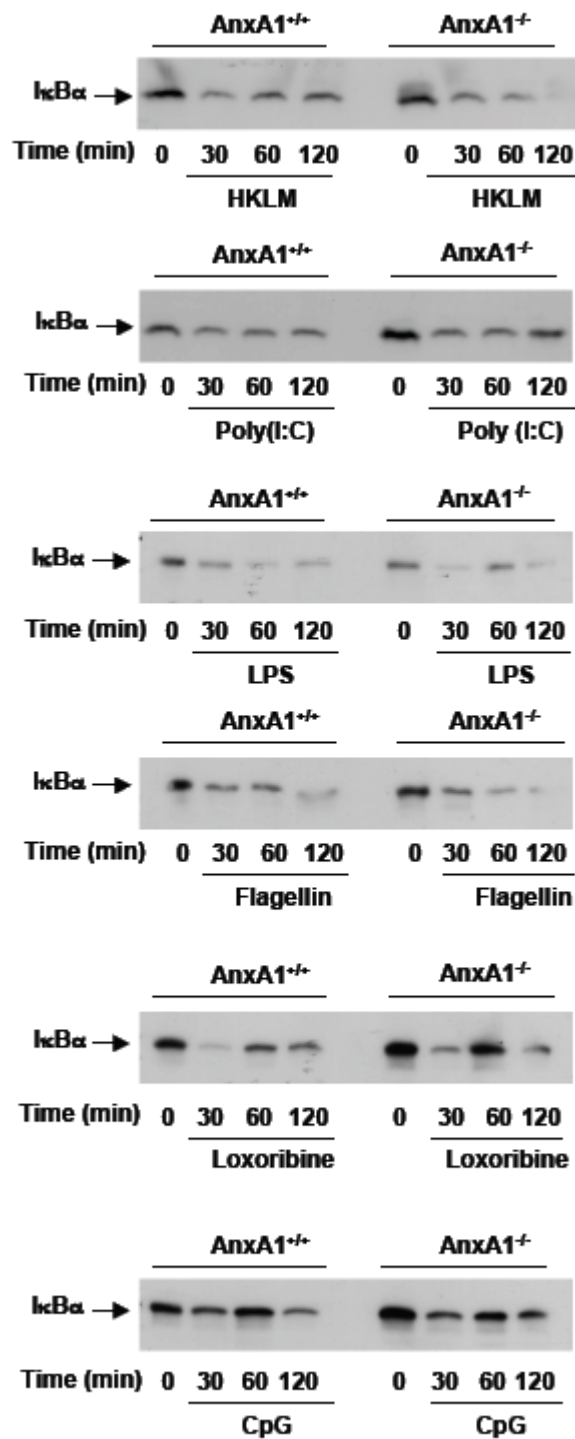
Figure 3.19: Profile of IκBα degradation in AnxA1^{-/-} BMDMs.

Western blotting analysis of IκBα degradation profile in AnxA1^{+/+} and AnxA1^{-/-} BMDMs treated with the TLR ligands for the indicated times.
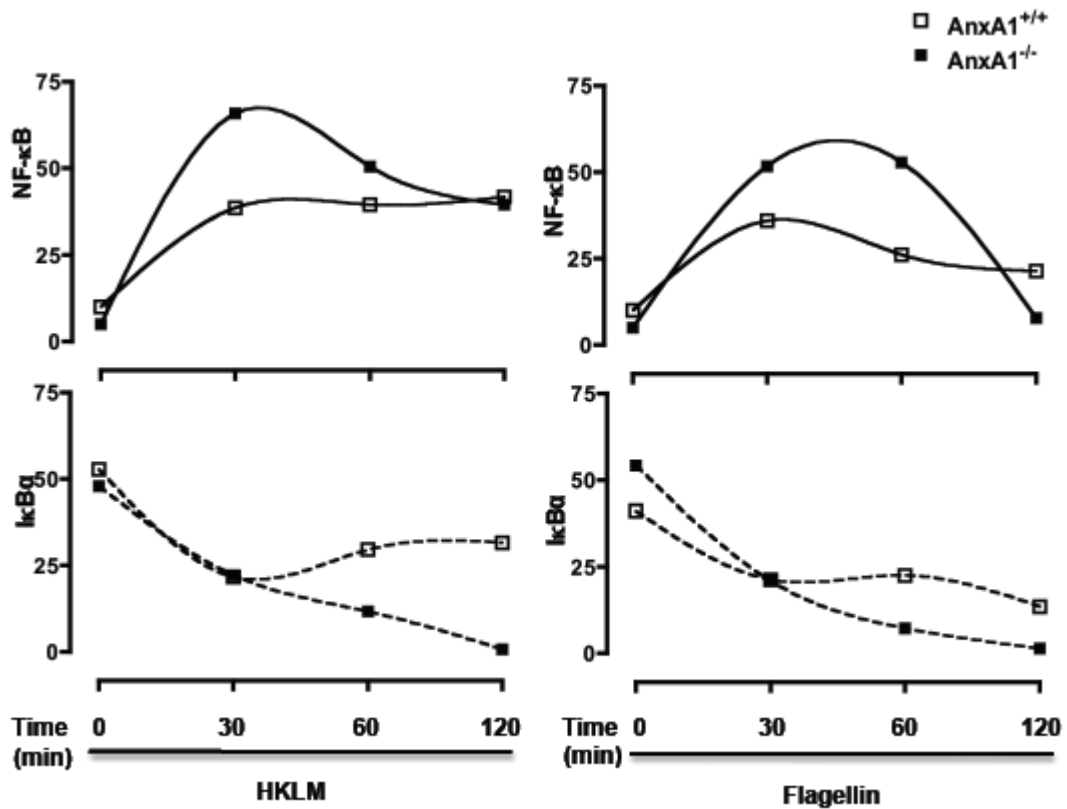
Figure 3.20: **Profile of NF-κB DNA binding and IκBα degradation by BMDMs in response to HKLM and flagellin.**

Graphs showing together the densitometric analysis of EMSA of NF-κB/DNA-binding activity (above) and western blot of IκBα expression (below) in AnxA1^{+/+} and AnxA1^{-/-} BMDMs stimulated with HKLM or flagellin for the indicated times. Data are from a single experiment representative of n=4 experiments.
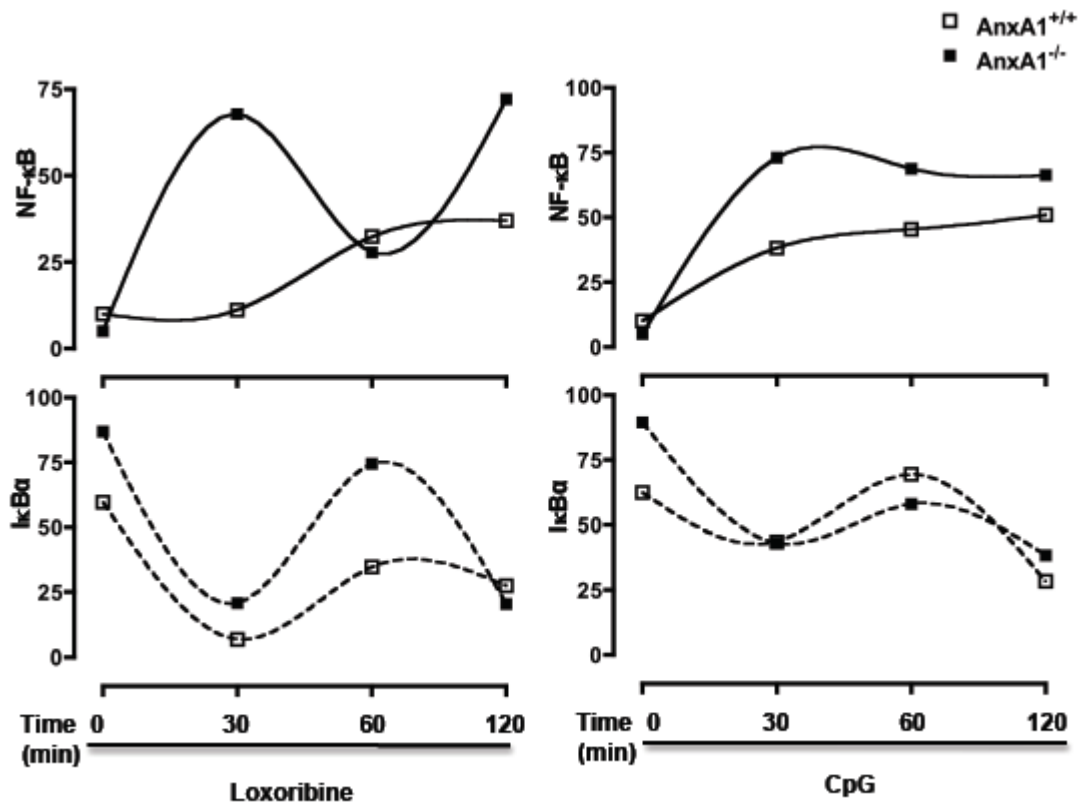
Figure 3.21: Profile of NF-κB DNA binding and IκBα degradation by BMDMs in response to loxoribine and CpG.

Graphs showing together the densitometric analysis of EMSA of NF-κB/DNA-binding activity (above) and western blot of IκBα expression (below) in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs stimulated with loxoribine or CpG for the indicated times. Data are from a single experiment representative of n=4 experiments.
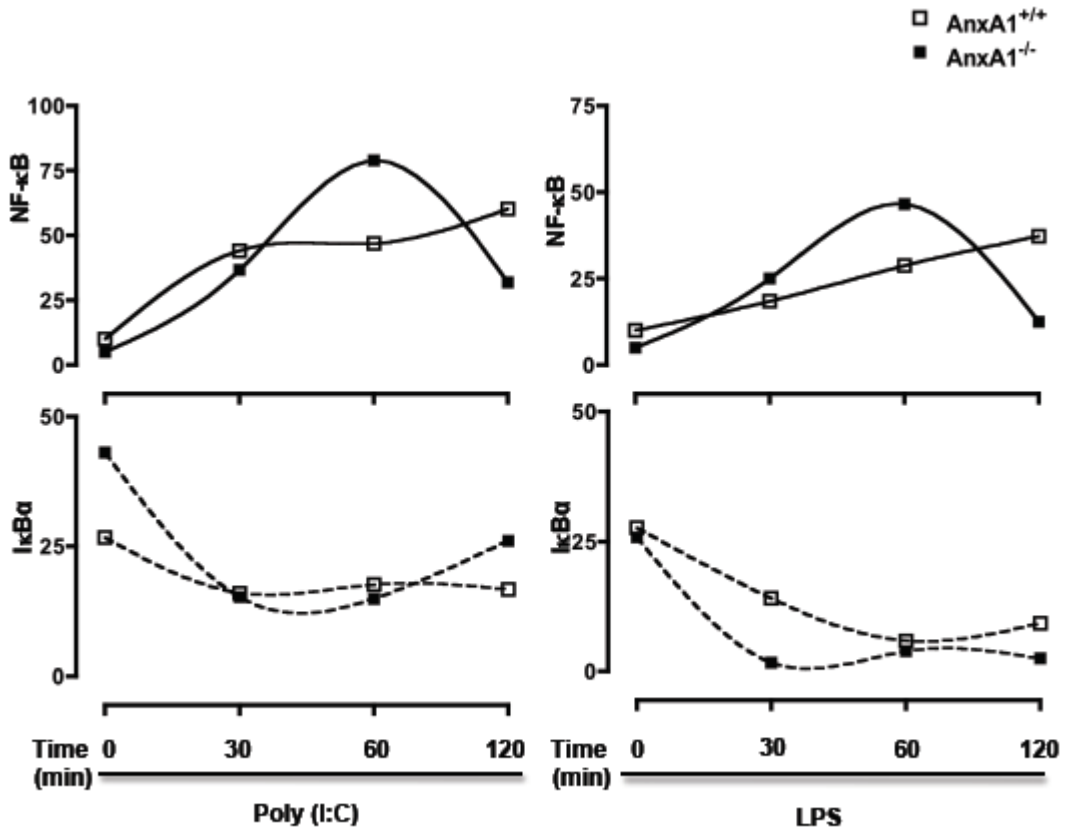
Figure 3.22: Profile of NF-κB DNA binding and IκBα degradation by BMDMs in response to poly (I:C) and LPS.

Graphs showing together the densitometric analysis of EMSA of NF-κB/DNA-binding activity (above) and western blot of IκBα expression (below) in AnxA1^{+/+} and AnxA1^{-/-} BMDMs stimulated with poly (I:C) or LPS for the indicated times. Data are from a single experiment representative of n=4 experiments.

3.3.7 Enhanced ERK1/2 activation in AnxA1^{-/-} BMDMs

Next, as an effort to explain our previous results, we attempted to determine the exact difference in the TLR signalling pathway and the FPR2 signalling pathway, between the AnxA1^{-/-} and AnxA1^{+/+} BMDMs by assessing several proteins involved in the TLR signalling pathway such as the MAP kinases ERK1/2, JNK, and p38. Unfortunately, despite our intense efforts, we were unable to resolve this using western blotting techniques. One possible reason for this is the presence of phosphatases in the 5% low-fat milk used throughout these experiments. Therefore, we tried a different approach by utilising a commercially available ELISA.

Firstly, we investigated whether differential activation of ERK1/2 could be involved in the increased cytokine release observed in AnxA1^{-/-} BMDMs after stimulation with TLR ligands. As expected (Kawai and Akira, 2007), all TLR agonists induced ERK1/2 activation, as measured by phosphorylation of ERK1/2 in AnxA1^{+/+} and AnxA1^{-/-} BMDMs, with a peak approximately at 30 min after stimulation (Fig 3.23). When the two types of cells were compared, we observed that the phosphorylation in the AnxA1^{-/-} BMDMs was enhanced at all time-points relative to the WT controls, indicating an increased ERK1/2 activation (Fig 3.23).

To validate this result, we incubated WT BMDMs with each tested TLR ligand alone or in combination with 2nM hr-AnxA1. As seen in Fig 3.24, the cells that were treated with the hr-AnxA1 exhibited less ERK1/2 phosphorylation compared to the cells that were treated with the TLR ligands alone.

Therefore, it has been shown that utilising an as yet unidentified mechanism, the presence of AnxA1 in these cells seems to reduce the phosphorylation of ERK1/2 induced by the TLR agonist cascade thereby leading to a less pronounced inflammatory response. This also may explain the increased ERK1/2 phosphorylation and proinflammatory response seen in the absence of AnxA1.
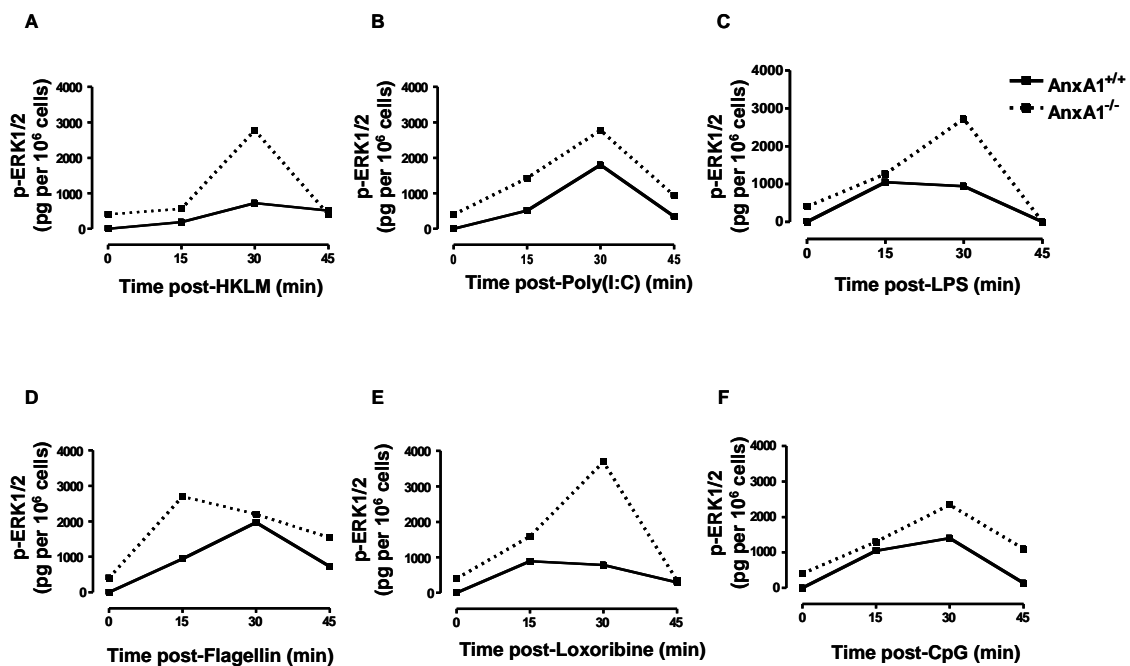
Figure 3.23: Increased phospho-ERK1/2 by AnxA1^{-/-} BMDMs.
Analysis by ELISA of the levels of phospho-ERK1/2 present in cell lysates of AnxA1^{+/+} and AnxA1^{-/-} BMDMs stimulated with the different TLR ligands for the indicated times. Graphs are from a single experiment representative of n=3 experiments.
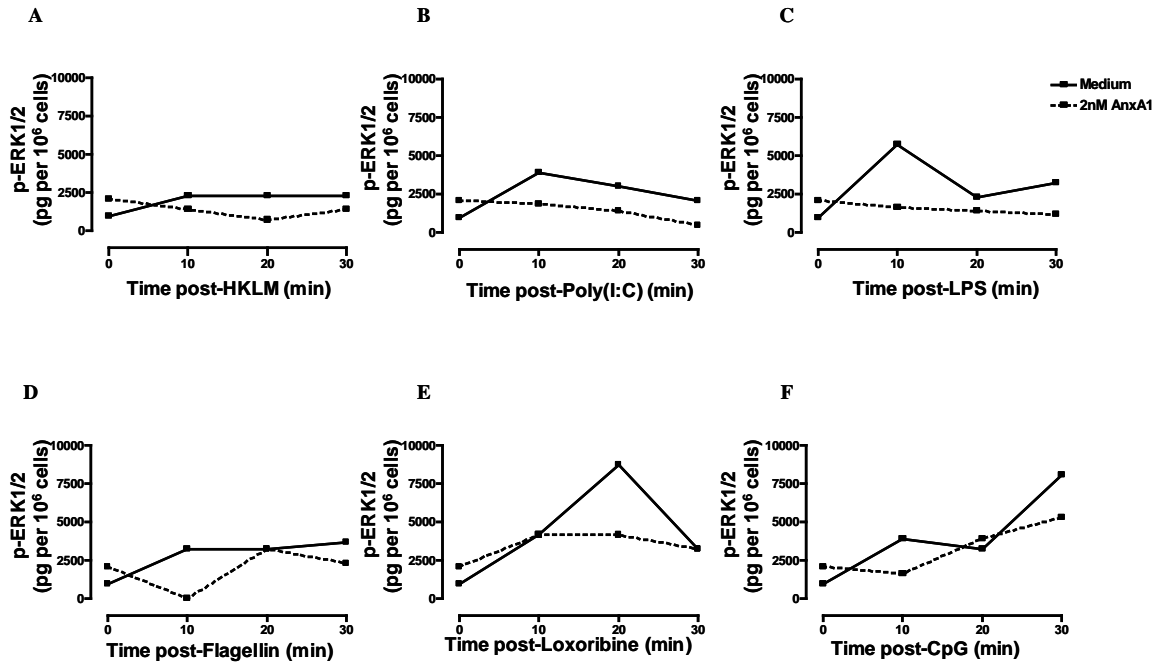
Figure 3.24: Decrease of phospho-ERK1/2 after treatment with AnxA1.

Analysis by ELISA of the levels of phospho-ERK1/2 of cell lysates of AnxA1^{+/+} BMDMs stimulated with the different TLR ligands and treated with 2nM AnxA1 or medium alone for the indicated times. Graphs are from a single experiment representative of n=2 experiments.

3.3.8 Analysis of MyD88- and TRIF-dependent gene expression in AnxA1^{-/-} BMDMS

When the gene expression profiles of wild-type cells and IRF-3 knockout cells were compared following viral infection, 3 sets of induced genes were revealed. These are IRF-3-dependent, IRF-3-independent, and IRF-3-augmented (not strictly dependent on IRF-3) direct response genes (Andersen et al., 2008). Since IRF-3 is a transcription factor involved in the TRIF-dependent pathway and not in the MyD88 pathway, we set to investigate the gene expression profiles of a set of these genes that belongs to these 3 categories (Fig 3.25) after stimulation of BMDMs with a strictly TRIF-dependent TLR ligand (poly (I:C)), a strictly MyD88-dependent TLR ligand (CpG) and a ligand which stimulates both pathways (LPS). Thereafter, Real-time PCR was performed to reveal the gene expression profiles of AnxA1^{+/+} and AnxA1^{-/-} BMDMs.

IFN-β1 and *Ifit2* (*ISG54*) are considered IRF3-dependent genes when TLR3 is stimulated (Andersen et al., 2008). When we stimulated BMDMs with poly (I:C), we observed a higher expression in AnxA1^{-/-} (Fig 3.26, 3.27). In addition, when BMDMs were stimulated with LPS, a different delayed kinetic was recorded in AnxA1^{-/-} cells (Fig 3.26, 3.27). On the other hand, when cells were stimulated with the MyD88-dependent TLR ligand CpG, no differences were seen between the two genotypes.

The IRF-3 dependent gene *NOS II* (Youn et al., 2005, Youn et al., 2008) was more highly expressed in   AnxA1^{-/-} BMDMs at the basal level (Fig 3.28). In addition, we observed a different profile of gene expression after stimulation with all the tested

TLR ligands when AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs were compared. After stimulation with poly (I:C), *NOS II* gene expression peaked at 3h in AnxA1$^{-/-}$ BMDMs. On the other hand, the gene expression was more gradual in AnxA1$^{+/+}$ BMDMs. Therefore, although at earlier time-points *NOS II* expression was higher in AnxA1$^{-/-}$, there were no differences between the two genotypes by 24h. After stimulation with LPS or CpG, AnxA1$^{-/-}$ BMDMs induced a peak of gene expression at 3h and thereafter gradually decreased. However, although at earlier time-points the gene expression had been enhanced in AnxA1$^{-/-}$ BMDMs, it was severely impaired at the following 24h-time-point. Overall, different kinetics were observed between the two genotypes post stimulation with all tested TLR ligands.

IL-6 gene expression was increased in AnxA1$^{-/-}$ cells under basal conditions (Fig 3.29). After stimulation with LPS or CpG, the gene expression of AnxA1$^{-/-}$ BMDMs peaked at 3h and was importantly higher than AnxA1$^{+/+}$ BMDMs (Fig 3.29). After stimulation with poly (I:C), although at 3h the gene expression had been slightly higher in AnxA1$^{-/-}$ BMDMs, the levels were similar at the following time-points (Fig 3.29).

TNF-α is considered an IRF3-independent gene (Andersen et al., 2008, Tian et al., 2005a, Tian et al., 2005b). When BMDMs were stimulated with poly (I:C), we did not observe major differences in gene expression between the two genotypes. On the other hand, when cells were stimulated with LPS or CpG, a delayed kinetic yielding a much higher gene expression at 6h was seen by AnxA1$^{-/-}$ BMDMs (Fig 3.30).

The CXCL10 gene (IRF-3 augmented gene) expression was exaggerated in AnxA1$^{-/-}$ BMDMs both at the basal level and after stimulation with all tested TLR ligands (Fig 3.31). However, the degree of upregulation of CXCL10 gene expression was lower in AnxA1$^{-/-}$ compared to AnxA1$^{+/+}$ BMDMs after stimulation with all the tested TLR ligands. This indicates that the enhanced gene expression post TLR-stimulation mainly reflects the increased basal level observed in AnxA1$^{-/-}$ BMDMs.

COX2 is considered an IRF3-dependent gene when triggering TLR3 (Youn et al., 2005, Youn et al., 2008) but a MyD88-dependent gene when triggering TLR4 (Bjorkbacka et al., 2004, Kawai et al., 2001). Post-poly(I:C) stimulation, an enhanced gene expression was seen in AnxA1$^{-/-}$ cells (Fig 3.32). After stimulation with LPS or CpG, a different kinetic was also observed (Fig 3.32).

<div style="border:1px solid black; padding:1em;">

SUMMARY

In this section the gene expression profiles of AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs were compared after stimulation with TRIF-dependent and MyD88-dependent TLR ligands.

- AnxA1 regulates the TLR signalling pathway at two levels: chiefly by blocking the MyD88 pathway and to a lesser extent by interacting with the IRF3-dependent TRIF pathway.

</div>
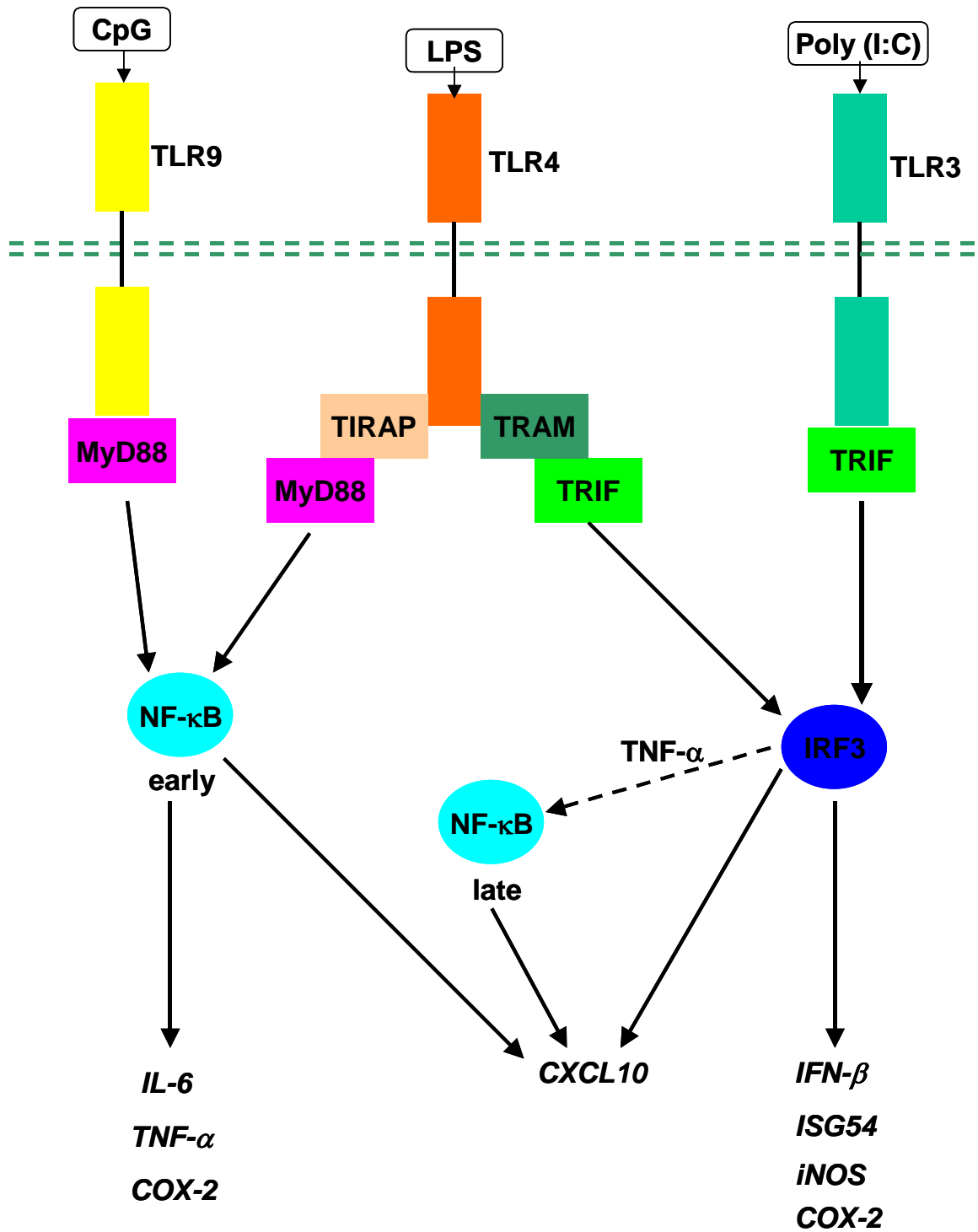
Figure 3.25: Diagram illustrating MyD88-dependent and IRF3-dependent genes post-stimulation with different TLR ligands.
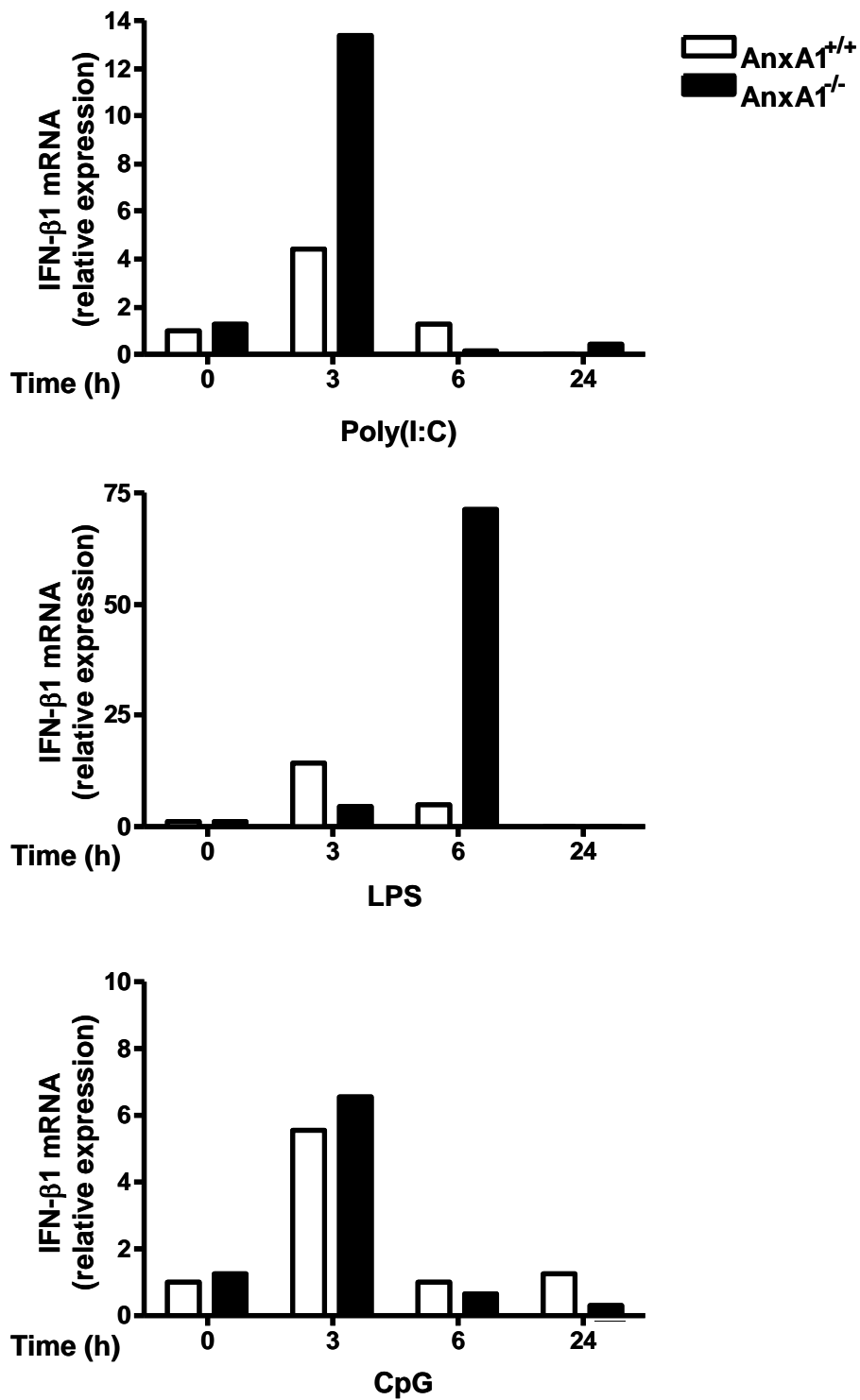
Figure 3.26: Analysis of *IFN-β1* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs.

Real-time PCR analysis of the *IFN-β* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
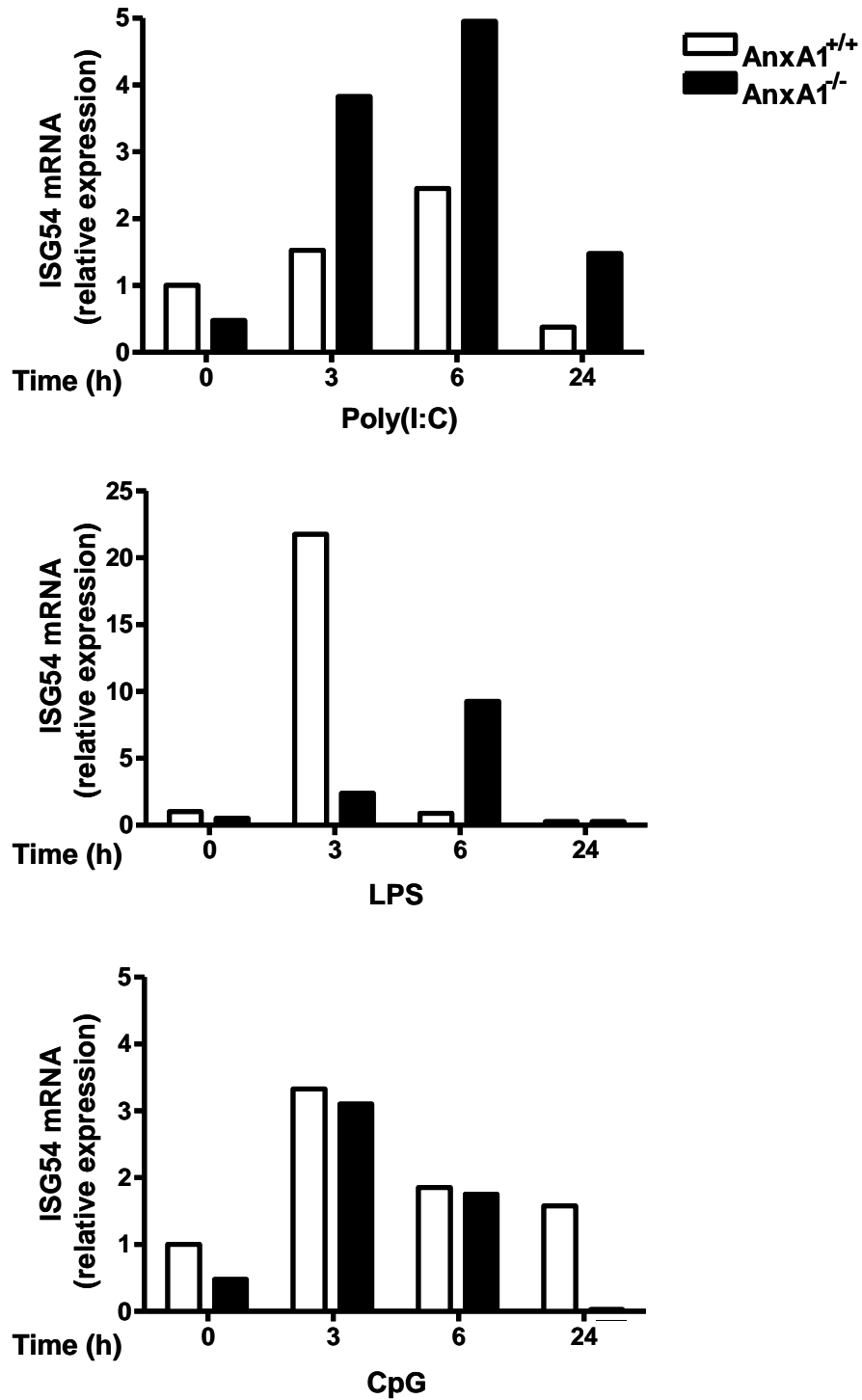
Figure 3.27: **Analysis of *ISG54* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs**

Real-time PCR analysis of the *ISG54* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
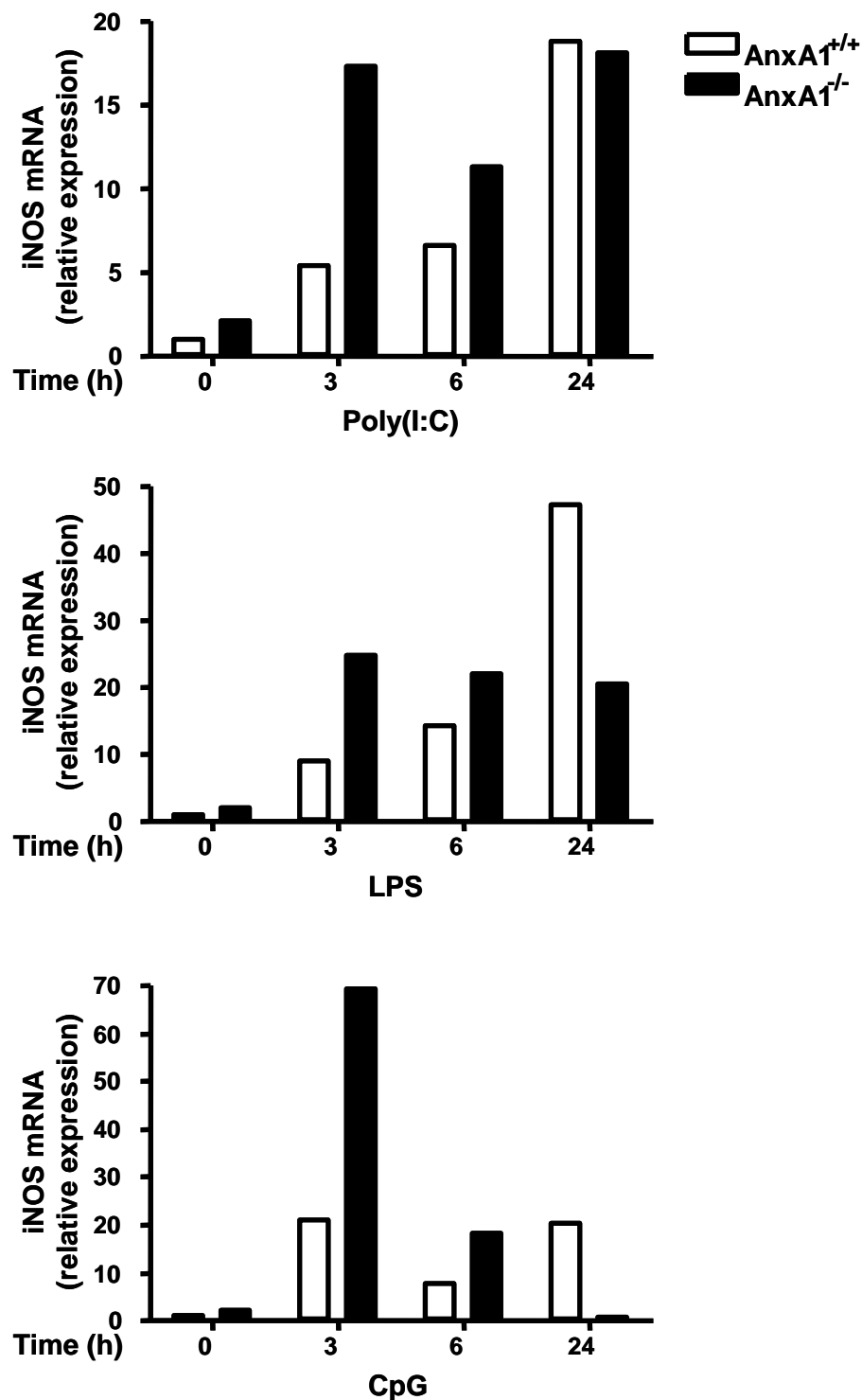
Figure 3.28: Analysis of iNOS gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs

Real-time PCR analysis of the iNOS gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
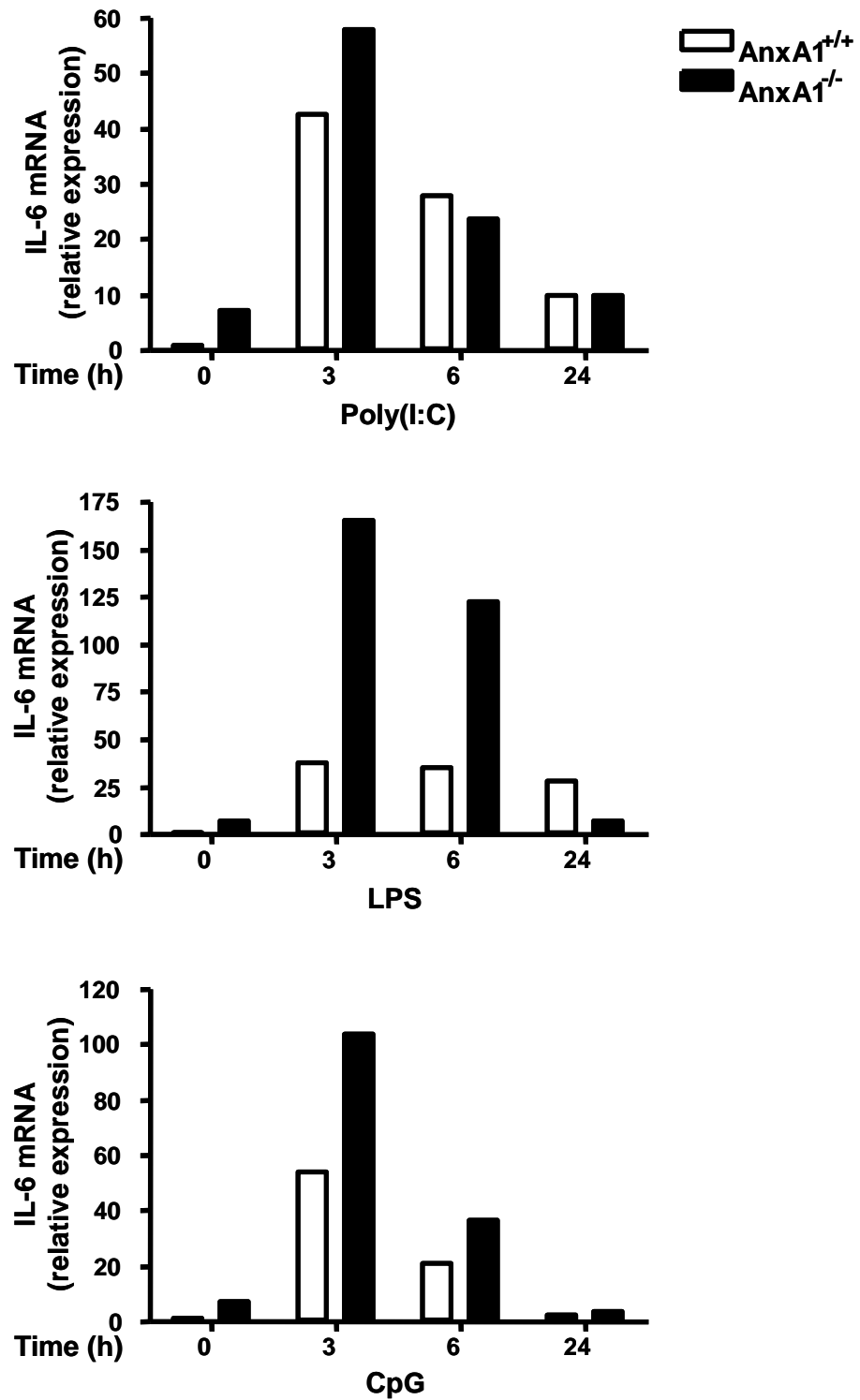
Figure 3.29: Analysis of IL-6 gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs

Real-time PCR analysis of the IL-6 gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
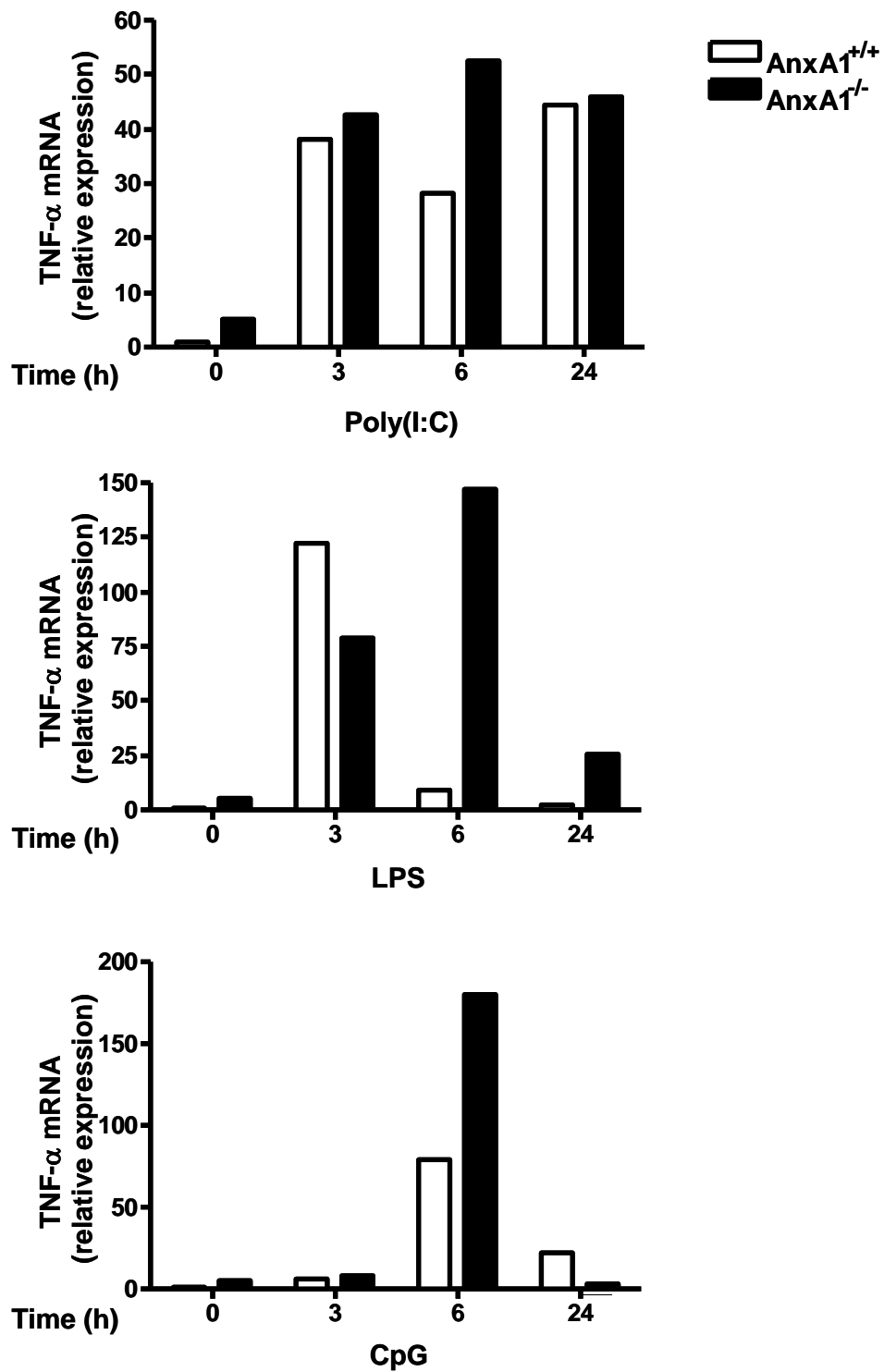
Figure 3.30: Analysis of *TNF-α* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs.

Real-time PCR analysis of the *TNF-α* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
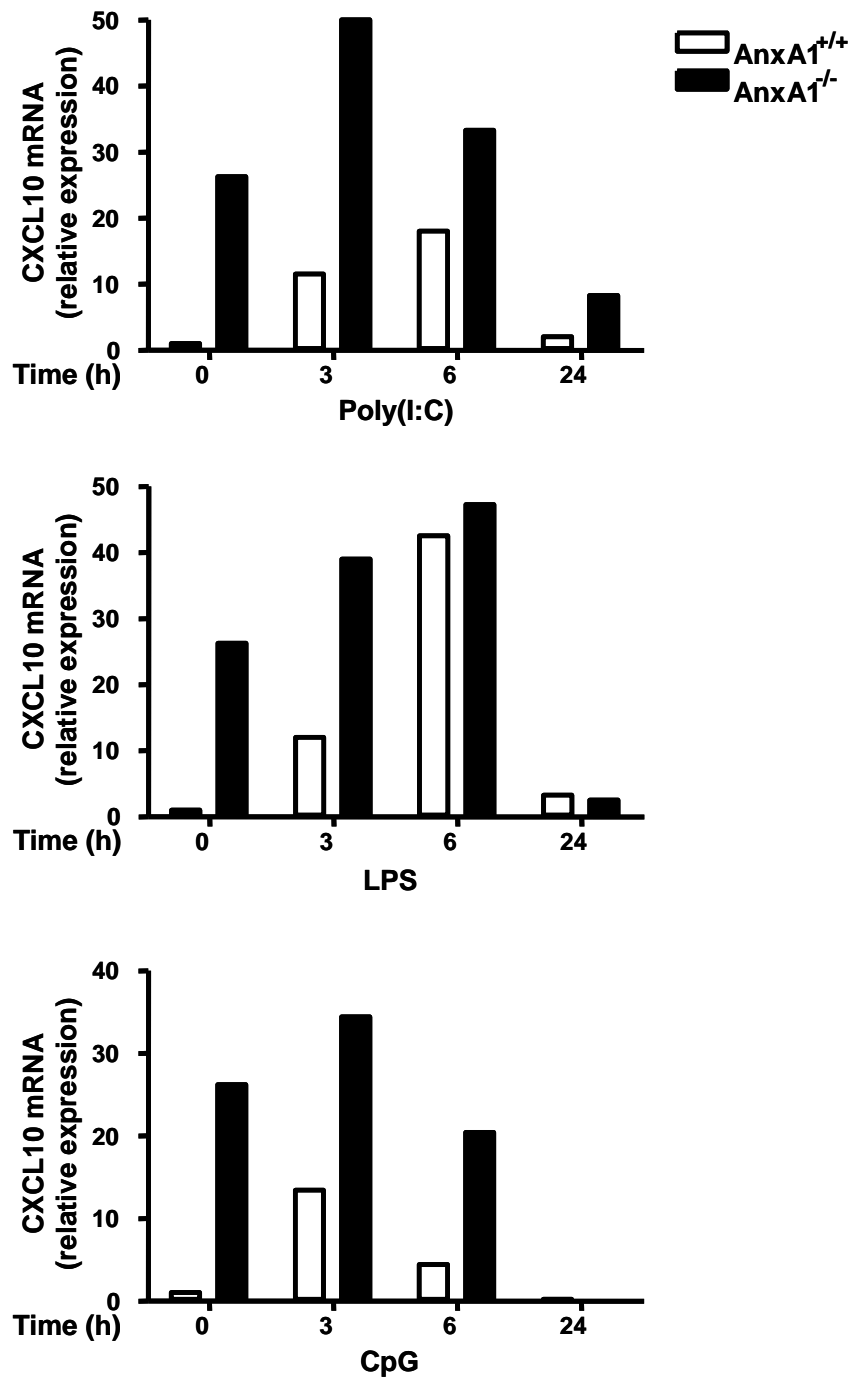
Figure 3.31: Analysis of CXCL10 (IP10) gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs. Real-time PCR analysis of the CXCL10 (IP10) gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.
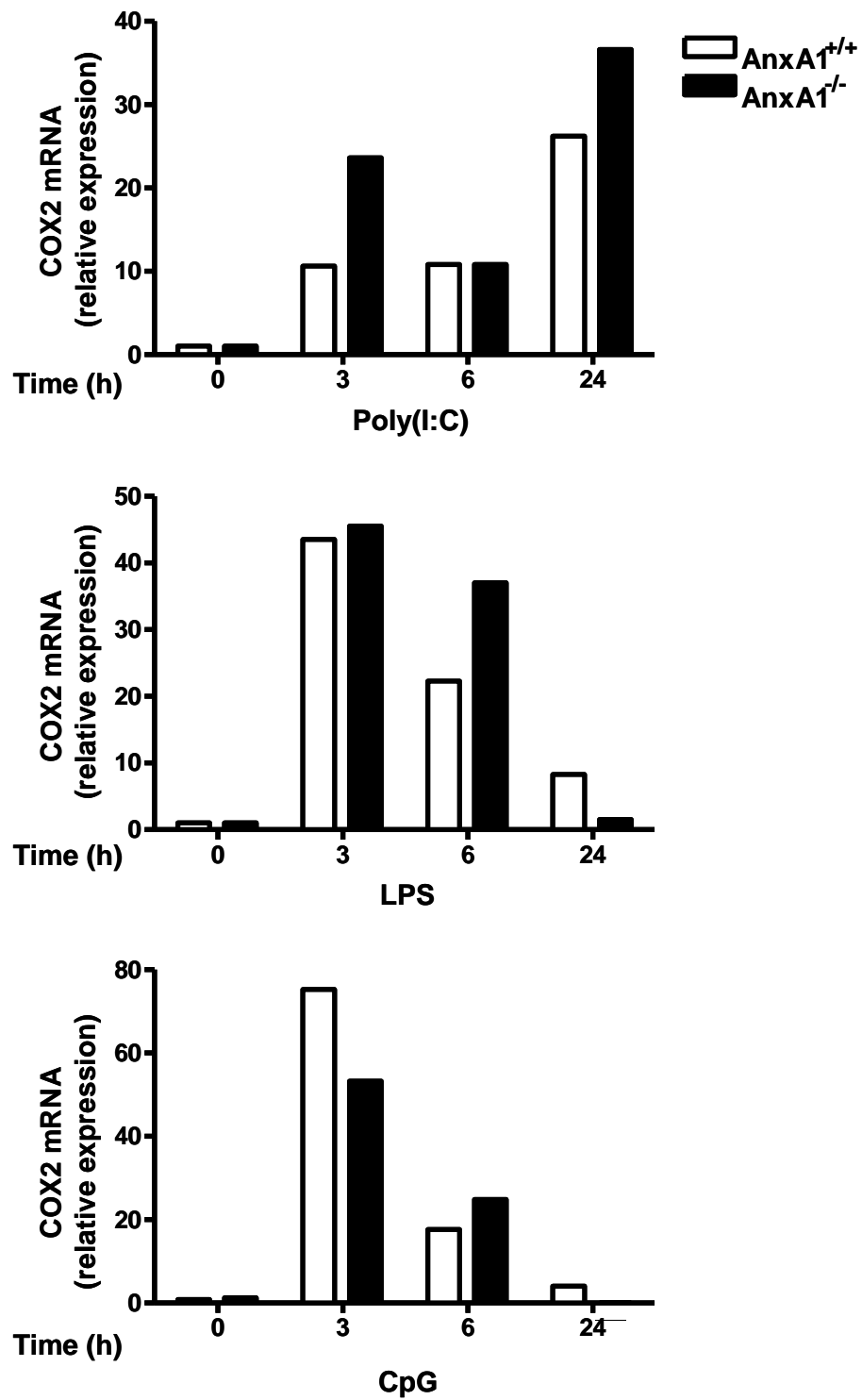
Figure 3.32: Analysis of *COX2* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs.

Real-time PCR analysis of the *COX2* gene expression in AnxA1^{+/+} and AnxA1^{-/-} BMDMs incubated with medium (control) or a TLR ligand (poly (I:C), LPS or CpG) for the indicated time-points. Values arise from a single experiment.

3.4 *In vivo* experiments: Poly (I:C)-induced Lethality

Treatment of mice with 2 different dosages of poly (I:C) (10μg/mouse or 20μg/mouse) induced early clinical signs of SIRS, which yielded a high rate of mortality in both AnxA1^{+/+} and AnxA1^{-/-} mice by 12h (Fig 3.33). However, when the percentage survival of the two genotypes was compared, we observed a 20% difference favouring the AnxA1^{-/-} mice, independent of the dosage of poly (I:C) administered (Fig 3.33). Therefore, in contrast to the administration of LPS, characterised by a high mortality rate of the AnxA1^{-/-} mice (Damazo et al., 2005), the intraperitoneal injection of the TRIF-mediated ligand poly (I:C) reveals a 20% increased survival by AnxA1^{-/-} mice.
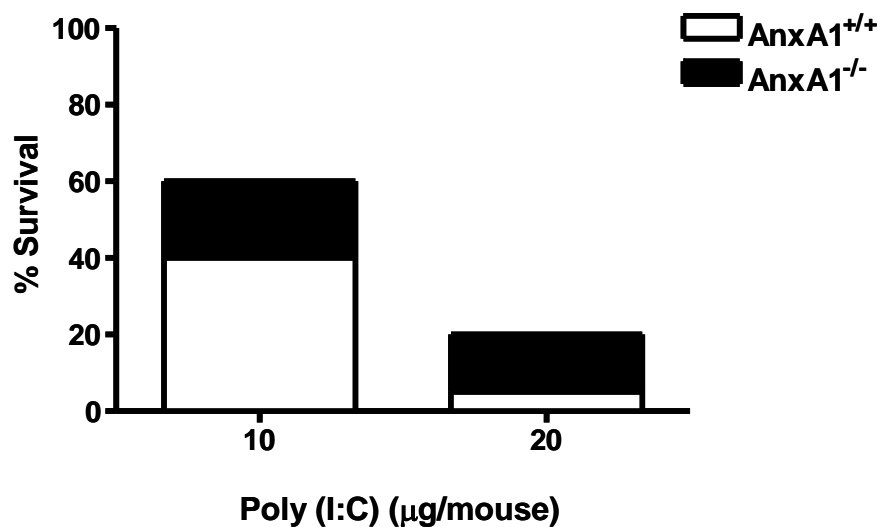
Figure 3.33: Increased survival of AnxA1^{-/-} mice post-administration of poly (I:C).
AnxA1^{+/+} and AnxA1^{-/-} C57/BL6 mice received 10μg/mouse or 20μg/mouse of poly (I:C) intraperitoneally at time 0 and survival rate was monitored at 12h-intervals up to 96h after poly (I:C).

SUMMARY

In this section the effect on poly (I:C) on lethality rate of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice was investigated

- AnxA1$^{-/-}$ mice showed a 20% increased survival post-poly (I:C) administration independent of the sublethal dosage used.

CHAPTER 4:

DISCUSSION

Introduction

Our group has shown an exquisite protective role of AnxA1 in experimental endotoxemia induced by the TLR4 agonist LPS (Damazo et al., 2005). In this thesis, we have extended these observations demonstrating a modulation of endogenous AnxA1 by TLR ligation. We have also compared *in vitro* the response of AnxA1$^{-/-}$ and AnxA1$^{+/+}$ macrophages after stimulation with different TLR ligands. We showed a different inflammatory response of AnxA1$^{-/-}$ macrophages to a panel of TLR agonists. Furthermore, we have investigated the pathophysiological mechanism by which AnxA1 could influence TLR signalling in macrophages. Overall, our studies provide insight into the role of AnxA1 as a protective mediator in several types of infection.

4.1. AnxA1 expression in BMDMs

AnxA1 is constitutively expressed in many different tissue specific macrophages i.e. peritoneal, alveolar, synovial, and microglial cells (Ambrose et al., 1992), and it increases following exposure to GCs (Ambrose et al., 1992, Cirino et al., 1993, De Caterina et al., 1993) and IL-6 (Solito et al., 1998). Our data clearly show the expression of the protein by BMDMs. The activation of different cell types with inflammatory stimuli increases expression and release of endogenous AnxA1 (D'Acquisto et al., 2007a, Damazo et al., 2005, Rescher et al., 2006). We have demonstrated the modulation and secretion of AnxA1 in to the extracellular matrix following stimulation of macrophages with TLR ligands. This novel observation

applies to all the tested TLR agonists, acting as they do through their different TLRs.

The N-terminal of AnxA1 is responsible for the biological action of the molecule (Cirino et al., 1993). After neutrophil activation this protein is externalized and cleaved by enzymes such as proteinase 3 (PR3) (Vong et al., 2007) and neutrophil elastase (HNE) (Rescher et al., 2006), yielding an N-terminal and the 33 kDa C-terminal. In our experiments, we observed both forms of AnxA1 (37/33 kDa) in the BMDMs under resting condition with the majority of the molecule to remain as the full-length 37 kDa form. However, after stimulation with all the TLR agonists under investigation (HKLM, poly (I:C), LPS, flagellin, loxoribine, CpG) additional cleavage occurred in 37 kDa AnxA1 and at 1 h the AnxA1 33 kDa predominated. At subsequent time-points, intracellular AnxA1 was decreased, indicating the secretion into the extracellular matrix. This was followed by resynthesis of the protein in the cytoplasm and return to the resting condition. This result could imply that when the macrophage detects a pathogen invasion through TLR ligation, it leads to a cleavage of the cytosolic 37 kDa AnxA1 in to its two domains followed by secretion to the extracellular milieu, therefore releasing the active N-terminal to interact with its receptor and possibly switching on an anti-inflammatory mechanism. This would be an unusual mechanism that has never been observed hitherto. It is possible, however, that full-length AnxA1, which is secreted by macrophages after stimulation with the TLR ligands, is cleaved extracellularly and then the N-terminal protein enters into the cells.

4.2. Treatment of macrophages with human recombinant AnxA1

Since we have proved that endogenous AnxA1 is secreted by the macrophage after TLR stimulation and therefore may act on FPR2 as an autocrine regulatory exogenous protein, it would be interesting to know if treatment of macrophages with the human recombinant protein has an anti-inflammatory effect.

Indeed, when both RAW and BMDMs were treated with hr-AnxA1, we observed decrease of IL-6 and TNF-α in the culture supernatant. This, however, was not always the case. For example hr-AnxA1 was not as effective post-stimulation with HKLM. Nevertheless, for most TLR ligands the recombinant protein inhibited both IL-6 and TNF-α.

The effect of hr-AnxA1 on the two cytokines showed a concentration-dependent pattern. Initially, the results were opposite to our expectations since the lowest concentration was the most effective, whereas the highest concentration usually did not show any effect. On the other hand, AnxA1 could exert an anti-inflammatory action only in very low concentrations, at conditions that mimic the physiological endogenous state. In addition, maybe the higher tested concentrations lead to a desensitisation of the FPR-family receptor responsible for the exogenous action of the protein.

Unfortunately, when the results of MyD88 pathway-dependent and TRIF pathway-dependent TLR ligands were compared in RAW cells or BMDMs, we were unable to conclude whether AnxA1 influence either of the two pathways. In both RAW

cells and BMDMs, treatment of macrophages with hr-AnxA1 at specific concentrations lowered the content of both IL-6 and TNF-α post-stimulation with poly (I:C), implying an influence of the protein on the TRIF-pathway. On the other hand, hr-AnxA1 also lowered the pro-inflammatory response seen after stimulation of macrophages with pure MyD88-dependent TLR ligands such as loxoribine and CpG. Does this mean that AnxA1 has an influence on both pathways? If this is true then we could not understand why a similar effect was not observed in the case of stimulation with HKLM.

In addition, we were unable to explain why different types of macrophages such as RAW and bone marrow-derived cells exhibited different responses after treatment with the human recombinant protein. Therefore, we focused our investigation at the comparison of wild-type and AnxA1 null macrophages. After all, the observation that initiated our investigation was the difference in lethality rate of wild-type and AnxA1 knockout mice after LPS administration (Damazo et al., 2005). Studying cells in which the protein was completely absent seemed at the time to be a conclusive direction for our study.

4.3. Comparison of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs

4.3.1. Markers of macrophage activation

A phenotypic characteristic under investigation has been the expression of markers of macrophage activation that are induced by TLRs. Since macrophages are APCs, we have chosen to examine MHC II and the co-stimulatory molecules

B7.1 (CD80) and B7.2 (CD86), which correspond to signal 1 and signal 2 of T-cell activation. MHC II interacts with the TCR of the CD4 T_H cells whereas CD80 and CD86 both bind to CD28 and to cytotoxic T-lymphocyte associated protein 3 (CTL-4) priming T-cells against presented antigens (Blander, 2008, Kaye, 1995, Mondino and Jenkins, 1994). Another marker under investigation has been the co-stimulatory molecule CD40, which binds to CD40 ligand (CD40L) on the surface of T_H cells and is required for the activation of APCs (Diehl et al., 2000). The role of AnxA1 in adaptive immune response as a modulator of T-cell activation has been established by our group (D'Acquisto et al., 2007a, D'Acquisto et al., 2007b). However, the role of AnxA1 in the macrophage antigen presentation had remained unexplored.

In light of these considerations, the current study showed a different phenotype in the unstimulated AnxA1$^{-/-}$ macrophages. This is in agreement with another study of our group, which investigated the role of AnxA1 in the dendritic cell, and indicated a heightened mature phenotype of AnxA1$^{-/-}$ dendritic cells (Huggins et al., 2009).

Several studies describe the upregulation of co-stimulatory molecules in macrophages by different TLR agonists (Edwards et al., 2006, Hoebe et al., 2003, Lee et al., 2003, Liu et al., 2006, Shen et al., 2008). It has been reported that the upregulation of co-stimulatory molecules in APCs induced by TLR ligands occurs by TRIF-dependent and TRIF-independent pathways involving an autocrine or paracrine loop (Hoebe et al., 2003). However, the capacity of AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs to upregulate these co-stimulatory molecules was similar after stimulation with all TLR agonists. Results shown by our experiments, such as the

increased expression of these markers by the AnxA1$^{-/-}$ macrophages after stimulation with most TLR agonists, may simply reflect the increased expression at the basal level. Indeed, AnxA1 could down-regulate co-stimulatory molecules, and subsequently antigen-presentation, in the macrophage. However, the finding that AnxA1$^{-/-}$ and AnxA1$^{+/+}$ macrophages exert a similar capacity to upregulate co-stimulatory molecules after stimulation with a panel of TLR agonists supports the notion that this effect is probably independent of TLR signalling.

The increased MHC II basal level of the AnxA1$^{-/-}$ macrophages indicated that AnxA1 may down-regulate MHC II expression. However, an impaired upregulation of MHC II in AnxA1$^{-/-}$ BMDMs was evident following stimulation with the TLR3 ligand poly (I:C), which ensures the presentation of less epitopes of the antigen to the T-cells. Since TLR3 is the receptor for viral double-stranded RNA (Alexopoulou et al., 2001), this finding supports a potentiating role of AnxA1 in antigen-presentation specifically in viral infections.

TLR4 is the only TLR that uses all adaptor proteins and may trigger responses through both the MyD88-dependent and the MyD88-independent/TRIF-dependent pathway (Yamamoto et al., 2003). However, the upregulation of co-stimulatory molecules in macrophages after activation of the TLR4 is dependent only on the adaptor protein TRIF and the secretion of type I IFNs that is a result of TRIF-dependent pathway, and therefore is independent of MyD88 (Hoebe et al., 2003, Shen et al., 2008). On the contrary, although TLR3 uses only TRIF as an adaptor protein, upregulation of co-stimulatory molecules after stimulation with poly (I:C) is only partly-dependent on the TLR3-TRIF axis, indicating that certain viruses

possess the capacity to upregulate co-stimulatory molecules independently of TLRs (Hoebe et al., 2003). In our experiments we could not identify any differences between AnxA1^{-/-} and AnxA1^{+/+} macrophages in the upregulation of co-stimulatory molecules after stimulation with poly (I:C) or LPS, although our result has indicated a much higher upregulation of the co-stimulatory molecules by poly (I:C) and LPS compared to the other MyD88-dependent ligands. We did find, however, a difference in the upregulation of MHC II after stimulation with poly (I:C) in the AnxA1^{-/-} macrophages. Could AnxA1 be interfering with a molecule of the TRIF-dependent pathway in the macrophage, changing MHC II expression and therefore antigen-presentation only in TLR3-dependent viral infections? Since the upregulation of MHC II by viruses is not strictly TRIF-dependent but takes place via a non-TLR pathway, our finding could support a role for AnxA1 in the macrophage antigen-presentation, which is independent of TLR signalling. However, a similar impaired MHC II upregulation by AnxA1^{-/-} BMDMs was also seen after treatment with LPS, supporting the hypothesis of an 'influence' of AnxA1 on the TRIF pathway.

4.3.2. Cytokine production

We, and others, have showed that following activation with different TLR ligands macrophages normally produce cytokines (Alexopoulou et al., 2001, Bagchi et al., 2007, Fang et al., 2004, Heil et al., 2003, Hemmi et al., 2000, Hoshino et al., 1999, Rowlett et al., 2008). To investigate whether the increased lethality of AnxA1^{-/-} mice after administration of LPS (Damazo et al., 2005), is due to a 'cytokine storm', we compared the production of IL-6 and TNF-α in AnxA1^{-/-} and AnxA1^{+/+}

BMDMs after stimulation with LPS. Indeed, we have demonstrated that the production of these cytokines was heightened in AnxA1$^{-/-}$ macrophages post-stimulation with LPS.

Moreover, we showed that other TLR agonists also result in the same increased pro-inflammatory reaction by AnxA1$^{-/-}$ macrophages, suggesting that the 'influence' of AnxA1 signalling pathway and TLR signalling pathway may take place at a point that is common to certain TLRs. Indeed, although our results regarding IL-6 were contradictory in the case of certain TLR ligands, the TNF-α results clearly showed an increased pro-inflammatory response in the AnxA1$^{-/-}$ BMDMs by all the MyD88-dependent ligands and by LPS which uses both MyD88 and TRIF as adaptor proteins. Therefore, the most plausible explanation would have been an interaction of AnxA1 at a certain point of the MyD88-dependent pathway. In conclusion, AnxA1 somehow seems to block specifically the MyD88-dependent pathway. Furthermore, we can expand our observations and support the notion that AnxA1 does not only have a protective role against Gram (-) bacterial infections but in other types of sepsis such as those of Gram (+) and viral origin.

TNF-α is considered a classical pro-inflammatory cytokine (Tracey and Cerami, 1994). Besides the fact that it is overproduced and released systemically during sepsis (Girardin et al., 1988), the first studies of exposure to human recombinant TNF revealed that it induces a syndrome indistinguishable from septic shock syndrome (Tracey et al., 1986). In addition, anti-TNF antibodies prevented the development of septic shock (Tracey et al., 1987). It has long been known that TNF-α is induced after stimulation of macrophages with TLR ligands such as LPS,

contributing to macrophage-mediated cytotoxicity (Zacharchuk et al., 1983). Therefore, the fact that in our experiments this cytokine was found to be increased in the AnxA1$^{-/-}$ BMDMs after stimulation with different TLR ligands is not surprising and it supports our previous *in vivo* observations (Damazo et al., 2005), signifying that AnxA1 is an endogenous protective anti-inflammatory factor in different infections.

Although the pro-inflammatory role of TNF-α remains unquestionable, this is not the case for IL-6. Indeed, IL-6 has been assigned both pro-inflammatory and anti-inflammatory characteristics since during an infection it directs the immunological switch from neutrophil to mononuclear cell population and therefore from innate to acquired immunity by influencing both arms of the immune response (Jones, 2005). In specific, it blocks neutrophil accumulation at sites of infection or inflammation (Xing et al., 1998), whereas it promotes trafficking of T-cells (McLoughlin et al., 2005) and rescues T-cells from entering apoptosis (Teague et al., 1997). Nevertheless, despite its established characterisation as a pro-inflammatory cytokine, a protective role of IL-6 in the development of septic shock has been reported (Barton and Jackson, 1993, Diao and Kohanawa, 2005). Therefore, the impaired release of IL-6 by the AnxA1$^{-/-}$ BMDMs at the basal level does not necessarily reflect a pro-inflammatory role for AnxA1 in the macrophage. These findings, however, suggest a different role of endogenous AnxA1 in the macrophage compared to other cell types such as the fibroblast, where a negative regulatory role of AnxA1 in the expression of IL-6 was revealed (Yang et al., 2006).

Besides the induction of co-stimulatory molecules by APCs, TLRs have another mechanism to control the adaptive immune response. Normally, regulatory T cells (T$_R$ cells) have the role of preventing activation of autoreactive T-cells. Upon recognition of PAMPs, APCs induce the production of IL-6, which blocks the suppressive effect of T$_R$ cells on the T cells (Pasare and Medzhitov, 2003). Therefore, during infection, where activation of the adaptive immune response is necessary, the secreted IL-6 by APCs renders T cells refractory to the suppressive activity of T$_R$ cells (Pasare and Medzhitov, 2003). In addition, AnxA1 increases the T$_H$1 arm of the adaptive immune response in apparent contrast to its established anti-inflammatory role in the neutrophil (D'Acquisto et al., 2007a, D'Acquisto et al., 2007b). Therefore, since the macrophage is an APC, it would not be surprising for AnxA1 to have distinct roles in relation to the TLR3-driven viral infections in macrophages by promoting adaptive immune responses through the increase of both IL-6 and MHC II.

TLR3 is the receptor responsible for recognition of double-stranded RNA, which is the result of viral replication within infected cells (Alexopoulou et al., 2001). The contrasting effects of poly (I:C) compared to the other TLR ligands in the induction of IL-6 could be explained by the fact that TLR3 differs from the other TLRs since it uses only TRIF as an adaptor protein and not MyD88, which is used by all the other TLRs (Yamamoto et al., 2003, Yamamoto et al., 2002). Although both MyD88-dependent signalling pathway and TRIF-dependent signalling pathway lead to NF-κB and MAPK activation, important differences exist in their 'downstream' signals. For example, RIP1 is an essential mediator of TLR3-induced NF-κB activation (Meylan et al., 2004). Ubiquitin ligase Peli 1 is needed

for the transmission of TRIF-dependent TLR signals through binding and ubiquitination of RIP1 (Chang et al., 2009). Therefore, Peli 1 mediates IKK-NF-κB activation in the TRIF-dependent pathway (Chang et al., 2009). On the other hand, EMSA did not reveal impaired NF-κB DNA binding after stimulation with poly (I:C) compared to the other TLR ligands, suggesting that probably NF-κB is not the key to the impaired IL-6 production observed in the AnxA1$^{-/-}$ BMDMs post-stimulation with poly (I:C). Therefore, AnxA1 seems to have a most distinct role especially in viral infections. The 'check-point' on the TRIF-dependent signalling pathway at which AnxA1 acts altering IL-6 expression still remains puzzling.

TRIF can activate the IFN-β promoter (Yamamoto et al., 2003) through the transcription factor IRF-3 (Yamamoto et al., 2003) and lead to the production of the signature cytokine of this pathway i.e. IFN-β. Therefore, IFN-β$^{-/-}$ macrophages presented diminished activation of the intracellular molecules STAT1 Tyr-701, STAT1 Ser-727, and Akt after stimulation with LPS (Thomas et al., 2006). In addition, treatment with exogenous recombinant IFN-β to IFN-β$^{-/-}$ macrophages increased levels of LPS-induced gene expression of monocyte chemotactic protein 5 (MCP5), iNOS, IP-10, and IL-12 p40 mRNA (Thomas et al., 2006). However, no modulation of IL-6 has been recorded in IFN-β$^{-/-}$ macrophages, indicating that the impaired IL-6 in AnxA1$^{-/-}$ macrophages after stimulation with poly (I:C) could not be caused by an 'influence' of AnxA1 on the levels of IFN-β. Indeed, comparison of gene expression profiles in cells from IRF-3$^{-/-}$ mice following viral infection, revealed that the gene for IL-6 is an IRF-3-independent direct response gene (Andersen et al., 2008).

Finally, what could be the pathophysiological mechanism in AnxA1$^{-/-}$ BMDMs that results in normal production of TNF-α and impaired IL-6 during viral infections? It has been reported that the production of IL-6 induced by certain TLR ligands is mediated by COX2 (Chen et al., 2006). Moreover, engagement of TLR3 by viral RNA or poly (I:C) increases the levels of PGE$_2$ by COX2 through a mechanism of cytosolic phospholipase A2 (cPLA2)-mediated arachidonic acid mobilization (Pindado et al., 2007, Steer et al., 2006). In addition, it has been reported that PGE$_2$ can upregulate the levels of IL-6 produced by alveolar macrophages (Williams et al., 2000). Conclusively, PGE$_2$ enhances the release of IL-6 in macrophages (Treffkorn et al., 2004) and therefore a modulation in COX2 and PGE$_2$ production could result in decreased IL-6, offering an explanation for our result. On the other hand, AnxA1 blocks PLA$_2$ activity (Wallner et al., 1986) and therefore indirectly the production of PGE$_2$ and therefore, it is expected that AnxA1$^{-/-}$ BMDMs would have increased levels of PGE$_2$ and subsequently IL-6. Moreover, we did not find statistically significant differences in the PGE$_2$ production between AnxA1$^{-/-}$ and AnxA1$^{+/+}$ BMDMs after stimulation of TLR3 that could support this hypothesis.

Nevertheless, another mechanism could exist that downregulates IL-6 in AnxA1$^{-/-}$ BMDMs, specific in response to viral infections, which is independent of PGE$_2$. For example, Palaga et al reported that several TLR agonists upregulate Notch1 protein expression in BMDMs, whereas inhibiting processing of Notch receptor by γ-secretase and blocking Notch signalling caused a decrease in IL-6 and NO production (Palaga et al., 2008), a similar phenotype to the AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C). It is believed that Notch signalling is required for IL-6

expression since the binding partner of Notch, CBF-1, can bind to and regulate the IL-6 gene (Kannabiran et al., 1997). However, poly (I:C) was not the only TLR agonist which was able to trigger up-regulation of Notch 1 (Palaga et al., 2008) suggesting that this is not an effect restricted to viral infections.

Finally, could the identification of TLR3–independent effects induced by poly (I:C) such as the upregulation of co-stimulatory molecules (Hoebe et al., 2003) and COX-2 expression (Steer et al., 2006) also apply to the poly (I:C)-mediated IL-6 production? Overall, it is possible that certain viruses can influence the IL-6 production of the macrophage using mechanisms independent of TLR3.

Another important observation has been the intriguing role of the TLR5 agonist flagellin. In both AnxA1^{-/-} and AnxA1^{+/+} BMDMs, the cytokine production induced by flagellin was severely impaired compared to that induced by the other TLR agonists. This could be explained by a lower expression of the TLR5 in BMDMs and was supported by previous studies that reported no production of IL-6 and TNF-α in BMDMs after stimulation with flagellin (Feuillet et al., 2006, Hawn et al., 2007), possibly due to an absence of TLR5 in these cells (Uematsu et al., 2006). Another mechanism, which could be responsible for a low level of expression of TLR5 without altering the TLR5 mRNA level, is the overexpression of TRIF (Choi et al.). Indeed, if TRIF is overexpressed by BMDMs, then the functionality of TLR5 at a post-translational level may be influenced, since TRIF induces TLR5 proteolytic degradation (Choi et al.). Alternatively, TRIF could be over-expressed in BMDMs but not in RAW macrophages. Therefore, there was a sufficient cytokine production in RAW cells after stimulation with flagellin despite the fact

that the concentration for stimulation was 1/10 of that used for BMDMs. Moreover, although it was originally thought that since flagellin uses the same adaptor protein as HKLM, LPS, loxoribine, and CpG, and should therefore induce a similar phenotype, the production of IL-6 was impaired by the AnxA1$^{-/-}$ macrophages at 24 h post-stimulation. Interestingly, a recent study has proven that flagellin can directly induce adaptive immune responses without previous activation of the innate immune response or TLR5 (Sanders et al., 2009). Therefore, like poly (I:C) acting independently of TLR3, the impaired IL-6 found in AnxA1$^{-/-}$ macrophages after stimulation with flagellin could be a TLR5-independent effect.

4.3.3. Nitric Oxide and PGE$_2$

It has been reported that certain TLR agonists such as poly (I:C) (Heitmeier et al., 1998, Snell et al., 1997), LPS (Hauschildt et al., 1990, Jones et al., 2008, Mulsch et al., 1993) and CpG (Li et al., 2005) induce the production of NO in macrophages. On the contrary, stimulation with HKLM or flagellin alone cannot induce the production of NO in macrophages but requires the addition of IFN-γ (Beckerman et al., 1993, Mizel et al., 2003). Nothing has been reported concerning NO in these cells after stimulation of TLR7 with loxoribine. In agreement with this, we did not observe a production of nitrites after stimulation with HKLM, flagellin or loxoribine. Moreover, the result of our experiment showed that after stimulation with poly (I:C), LPS or CpG there was a significant production of NO.

The production of NO after stimulation of TLR4 and TLR9 was not statistically different in the AnxA1$^{-/-}$ compared to the AnxA1$^{+/+}$ BMDMs. On the other hand,

poly (I:C) significantly impaired production of nitrites by AnxA1$^{-/-}$ BMDMs compared to AnxA1$^{+/+}$ BMDMs. This result could offer another important observation that focuses again on the TLR3 ligand poly (I:C). Again, a possible involvement of AnxA1 at a certain point of TRIF-dependent TLR-signalling pathway, could explain these differences.

Contrasting effects were recorded by the western blotting of the enzyme iNOS in comparison to the Griess reaction for detection of production of nitrites (data not shown). As expected, we did not detect an upregulation of iNOS expression after stimulation with flagellin. However, we observed an increase in expression of the enzyme after stimulation of the AnxA1$^{-/-}$ BMDMs with HKLM and loxoribine, which was not accompanied by an increase in the level of secreted NO in the supernatant.

After stimulation with poly (I:C), the upregulation of iNOS expression was increased in the AnxA1$^{-/-}$ BMDMs (data not shown), whereas we recorded an impaired production of NO. If decreased NO in the supernatant of AnxA1$^{-/-}$ BMDMs accompanied by increased iNOS expression in the cytoplasm after stimulation with the TLR3 ligand poly (I:C) is indeed correct, then a different pathophysiological mechanism could offer an explanation. For example, there is a group of hydrolytic enzymes namely arginases, which can compete with NO synthases for their common substrate arginine (Modolell et al., 1995, Munder et al., 1999). A study by Kasmi *et al* has reported that intracellular pathogens may induce expression of Arginase 1 (Arg1) in mouse macrophages through the TLR pathway depending on the adaptor protein MyD88 (El Kasmi et al., 2008). Besides intracellular infections,

macrophages lacking Arg1 produce more NO after stimulation of TLR4 with LPS (El Kasmi et al., 2008, Ryan et al., 1980, Sonoki et al., 1997), suggesting that regulation of Arg1 is a mechanism restricting macrophage NO production common to different types of TLR stimulation. If we assume that TLR3 stimulation could also induce Arg1 through a MyD88-independent mechanism, an increased Arg1 expression could be associated with a decreased NO production without necessarily being accompanied by an analogous increase in the iNOS expression. Therefore, AnxA1 could be implicated in this MyD88-independent pathway blocking the induction of Arg1 and therefore leading to an increased production of NO in the macrophage. Indeed, if this hypothesis is true, our result of decreased NO production and increased iNOS expression in the AnxA1$^{-/-}$ BMDMs could not be a random finding.

Several studies have reported activation of the enzyme COX2 and subsequent production of PGE$_2$ after stimulation of macrophages with different TLR ligands such as poly (I:C) (Pindado et al., 2007, Steer et al., 2006), peptidoglycan (PGN) (Chen et al., 2004, Chen et al., 2009a), LPS (Buczynski et al., 2007, Uematsu et al., 2002) or CpG DNA (Yeo et al., 2003). However, the signalling pathways resulting in the induction of COX2 after stimulation of each corresponding TLR are different in important aspects. Although disparities between different TLR ligands may exist, it is a fact that several molecules influence the induction of COX2 negatively or positively. Therefore, both MyD88-dependent and TRIF-dependent pathways may lead to NFκB-dependent or independent activation of COX2, whereas a TLR-independent mechanism specifically for the TLR3 ligand double-stranded RNA has also been described (Steer et al., 2006).

In our experiments, HKLM, poly (I:C), and LPS induced COX2 expression and PGE$_2$ production in both AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs in a statistically similar way. On the other hand, we were unable to detect induction of COX2 after stimulation with CpG whereas there was detection of PGE$_2$ in the corresponding culture supernatant (data not shown). Flagellin and loxoribine did not induce the enzyme or its product. The fact that there are no studies describing induction of COX2 by neither flagellin nor loxoribine supports these results.

The detection of PGE$_2$ after stimulation with poly (I:C) did not suggest any statistically important differences between AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs. However, stimulation of TLR3 revealed several differences in comparison to the other TLRs regarding IL-6 and NO production, and a difference in PGE$_2$ between AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs was also expected. Firstly, we identified impaired NO production in AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C) and NOS2 and COX2 signalling pathways may influence each other. Specifically, it has been reported that induction of iNOS in these cells takes place via a cPLA$_2$/COX2 pathway (Pindado et al., 2007). Interestingly, if cPLA$_2$ is involved in TLR3-induced COX2, it is surprising that there is no difference in PGE$_2$ in the AnxA1$^{-/-}$ BMDMs since AnxA1 blocks cPLA$_2$ activity (Cirino et al., 1987). Finally, a modulation in COX2 and PGE$_2$ production, specifically impairment in PGE$_2$ production, could result decreased IL-6, offering an explanation to our previously described observations concerning cytokine production (Treffkorn et al., 2004).

4.3.4. TLR expression

The exact relationship between the number of TLRs expressed by a cell to its response after addition of the ligand still remains obscure. However, LPS tolerant macrophages exhibit decreased inflammatory cytokine production, which correlates with downregulation of surface TLR4 (Nomura et al., 2000). Therefore, differential expression of the TLRs on the surface of macrophages could explain the differences in cytokine production between AnxA1$^{-/-}$ and AnxA1$^{+/+}$ macrophages i.e. an increased receptor expression in the AnxA1$^{-/-}$ macrophages could induce an exaggerated inflammatory reaction. Unfortunately, we were unable to detect the expression of any TLR protein due to the lack of reliable antibodies. Nevertheless, we did observe an increased mRNA expression of all TLRs in the absence of AnxA1. However, this phenotype was common to all TLRs including the TLR3, which presented certain differences to the other TLRs in the regulation of MHC II, IL-6 and NO in the AnxA1$^{-/-}$ BMDMs after stimulation with its ligand poly (I:C). On the other hand, even if the macrophage response to TLR stimuli can be explained by modulation of the number of TLRs expressed by this cell, the mechanism by which AnxA1 down-regulates the transcription of these receptors still remains unresolved. On top of this, there is a complicated discrepancy between mRNA expression and responsiveness to TLR ligands and the expression of a TLR by macrophages does not always correspond to cell activation after stimulation of this receptor. For example, neutrophils express TLR9 but are not stimulated by CpG (Hornung et al., 2002).

4.3.5. NF-κB DNA-binding

TLR signalling through both MyD88-dependent and TRIF-dependent pathways leads to dissociation of NF-κB from the IκB proteins and its nuclear translocation, in order to bind to the promoters of target genes (Kawai and Akira, 2007). Several studies have demonstrated normal NF-κB activation after stimulation with a panel of TLR ligands in different types of macrophages (Alexopoulou et al., 2001, Bagchi et al., 2007, Hemmi et al., 2000, Muller et al., 2001, Yamamoto et al., 2003). NF-κB activation regulates several genes leading to the production of proteins including the IκBα, which in turn inhibits NF-κB allowing a fast turn-off of the NF-κB response. On the other hand, IκBβ and IκBε remain at low levels, since their impact on NF-κB activity is unidirectional, meaning that they inhibit NF-κB but NF-κB does not regulate their production. In wt cells, the IκBα cycle leads to the production of an oscillatory pattern of NF-κB activity observed by EMSA (Nelson et al., 2004), and IκBβ and IκBε are responsible for dampening the system's oscillatory potential (Hoffmann et al., 2002). Therefore, in mouse knockout cells lacking the IκBβ and IκBε isoforms, undampened NF-κB oscillations are produced (Hoffmann et al., 2002) (Fig 4.1).

As expected, after stimulation with a panel of TLR ligands, we observed normal oscillatory NF-κB activation leading to the production of several pro-inflammatory mediators such as IL-6 and TNF-α by AnxA1$^{+/+}$ BMDMs. Conversely, after stimulation with LPS, we observed a linear increase of NF-κB activation as

described by Covert et al (Covert et al., 2005), possibly due to interaction of the two signalling pathways.

Since AnxA1$^{-/-}$ BMDMs exhibited an augmented pro-inflammatory state after stimulation of the MyD88-dependent signalling pathway, we expected to see increased NF-κB activation. Indeed, our results clearly showed that although AnxA1$^{-/-}$ BMDMs started with an impaired NF-κB activation at the basal level, 30min after stimulation with all MyD88-dependent ligands, they presented with an increased NF-κB binding ability, which could subsequently correspond to the enhanced transcription of certain pro-inflammatory genes. Indeed, we observed NF-κB oscillations of higher amplitude by AnxA1$^{-/-}$ BMDMs. These undampened oscillations have also been described in cells lacking the IκBβ and IκBε isoforms, which normally dampen the system's oscillatory potential (Hoffmann et al., 2002). However, although an influence of AnxA1 in one or both of these IκB isoforms would have been very tempting, we did not manage to support this hypothesis by performing western blotting in cytoplasmic extracts of BMDMs stimulated with different TLR ligands (results not shown). On the other hand, a dual role for IκBβ has been reported (Rao et al.). Specifically, although initially post-LPS stimulation IκBβ degrades, contributing to the initial expression of TNF-α, then newly synthesized hypophosphorylated IκBβ acts through p65:c-Rel dimers, binds to the TNF-α promoter, and enhances TNF-α transcription (Rao et al.). Therefore, if AnxA1 could block IκBβ utilising an as yet unidentified mechanism, it could also control indirectly the transcription of TNF-α. Our preliminary results obtained by western blotting after stimulation with LPS, revealed an increased IκBβ expression

by AnxA1$^{-/-}$ cells (data not shown), which could also induce enhanced TNF-α production.

As described previously, after stimulation with the TLR3 ligand poly (I:C), we observed normal TNF-α production and impaired IL-6 production by AnxA1$^{-/-}$ BMDMs. Consistent with this, at 30min post-stimulation with poly (I:C) we did not observe any difference in the NF-κB DNA-binding by the AnxA1$^{-/-}$ BMDMs, in contrast to the increased NF-κB activation seen by the MyD88-dependent ligands. However, 60min post-stimulation of TRIF, the NF-κB DNA-binding was enhanced in AnxA1$^{-/-}$ BMDMs. The TRIF pathway requires a time delay to establish NF-κB activation compared to the MyD88 pathway, because it requires protein synthesis. In specific, the transcription factor IRF3 mediates the activation of TNF-α, which in turn binds its receptors on the cell leading to the activation of NF-κB (Fig 4.2) (Covert et al., 2005). This different NF-κB activation/ IκBα degradation kinetic, seen in AnxA1$^{-/-}$ BMDMs after stimulation of TRIF, implies an involvement of AnxA1 in the TRIF-pathway as well. Nevertheless, this enhanced delayed NF-κB activation could be responsible for different phenotype characteristics by these cells but not for the production of TNF-α. Again, this could support a different pathophysiological role of AnxA1 in viral infections.
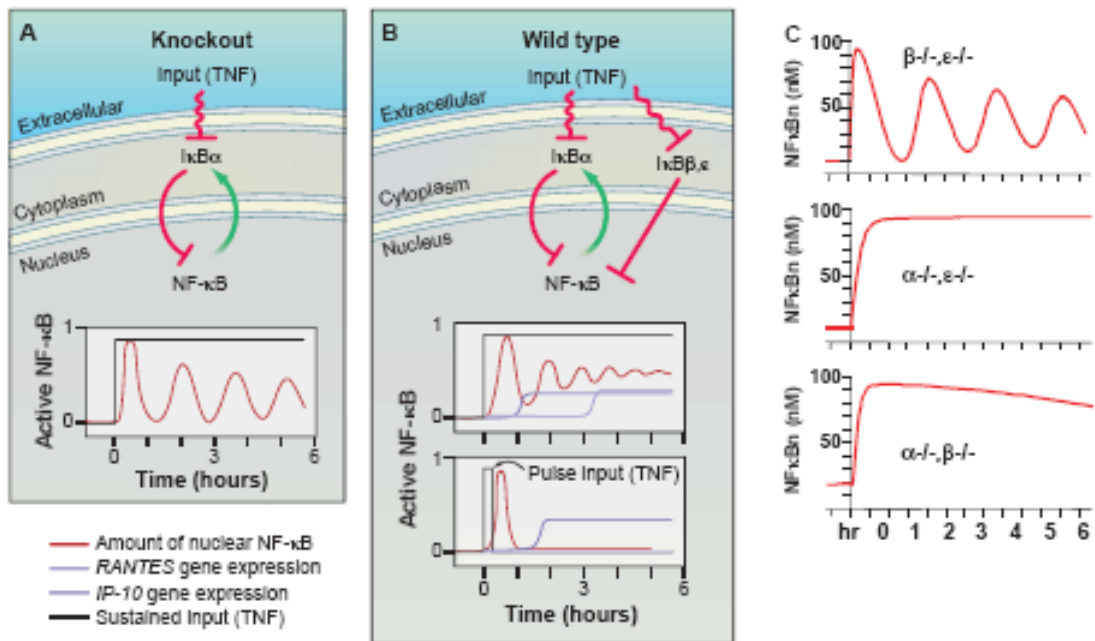
Figure 4.1: A computational model based on genetically reduced systems. The NF-κB signalling pathway in mouse knockout cells lacking IκBβ and IκBε (A) and in WT cells (B). Taken from (Ting and Endy, 2002). Computational modelling of each genetically simplified signalling module resulting in characteristic kinetics of the NF-κB response (C). Taken from (Hoffmann et al., 2002).
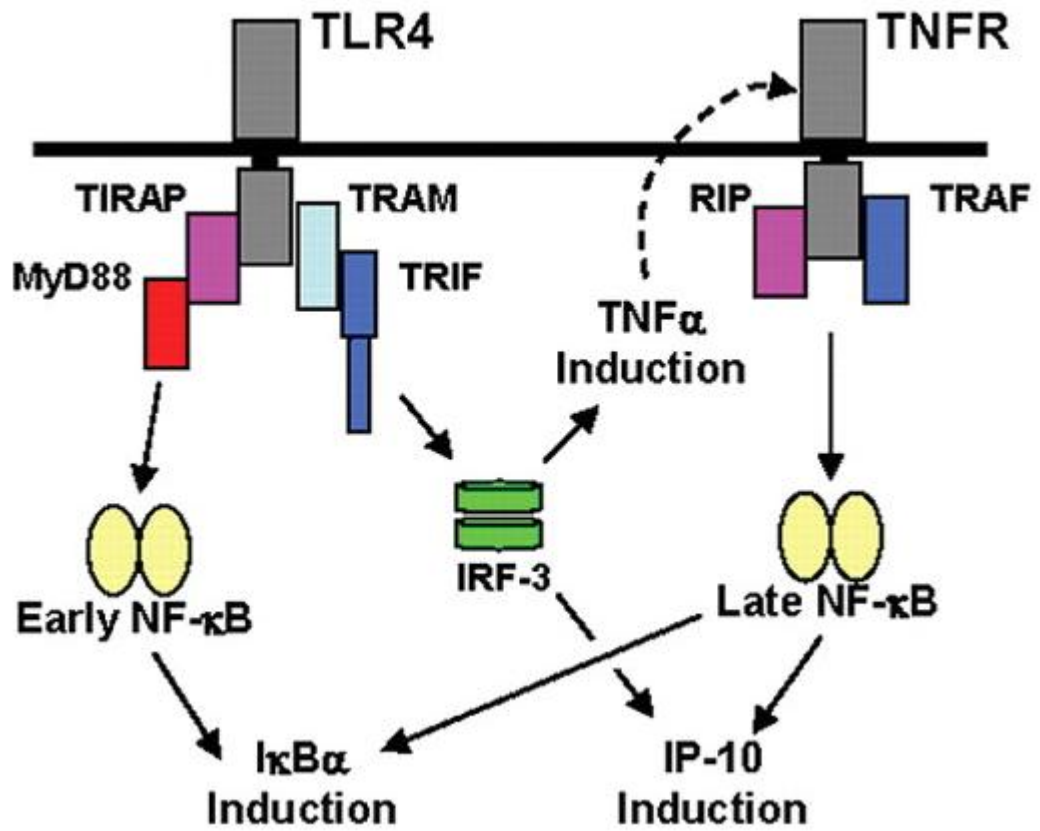
Figure 4.2: **MyD88-independent pathway activation of NF-κB requires IRF3-mediated expression of TNF-α.** Scheme of the pathway for activation of NF-κB by means of TRIF. Taken from (Covert et al., 2005).

4.3.6 ERK1/2 activation

All TLR ligands acting through TLR MyD88-dependent and/or TRIF-dependent signalling pathways lead to activation of MAPKs (Kawai and Akira, 2007). Similarly, the FPR signalling pathway activates the MAPK ERK (Selvatici et al., 2006). We have proved that AnxA1 is secreted from the macrophages after stimulation with all the different TLR ligands. In macrophages, FPR2 is expressed and is functional (Devosse et al., 2009). Therefore, after its secretion, AnxA1 could act on FPR2 receptors activating the corresponding signalling pathway (Perretti et al., 2001, Walther et al., 2000). Overall, the MAPK ERK is a common 'checkpoint' in the two signalling pathways (Fig 4.3), which after TLR stimulation are both activated in the macrophage, and therefore it can be a candidate molecule for the possible interaction of the two signalling pathways. Indeed, if AnxA1 has an effect on ERK phosphorylation after TLR stimulation, this could offer an explanation to the exaggerated pro-inflammatory response that we observed in AnxA1^{-/-} BMDMs.

A study by Alldridge *et al.* revealed a dysregulation of the MAPK ERK pathway by AnxA1 in a macrophage cell line (Alldridge et al., 1999). In specific, clones with decreased AnxA1 expression showed prolonged ERK activity following LPS treatment, whereas changes in AnxA1 expression had no effect on p38 or JNK (Alldridge et al., 1999). In agreement with this view, we observed increased phosphorylation of ERK1/2 in AnxA1^{-/-} BMDMs after stimulation with different TLR ligands. These data are also supported by the study of Yang et al. (Yang et al., 2009), who stimulated macrophages with LPS and demonstrated that AnxA1 is a negative regulator of IL-6 and TNF-α by acting through MAPK phosphorylation and

regulation of the protein Glucocorticoid induced leucine zipper (GILZ) (Yang et al., 2009). Our data suggest that the downregulation of phospho-ERK1/2 by AnxA1 is not only limited to the LPS stimulation but it can be expanded to all the other TLR ligands as well.

However, the fact that TLR3, which induced impaired IL-6 production in the AnxA1^{-/-} macrophages, also showed a similar ERK phenotype with the other TLRs, could suggest a different pathophysiological mechanism, independent of ERK phosphorylation. In this case, a difference in the TRIF-dependent pathway due to an interaction of AnxA1 at a certain point of the signalling, could offer a more reliable explanation, which applies specifically to viral infections. Finally, if AnxA1, which is secreted by the macrophage, activates the FPR signalling pathway, phosphorylation of ERK should also be induced. Therefore, our finding of increased ERK phosphorylation in the absence of AnxA1 is contradictory, since it supports the notion that AnxA1 is a negative regulator of ERK phosphorylation. Overall, the question of how AnxA1 can be a negative regulator of ERK since it induces its phosphorylation after interaction with FPR2 remains unanswered.
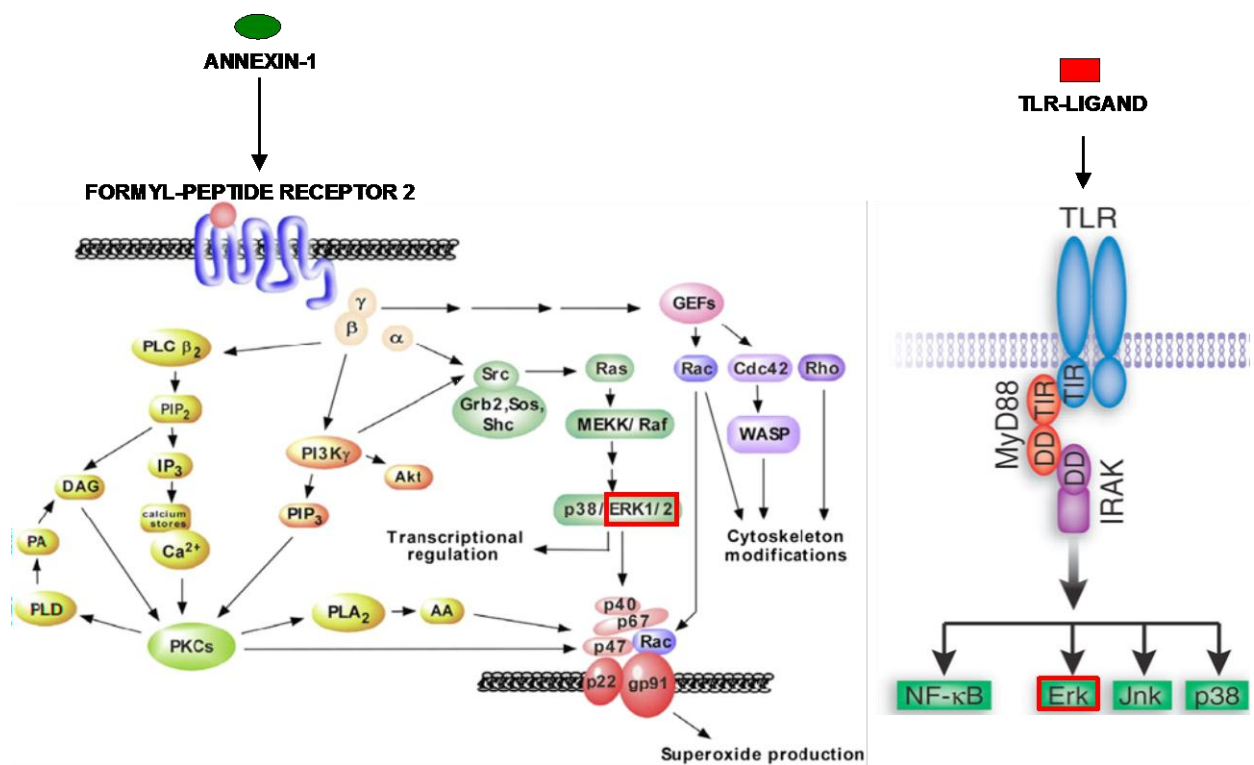
Figure 4.3: Both FPR2 and TLR-signalling pathways lead to the activation of ERK1/2.

Schematic demonstration of the FPR2 signalling pathway after stimulation with AnxA1 leads to activation of ERK1/2 (left). TLR stimulation also induces ERK1/2 activation (right). Adapted from (Rabiet et al., 2007).

4.3.7 Gene expression profile

During a viral infection triggering TLR3, the activated genes can be divided in 3 sets: IRF-3-dependent, IRF-3-independent, and IRF-3-augmented direct response genes (Andersen et al., 2008). For example, the *IFN-β1*, *ISG54*, *NOS II* genes are considered IRF3-dependent, the *IL-6* and *TNF-α* genes IRF3-independent, and the *CXCL10/ IP10* gene IRF3-augmented (not strictly dependent on IRF-3). Therefore, by using TLR ligands stimulating only the MyD88-dependent pathway, the TRIF-dependent pathway or both, we tried to investigate whether AnxA1 had any effect in any of these pathways.

CpG, which stimulates the MyD88 pathway only, showed enhanced *IL-6* gene expression in AnxA1$^{-/-}$ cells, suggesting that AnxA1 may interfere at a certain point of this pathway blocking it. On the contrary, we did not find important differences in the AnxA1$^{-/-}$ BMDMs regarding the *IL-6* gene expression after stimulation with the TRIF-dependent ligand poly (I:C), whereas the levels of IL-6 in the supernatant of AnxA1$^{-/-}$ BMDMs post-stimulation with poly (I:C), were severely impaired. Therefore, an interaction of AnxA1 on the TRIF pathway influencing IL-6 cannot be excluded. When triggering TLR3, the *IL-6* gene is considered an IRF3-independent gene (Andersen et al., 2008, Thomas et al., 2006). Yet TRIF knockout macrophages show a completely abrogated IL-6 production post-stimulation with LPS (Shen et al., 2008), suggesting that in the case of TLR4, TRIF is considered indispensable for the production of IL-6, and that it probably synergises with MyD88. In addition, a different result was described for dendritic cells (Shen et al., 2008), indicating that the signalling pathway leading to IL-6

production may be different for each cell type (Fig 4.4). Nevertheless, the TRIF pathway activated after TLR3 stimulation may not be identical to the TRIF pathway activated after TLR4 stimulation, since in the second case the adaptor protein TRAM is also involved (Fitzgerald et al., 2003b). TRIF signalling includes activation of IRF3 but also another, yet unidentified pathway (Fig 4.5) (Kawai et al., 2001). If indeed the production of IL-6 in the macrophage after stimulation with poly (I:C) is IRF-3-independent, then the similar IL-6 gene expression in the AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C) supports the notion that this IRF-3-independent pathway is not influenced by AnxA1. It does not exclude, however, an interaction of AnxA1 on the IRF-3-dependent TLR3 signalling pathway. However, even if we assume that AnxA1 does exert an effect on the IRF-3 pathway, we still cannot understand how this influence could change the IL-6 production by the macrophage.

CpG, by activating only the MyD88-dependent pathway, results in enhanced *TNF-α* gene expression in AnxA1$^{-/-}$ BMDMs, supporting again the blockage of MyD88 pathway by AnxA1. On the other hand, since the *iNOS* gene is considered an IRF3-dependent gene when triggering TLR3 (Youn et al., 2005, Youn et al., 2008), it is surprising the fact that we observe different kinetics of gene expression in AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C). This suggests that AnxA1 somehow does influence the IRF3-mediated gene expression. This hypothesis is also supported by the enhanced *IFN-β1*, *ISG54*, *CXCL10/IP10* genes expression in AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C), since they all are considered IRF3-dependent or augmented genes (Andersen et al., 2008). In addition, the different kinetic in COX2 gene expression after stimulation with the 3 different TLR

ligands, supports again an interaction in both pathways since COX2 gene is considered IRF-3-dependent when triggering TLR3 (Youn et al., 2005, Youn et al., 2008) and MyD88-dependent when triggering TLR4 (Bjorkbacka et al., 2004, Kawai et al., 2001).

Conclusively, AnxA1 seems to act on both MyD88-dependent and TRIF-dependent pathways. Specifically for the TRIF-pathway, it probably influences the transcription factor IRF-3 pathway. Since IRF-3 activation leads to TNF-α production, which after secretion binds to the cell receptors activating NF-κB, the activation of NF-κB is also influenced. Indeed, we did observe an increased delayed NF-κB DNA binding post-poly (I:C) stimulation in AnxA1$^{-/-}$ cells, further supporting this hypothesis. Therefore, the levels of several gene products of the IRF-3 transcription pathway should also be different in AnxA1$^{-/-}$ cells. To further confirm this hypothesis, it would be interesting to measure the production of IFN-β in these cells after stimulation with poly (I:C).

The influence of AnxA1 on the TLR signalling pathway includes two levels of regulation: mainly by blocking the MyD88 pathway and to a lesser extent by interacting with the IRF3-dependent TRIF pathway.
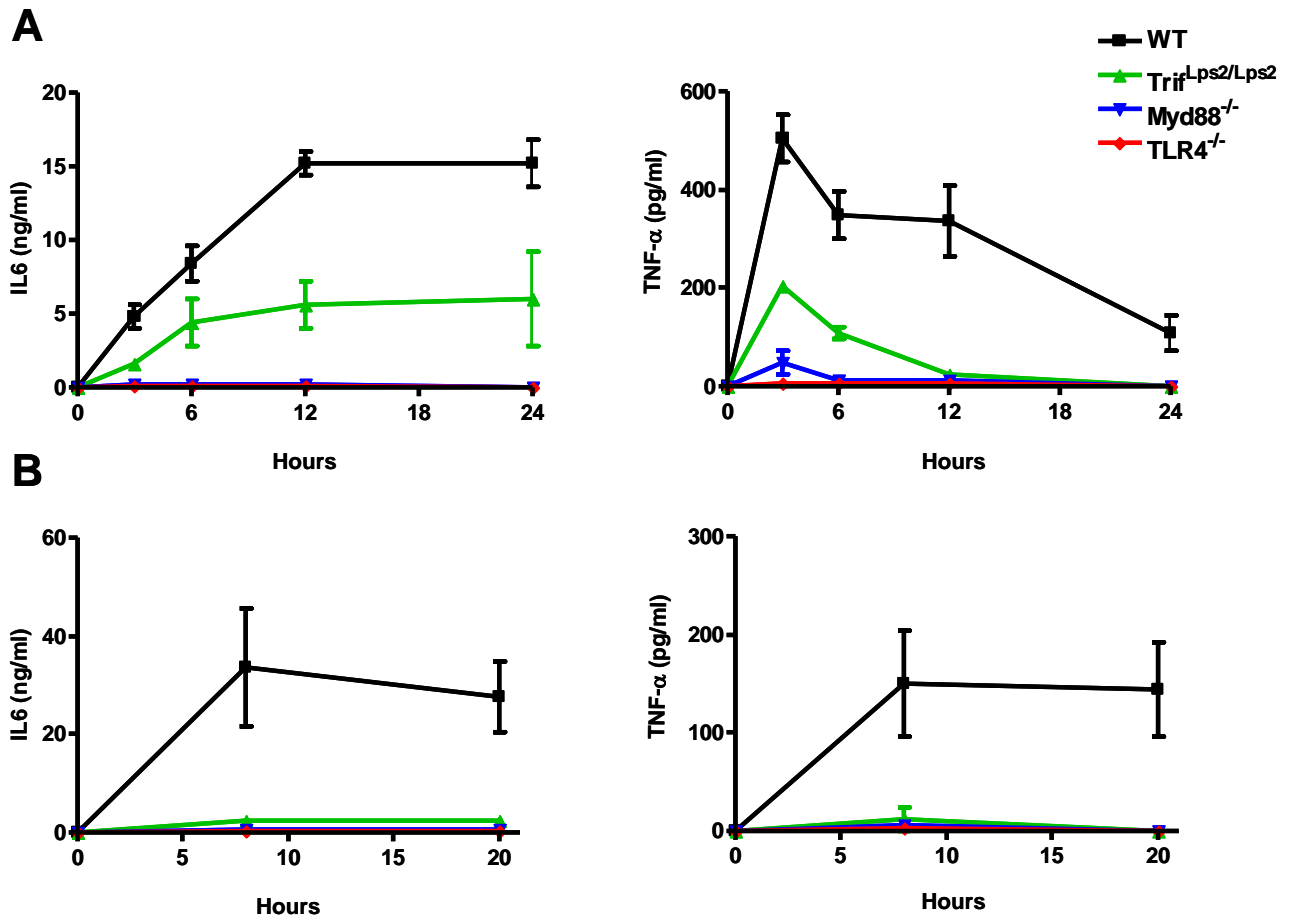
Figure 4.4: Impact of TRIF or MyD88 signalling on proinflammatory cytokines in bone marrow-derived dendritic cells (BMDCs) (A) and glycolate-elicited peritoneal macrophages (TGC-PECs) (B).

Redrawn from (Shen et al., 2008).
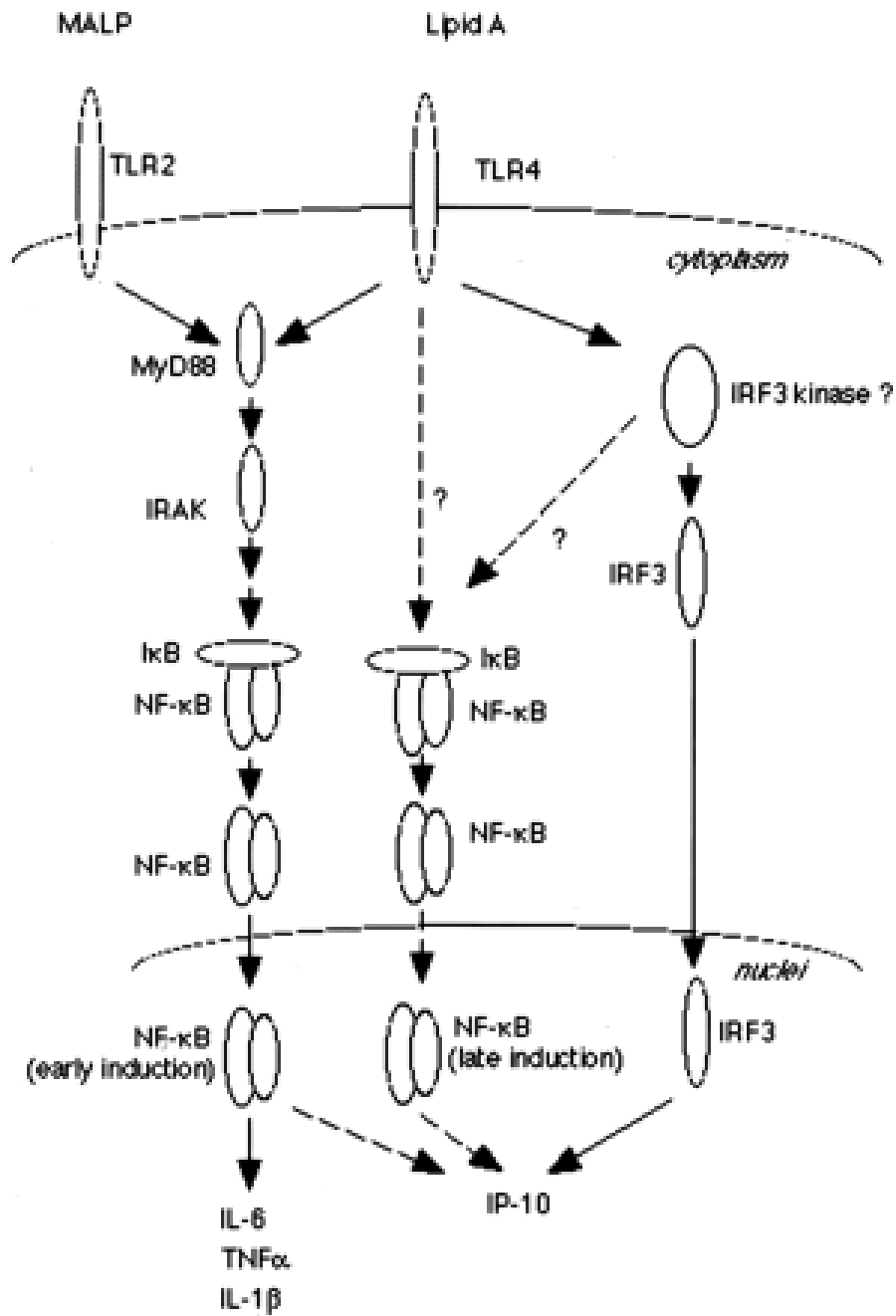
Figure 4.5: Model of the signalling pathways though TLR2 and TLR4.

Taken from (Kawai et al., 2001).

4.4 In vivo experiments

In contrast to the administration of LPS and the increased lethality rate of AnxA1$^{-/-}$ mice (Damazo et al., 2005), the intraperitoneal injection of sublethal doses of poly (I:C) revealed different results. The AnxA1$^{-/-}$ mice showed a 20% increased survival post-poly (I:C) administration independent of the sublethal dosage used. Therefore, the absence of AnxA1 in this case showed beneficial results, which were in the opposite direction to those seen following the stimulation of TLR4.

If the AnxA1$^{-/-}$ mice die more easily after LPS administration due to the induced 'cytokine storm', then definitely the *in vitro* experiments with the BMDMs support this hypothesis. Indeed, AxA1$^{-/-}$ BMDMs showed increased TNF-α and IL-6 production after stimulation with LPS. On the other hand, there was no difference in TNF-α by AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C). Therefore, we did not expect to detect a 20% survival advantage by the AnxA1$^{-/-}$ mice after administration of poly (I:C). Does this mean that the presence of AnxA1 instead of being 'protective/ beneficial' can be a disadvantage in the case of viral infections? Perhaps the pathophysiological mechanism involved is this case is more complicated. Indeed, it is now known that the initial innate immune responses are suppressed by the adaptive immune system and specifically by conventional T cells (Kim et al., 2007). Therefore, when Rag-deficient mice, which lack all lymphocytes, were injected with a sublethal dose of poly (I:C), they experienced a very rapid death characterised by increased levels of IFN-γ and TNF-α (Kim et al., 2007). On the contrary, the WT mice survived because their T cells suppressed the early inflammatory response by innate cells in a contact-and MHC II-

dependent manner (Kim et al., 2007). Poly (I:C) is considered a T_H1 stimulus (Longhi et al., 2009). Balb/c mice present a predominantly T_H2 phenotype (Watanabe et al., 2004). In addition to, T cells from AnxA1$^{-/-}$ mice show an increased T_H2 phenotype (D'Acquisto et al., 2007b). This means that the AnxA1$^{-/-}$ Balb/c mice used in our *in vivo* experiments, had a highly T_H2 adaptive immune response and that with the poly (I:C) administration directed their T_H-response towards a T_H1 phenotype. Possibly, the initial increased T_H2 phenotype of the AnxA1$^{-/-}$ mice might have helped them to balance more easily the adaptive immune response, and therefore has given them a small survival advantage compared to their WT littermates. Moreover, we did observe impaired IL-6 production by AnxA1$^{-/-}$ BMDMs after stimulation with poly (I:C). If we assume that AnxA1$^{-/-}$ mice induce an analogous impaired IL-6 production post-administration of poly (I:C), then the suppression of T cells by T_R cells in this case is also increased (Pasare and Medzhitov, 2003) and the adaptive immune responses more tightly controlled, possibly giving them a small survival advantage.

4.5 Conclusions

We believe that when the macrophage detects a pathogen invasion through its TLRs, it secretes AnxA1 in to the extracellular milieu, in order to interact with its receptor. This pathophysiological mechanism possibly switches on an anti-inflammatory mechanism in Gram (+), Gram (-), and certain viral infections and is partly caused by down-regulation of ERK1/2 activation and NF-κB activation by AnxA1. Therefore, AnxA1 seems to block the MyD88-pathway utilising an as yet unidentified mechanism and this results in increased pro-inflammatory response and lethality of AnxA1$^{-/-}$ mice after administration of TLR ligands triggering this pathway such as LPS (Damazo et al., 2005). On the other hand, the role of AnxA1 in the TRIF-pathway and against viruses triggering TLR3 remains intriguing. In this case, AnxA1 seems to have the opposite effect by enhancing the upregulation of MHC II, IL-6, and NO and resulting in a survival advantage of AnxA1$^{-/-}$ mice post administration of poly (I:C). In conclusion, more detailed investigations are warranted in order to resolve the exact point in the TLR signalling pathway at which AnxA1 is implicated. Overall, we here present further results that support the idea that AnxA1 is a modulator of inflammation with therapeutic potential in the fight against infectious diseases.

Finally, our study was mostly based on the comparison of AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs and the assumption that the only difference in these two types of cells is the expression of AnxA1. Therefore, the inconsistencies in our findings regarding cytokine production could be due to the fact that there are other differences in these KO macrophages, which alter our observations. Indeed, the study of the

AnxA1$^{-/-}$ mouse has revealed that there are such compensatory mechanisms. More specifically, knockout of AnxA1 may lead to up-regulation of other annexins such as AnxA2 (Hannon et al., 2003). AnxA2 has proven to have a pro-inflammatory effect in the macrophages such as to increase the IL-6 and TNF-α content (Swisher et al., 2007). In addition, AnxA2 seems to modulate macrophage function through TLR4 (Swisher et al., 2010). Therefore, the comparison of AnxA1$^{+/+}$ to AnxA1$^{-/-}$ BMDMs to study the possible anti-inflammatory role of AnxA1 could be a problematic set-up to begin with. Alternatively, the treatment of macrophages with the human recombinant protein could be a more reliable methodology to examine the role of AnxA1 in these cells. Indeed, our very preliminary results of comparing the expression of AnxA2 in AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs has revealed a small increase in the expression of AnxA2 in AnxA1$^{-/-}$ cells, supporting this hypothesis (data not shown). It would be interesting to examine the use of an AnxA2 neutralising antibody in AnxA1$^{-/-}$ BMDMs before treating them with the different TLR ligands.

Summarising, endogenous AnxA1 seems to have a homeostatic role, by influencing mainly the MyD88-dependent pathway and to a lesser extent in the opposite direction the TRIF-dependent pathway both *in vitro* and *in vivo*. Conclusively, endogenous AnxA1 has a tonic influence, acting as a molecular tuner of the TLR signalling pathways with specificity per different TLR.

4.6 Future work

A series of experiments could be added to the work of this Thesis to complement our investigation on the exact role of AnxA1 in TLR signalling.

Firstly, AnxA1$^{-/-}$ BMDMs could be treated with hr-AnxA1 followed by measurement of IL-6 and TNF-α in the supernatant, ERK-phosphorylation and EMSA for NF-κB DNA-binding activity, in order to investigate whether the phenotype can be rescued.

The TLR signalling pathways (MyD88-dependent and MyD88-independent) need to be dissected in order to identify the exact molecules in TLR signalling pathway at which AnxA1 could exert an effect. These could include all the adaptor proteins (MyD88, TRIF, TRAM, TIRAP/MAL, SARM), TRAF3 and TRAF6, IKKs and IκBs, IRFs, RIP-1, TAB2 and TAB3, TAK1, TBK1, and Pellino-1. This analysis should give priority to molecules that are also part of the FPR2 signalling pathway such as MAPKs and Akt. Coprecipitation experiments of AnxA1 and the protein(s) of interest can take place to investigate possible interactions. Alternatively, we could transfect WT and AnxA1$^{-/-}$ BMDMs with plasmids overexpressing the protein of interest followed by stimulation with TLR ligands and measurement of IL-6/TNF-α mRNA synthesis and protein secretion, ERK phoshorylation and NF-κB DNA-binding activity. In addition, WT and AnxA1$^{-/-}$ BMDMs may be transfected with NF-κB luciferase reporter gene followed with co-transfection with plasmid of the protein of interest (e.g. different adaptor proteins). Thereafter, stimulation with the different TLR ligands and luciferase reporter assay will show if overexpression of

any of these proteins how much will influence NF-κB activation in WT and AnxA1$^{-/-}$ BMDMs.

Western blotting and Real-time PCR for IκBα, IκBβ, IκBε in cell lysates from WT and AnxA1$^{-/-}$ BMDMs stimulated with TLR ligands will reveal the possible involvement of these molecules in the effect of AnxA1 on TLR signalling. WB and Real-time PCR can also be performed for other proteins of interest such as the different adaptor proteins. To confirm the involvement of AnxA1 to the TRIF-pathway, we could measure the levels of IFN-β in the supernatant of WT and AnxA1$^{-/-}$ BMDMs stimulated with poly (I:C) or LPS. The involvement of IRFs can be investigated by performing EMSA for each IRF DNA binding activity with depletion analysis using siRNA for IRFs. In order to see if the effect of AnxA1 is mediated through ERK activation, we could use an ERK inhibitor and repeat the previous experimental procedure. To verify that gene transcription mediates our observations, we could use inhibitors such as Actinomycin D followed by Real-time PCR for *IL-6* and *TNF-α*.

FPR2 null mice could be used as a source for the isolation and generation of BMDMs may be used to investigate whether the effect of AnxA1 on TLR signalling is mediated through this receptor. After stimulation of these cells with TLR ligands, IP/IB for AnxA1 in the supernatant will firstly show if AnxA1 is secreted, and repetition of the experimental procedures performed in this thesis will compare WT and FPR2$^{-/-}$ BMDMs. Alternatively, Boc2 (a pan antagonist for the FPR-family receptors) may be given to WT or AnxA1$^{-/-}$ BMDMs consecutively with the TLR ligands.

All our *in vitro* observations should be confirmed *in vivo* after the administration of different TLR ligands such as LPS or poly (I:C) to WT and AnxA1$^{-/-}$ mice. For example, the concentration of different cytokines such as IL-6, TNF-α, IFN-β can be measured in plasma. Histological samples for analysis may also be taken.

The experiments on BMDMs may be repeated using neutralising antibody against Annexin-A2 in order to investigate whether our observations in AnxA1$^{-/-}$ BMDMs are indeed findings due to the absence of AnxA1.

Specific viruses and live Gram (+) and Gram (-) bacteria representative of each TLR ligand may be used *in vivo* to infect AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice or *in vitro* to AnxA1$^{+/+}$ and AnxA1$^{-/-}$ BMDMs. Different outcomes, for example viral titers can be added to our investigation.

Finally, we could add to our research other measurements such as IL-10 secretion in the supernatant or certain protein(s) expression involved in the inflammatory process e.g. MIF or HMGB1.

CHAPTER 5:

BIBLIOGRAPHY

ABBAS, A. K., MURPHY, K. M. & SHER, A. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383**,** 787-93.

ABRAHAM, E., LATERRE, P. F., GARG, R., LEVY, H., TALWAR, D., TRZASKOMA, B. L., FRANCOIS, B., GUY, J. S., BRUCKMANN, M., REA-NETO, A., ROSSAINT, R., PERROTIN, D., SABLOTZKI, A., ARKINS, N., UTTERBACK, B. G. & MACIAS, W. L. 2005. Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. *N Engl J Med,* 353**,** 1332-41.

ABRAHAM, E., REINHART, K., OPAL, S., DEMEYER, I., DOIG, C., RODRIGUEZ, A. L., BEALE, R., SVOBODA, P., LATERRE, P. F., SIMON, S., LIGHT, B., SPAPEN, H., STONE, J., SEIBERT, A., PECKELSEN, C., DE DEYNE, C., POSTIER, R., PETTILA, V., ARTIGAS, A., PERCELL, S. R., SHU, V., ZWINGELSTEIN, C., TOBIAS, J., POOLE, L., STOLZENBACH, J. C. & CREASEY, A. A. 2003. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. *JAMA,* 290**,** 238-47.

ADIB-CONQUY, M., ADRIE, C., FITTING, C., GATTOLLIAT, O., BEYAERT, R. & CAVAILLON, J. M. 2006. Up-regulation of MyD88s and SIGIRR, molecules inhibiting Toll-like receptor signalling, in monocytes from septic patients. *Crit Care Med,* 34**,** 2377-85.

AGNESE, D. M., CALVANO, J. E., HAHM, S. J., COYLE, S. M., CORBETT, S. A., CALVANO, S. E. & LOWRY, S. F. 2002. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis,* 186**,** 1522-5.

AKIRA, S. & HOSHINO, K. 2003. Myeloid differentiation factor 88-dependent and - independent pathways in toll-like receptor signalling. *J Infect Dis,* 187 Suppl 2**,** S356-63.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* 413**,** 732-8.

ALEXOPOULOU, L., THOMAS, V., SCHNARE, M., LOBET, Y., ANGUITA, J., SCHOEN, R. T., MEDZHITOV, R., FIKRIG, E. & FLAVELL, R. A. 2002. Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. *Nat Med,* 8**,** 878-84.

ALIPRANTIS, A. O., YANG, R. B., MARK, M. R., SUGGETT, S., DEVAUX, B., RADOLF, J. D., KLIMPEL, G. R., GODOWSKI, P. & ZYCHLINSKY, A. 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science,* 285**,** 736-9.

ALLDRIDGE, L. C., HARRIS, H. J., PLEVIN, R., HANNON, R. & BRYANT, C. E. 1999. The annexin protein lipocortin 1 regulates the MAPK/ERK pathway. *J Biol Chem,* 274**,** 37620-8.

ALVAREZ-MARTINEZ, M. T., MANI, J. C., PORTE, F., FAIVRE-SARRAILH, C., LIAUTARD, J. P. & SRI WIDADA, J. 1996. Characterization of the interaction between annexin I and profilin. *Eur J Biochem,* 238**,** 777-84.

ALVAREZ-MARTINEZ, M. T., PORTE, F., LIAUTARD, J. P. & SRI WIDADA, J. 1997. Effects of profilin-annexin I association on some properties of both profilin and annexin I: modification of the inhibitory activity of profilin on actin polymerization and inhibition of the self-association of annexin I and its interactions with liposomes. *Biochim Biophys Acta,* 1339**,** 331-40.

AMBROSE, M. P., BAHNS, C. L. & HUNNINGHAKE, G. W. 1992. Lipocortin I production by human alveolar macrophages. *Am J Respir Cell Mol Biol,* 6**,** 17-21.

AN, H., XU, H., YU, Y., ZHANG, M., QI, R., YAN, X., LIU, S., WANG, W., GUO, Z., QIN, Z. & CAO, X. 2002. Up-regulation of TLR9 gene expression by LPS in mouse macrophages via activation of NF-kappaB, ERK and p38 MAPK signal pathways. *Immunol Lett,* 81**,** 165-9.

ANDERSEN-NISSEN, E., SMITH, K. D., STROBE, K. L., BARRETT, S. L., COOKSON, B. T., LOGAN, S. M. & ADEREM, A. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A,* 102**,** 9247-52.

ANDERSEN, J., VANSCOY, S., CHENG, T. F., GOMEZ, D. & REICH, N. C. 2008. IRF-3-dependent and augmented target genes during viral infection. *Genes Immun,* 9**,** 168-75.

ARAS, O., SHET, A., BACH, R. R., HYSJULIEN, J. L., SLUNGAARD, A., HEBBEL, R. P., ESCOLAR, G., JILMA, B. & KEY, N. S. 2004. Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood,* 103**,** 4545-53.

ARBOUR, N. C., LORENZ, E., SCHUTTE, B. C., ZABNER, J., KLINE, J. N., JONES, M., FREES, K., WATT, J. L. & SCHWARTZ, D. A. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet,* 25**,** 187-91.

ARMSTRONG, L., MEDFORD, A. R., HUNTER, K. J., UPPINGTON, K. M. & MILLAR, A. B. 2004. Differential expression of Toll-like receptor (TLR)-2 and TLR-4 on monocytes in human sepsis. *Clin Exp Immunol,* 136**,** 312-9.

ARUR, S., UCHE, U. E., REZAUL, K., FONG, M., SCRANTON, V., COWAN, A. E., MOHLER, W. & HAN, D. K. 2003. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell,* 4**,** 587-98.

ASAI, Y., HASHIMOTO, M., FLETCHER, H. M., MIYAKE, K., AKIRA, S. & OGAWA, T. 2005. Lipopolysaccharide preparation extracted from Porphyromonas gingivalis lipoprotein-deficient mutant shows a marked decrease in toll-like receptor 2-mediated signalling. *Infect Immun,* 73**,** 2157-63.

ASSUNCAO-MIRANDA, I., AMARAL, F. A., BOZZA, F. A., FAGUNDES, C. T., SOUSA, L. P., SOUZA, D. G., PACHECO, P., BARBOSA-LIMA, G., GOMES, R. N., BOZZA, P. T., DA POIAN, A. T., TEIXEIRA, M. M. & BOZZA, M. T. 2010. Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection. *Faseb J,* 24**,** 218-28.

BAEUERLE, P. A. & BALTIMORE, D. 1996. NF-kappa B: ten years after. *Cell,* 87**,** 13-20.

BAGCHI, A., HERRUP, E. A., WARREN, H. S., TRIGILIO, J., SHIN, H. S., VALENTINE, C. & HELLMAN, J. 2007. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol,* 178**,** 1164-71.

BARTON, B. E. & JACKSON, J. V. 1993. Protective role of interleukin 6 in the lipopolysaccharide-galactosamine septic shock model. *Infect Immun,* 61**,** 1496-9.

BARTON, G. M., KAGAN, J. C. & MEDZHITOV, R. 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol,* 7**,** 49-56.

BASSLER, B. L. 2002. Small talk. Cell-to-cell communication in bacteria. *Cell,* 109**,** 421-4.

BEAUMONT, C. & DELABY, C. 2009. Recycling iron in normal and pathological states. *Semin Hematol,* 46**,** 328-38.

BECKERMAN, K. P., ROGERS, H. W., CORBETT, J. A., SCHREIBER, R. D., MCDANIEL, M. L. & UNANUE, E. R. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to Listeria monocytogenes. *J Immunol,* 150**,** 888-95.

BEESON, P. B. 1947a. Tolerance to Bacterial Pyrogens : I. Factors Influencing Its Development. *J Exp Med,* 86**,** 29-38.

BEESON, P. B. 1947b. Tolerance to Bacterial Pyrogens : Ii. Role of the Reticulo-Endothelial System. *J Exp Med,* 86**,** 39-44.

BERG, D. J., ZHANG, J., LAURICELLA, D. M. & MOORE, S. A. 2001. Il-10 is a central regulator of cyclooxygenase-2 expression and prostaglandin production. *J Immunol,* 166**,** 2674-80.

BERNARD, G. R., VINCENT, J. L., LATERRE, P. F., LAROSA, S. P., DHAINAUT, J. F., LOPEZ-RODRIGUEZ, A., STEINGRUB, J. S., GARBER, G. E., HELTERBRAND, J. D., ELY, E. W. & FISHER, C. J., JR. 2001. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med,* 344**,** 699-709.

BERNHAGEN, J., CALANDRA, T., MITCHELL, R. A., MARTIN, S. B., TRACEY, K. J., VOELTER, W., MANOGUE, K. R., CERAMI, A. & BUCALA, R. 1993. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature,* 365**,** 756-9.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature,* 441**,** 235-8.

BEUTLER, B., JIANG, Z., GEORGEL, P., CROZAT, K., CROKER, B., RUTSCHMANN, S., DU, X. & HOEBE, K. 2006. Genetic analysis of host resistance: Toll-like receptor signalling and immunity at large. *Annu Rev Immunol,* 24**,** 353-89.

BEUTLER, B., MILSARK, I. W. & CERAMI, A. C. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science,* 229**,** 869-71.

BJORKBACKA, H., FITZGERALD, K. A., HUET, F., LI, X., GREGORY, J. A., LEE, M. A., ORDIJA, C. M., DOWLEY, N. E., GOLENBOCK, D. T. & FREEMAN, M. W. 2004. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signalling cascades. *Physiol Genomics,* 19**,** 319-30.

BLANDER, J. M. 2008. Phagocytosis and antigen presentation: a partnership initiated by Toll-like receptors. *Ann Rheum Dis,* 67 Suppl 3**,** iii44-9.

BONE, R. C., BALK, R. A., CERRA, F. B., DELLINGER, R. P., FEIN, A. M., KNAUS, W. A., SCHEIN, R. M. & SIBBALD, W. J. 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest,* 101**,** 1644-55.

BOONE, D. L., TURER, E. E., LEE, E. G., AHMAD, R. C., WHEELER, M. T., TSUI, C., HURLEY, P., CHIEN, M., CHAI, S., HITOTSUMATSU, O., MCNALLY, E., PICKART, C. & MA, A. 2004. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nat Immunol,* 5**,** 1052-60.

BOONSTRA, A., RAJSBAUM, R., HOLMAN, M., MARQUES, R., ASSELIN-PATUREL, C., PEREIRA, J. P., BATES, E. E., AKIRA, S., VIEIRA, P., LIU, Y. J., TRINCHIERI, G. & O'GARRA, A. 2006. Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J Immunol,* 177**,** 7551-8.

BOROVIKOVA, L. V., IVANOVA, S., ZHANG, M., YANG, H., BOTCHKINA, G. I., WATKINS, L. R., WANG, H., ABUMRAD, N., EATON, J. W. & TRACEY, K. J. 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature,* 405**,** 458-62.

BRINT, E. K., XU, D., LIU, H., DUNNE, A., MCKENZIE, A. N., O'NEILL, L. A. & LIEW, F. Y. 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signalling and maintains endotoxin tolerance. *Nat Immunol,* 5**,** 373-9.

BROIDE, D., SCHWARZE, J., TIGHE, H., GIFFORD, T., NGUYEN, M. D., MALEK, S., VAN UDEN, J., MARTIN-OROZCO, E., GELFAND, E. W. & RAZ, E. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol,* 161**,** 7054-62.

BROWNSTEIN, C., DEORA, A. B., JACOVINA, A. T., WEINTRAUB, R., GERTLER, M., KHAN, K. M., FALCONE, D. J. & HAJJAR, K. A. 2004. Annexin II mediates plasminogen-dependent matrix invasion by human monocytes: enhanced expression by macrophages. *Blood,* 103**,** 317-24.

BUCZYNSKI, M. W., STEPHENS, D. L., BOWERS-GENTRY, R. C., GRKOVICH, A., DEEMS, R. A. & DENNIS, E. A. 2007. TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *J Biol Chem,* 282**,** 22834-47.

CALANDRA, T. & BUCALA, R. 1995. Macrophage migration inhibitory factor: a counter-regulator of glucocorticoid action and critical mediator of septic shock. *J Inflamm,* 47**,** 39-51.

CALANDRA, T., ECHTENACHER, B., ROY, D. L., PUGIN, J., METZ, C. N., HULTNER, L., HEUMANN, D., MANNEL, D., BUCALA, R. & GLAUSER, M. P. 2000. Protection from septic shock by neutralization of macrophage migration inhibitory factor. *Nat Med,* 6**,** 164-70.

CALANDRA, T., FROIDEVAUX, C., MARTIN, C. & ROGER, T. 2003. Macrophage migration inhibitory factor and host innate immune defenses against bacterial sepsis. *J Infect Dis,* 187 Suppl 2**,** S385-90.

CALANDRA, T. & ROGER, T. 2003. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol,* 3**,** 791-800.

CAO, Z., XIONG, J., TAKEUCHI, M., KURAMA, T. & GOEDDEL, D. V. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature,* 383**,** 443-6.

CAVASSANI, K. A., ISHII, M., WEN, H., SCHALLER, M. A., LINCOLN, P. M., LUKACS, N. W., HOGABOAM, C. M. & KUNKEL, S. L. 2008. TLR3 is an

endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med,* 205**,** 2609-21.

CEDERHOLM, A. & FROSTEGARD, J. 2005. Annexin A5 in cardiovascular disease and systemic lupus erythematosus. *Immunobiology,* 210**,** 761-8.

CHANG, M., JIN, W. & SUN, S. C. 2009. Peli1 facilitates TRIF-dependent Toll-like receptor signalling and proinflammatory cytokine production. *Nat Immunol,* 10**,** 1089-95.

CHASSEROT-GOLAZ, S., VITALE, N., UMBRECHT-JENCK, E., KNIGHT, D., GERKE, V. & BADER, M. F. 2005. Annexin 2 promotes the formation of lipid microdomains required for calcium-regulated exocytosis of dense-core vesicles. *Mol Biol Cell,* 16**,** 1108-19.

CHEN, B. C., CHANG, Y. S., KANG, J. C., HSU, M. J., SHEU, J. R., CHEN, T. L., TENG, C. M. & LIN, C. H. 2004. Peptidoglycan induces nuclear factor-kappaB activation and cyclooxygenase-2 expression via Ras, Raf-1, and ERK in RAW 264.7 macrophages. *J Biol Chem,* 279**,** 20889-97.

CHEN, B. C., KANG, J. C., LU, Y. T., HSU, M. J., LIAO, C. C., CHIU, W. T., YEH, F. L. & LIN, C. H. 2009a. Rac1 regulates peptidoglycan-induced nuclear factor-kappaB activation and cyclooxygenase-2 expression in RAW 264.7 macrophages by activating the phosphatidylinositol 3-kinase/Akt pathway. *Mol Immunol,* 46**,** 1179-88.

CHEN, B. C., LIAO, C. C., HSU, M. J., LIAO, Y. T., LIN, C. C., SHEU, J. R. & LIN, C. H. 2006. Peptidoglycan-induced IL-6 production in RAW 264.7 macrophages is mediated by cyclooxygenase-2, PGE2/PGE4 receptors, protein kinase A, I kappa B kinase, and NF-kappa B. *J Immunol,* 177**,** 681-93.

CHEN, K., HUANG, J., LIU, Y., GONG, W., CUI, Y. & WANG, J. M. 2009b. Synergy of TRIF-dependent TLR3 and MyD88-dependent TLR7 in up-regulating expression of mouse FPR2, a promiscuous G-protein-coupled receptor, in microglial cells. *J Neuroimmunol,* 213**,** 69-77.

CHEN, K., ZHANG, L., HUANG, J., GONG, W., DUNLOP, N. M. & WANG, J. M. 2008. Cooperation between NOD2 and Toll-like receptor 2 ligands in the up-regulation of mouse mFPR2, a G-protein-coupled Abeta42 peptide receptor, in microglial cells. *J Leukoc Biol,* 83**,** 1467-75.

CHI, H., BARRY, S. P., ROTH, R. J., WU, J. J., JONES, E. A., BENNETT, A. M. & FLAVELL, R. A. 2006. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proc Natl Acad Sci U S A,* 103**,** 2274-9.

CHIANG, C. & BEACHY, P. A. 1994. Expression of a novel Toll-like gene spans the parasegment boundary and contributes to hedgehog function in the adult eye of Drosophila. *Mech Dev,* 47**,** 225-39.

CHIN, AC., FOURNIER, B., PEATMAN, EJ., REAVES, TA., LEE, WY., PARKOS, CA. 2009. CD47 and TLR-2 cross-talk regulates neutrophil transmigration. *J Immunol*, 183, 5957-5963

CHOI, Y. J., IM, E., POTHOULAKIS, C. & RHEE, S. H. TRIF modulates TLR5-dependent responses by inducing proteolytic degradation of TLR5. *J Biol Chem,* 285**,** 21382-90.

CHUANG, T. H. & ULEVITCH, R. J. 2004. Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat Immunol,* 5**,** 495-502.

CIRINO, G., CICALA, C., SORRENTINO, L., CILIBERTO, G., ARPAIA, G., PERRETTI, M. & FLOWER, R. J. 1993. Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol,* 108**,** 573-4.

CIRINO, G., FLOWER, R. J., BROWNING, J. L., SINCLAIR, L. K. & PEPINSKY, R. B. 1987. Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. *Nature,* 328**,** 270-2.

CIRINO, G., PEERS, S. H., FLOWER, R. J., BROWNING, J. L. & PEPINSKY, R. B. 1989. Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc Natl Acad Sci U S A,* 86**,** 3428-32.

CLARK, S. R., MA, A. C., TAVENER, S. A., MCDONALD, B., GOODARZI, Z., KELLY, M. M., PATEL, K. D., CHAKRABARTI, S., MCAVOY, E., SINCLAIR, G. D., KEYS, E. M., ALLEN-VERCOE, E., DEVINNEY, R., DOIG, C. J., GREEN, F. H. & KUBES, P. 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med,* 13**,** 463-9.

COVERT, M. W., LEUNG, T. H., GASTON, J. E. & BALTIMORE, D. 2005. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. *Science,* 309**,** 1854-7.

CREWS, C. M., ALESSANDRINI, A. & ERIKSON, R. L. 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science,* 258**,** 478-80.

CUADROS, M. A. & NAVASCUES, J. 1998. The origin and differentiation of microglial cells during development. *Prog Neurobiol,* 56**,** 173-89.

CUI, Y. H., LE, Y., GONG, W., PROOST, P., VAN DAMME, J., MURPHY, W. J. & WANG, J. M. 2002. Bacterial lipopolysaccharide selectively up-regulates the function of the chemotactic peptide receptor formyl peptide receptor 2 in murine microglial cells. *J Immunol,* 168**,** 434-42.

CUNHA, F. Q., MONCADA, S. & LIEW, F. Y. 1992. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. *Biochem Biophys Res Commun,* 182**,** 1155-9.

CZERMAK, B. J., SARMA, V., PIERSON, C. L., WARNER, R. L., HUBER-LANG, M., BLESS, N. M., SCHMAL, H., FRIEDL, H. P. & WARD, P. A. 1999. Protective effects of C5a blockade in sepsis. *Nat Med,* 5**,** 788-92.

D'ACQUISTO, F., MERGHANI, A., LECONA, E., ROSIGNOLI, G., RAZA, K., BUCKLEY, C. D., FLOWER, R. J. & PERRETTI, M. 2007a. Annexin-1 modulates T-cell activation and differentiation. *Blood,* 109**,** 1095-102.

D'ACQUISTO, F., PASCHALIDIS, N., SAMPAIO, A. L., MERGHANI, A., FLOWER, R. J. & PERRETTI, M. 2007b. Impaired T cell activation and increased Th2 lineage commitment in Annexin-1-deficient T cells. *Eur J Immunol,* 37**,** 3131-42.

DAHL, M. E., DABBAGH, K., LIGGITT, D., KIM, S. & LEWIS, D. B. 2004. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol,* 5**,** 337-43.

DAHLGREN, C., CHRISTOPHE, T., BOULAY, F., MADIANOS, P. N., RABIET, M. J. & KARLSSON, A. 2000. The synthetic chemoattractant Trp-Lys-Tyr-Met-Val-DMet activates neutrophils preferentially through the lipoxin A(4) receptor. *Blood,* 95**,** 1810-8.

DAMAZO, A. S., YONA, S., D'ACQUISTO, F., FLOWER, R. J., OLIANI, S. M. & PERRETTI, M. 2005. Critical protective role for annexin 1 gene expression in the endotoxemic murine microcirculation. *Am J Pathol,* 166**,** 1607-17.

DAUBEUF, B., MATHISON, J., SPILLER, S., HUGUES, S., HERREN, S., FERLIN, W., KOSCO-VILBOIS, M., WAGNER, H., KIRSCHNING, C. J., ULEVITCH, R. & ELSON, G. 2007. TLR4/MD-2 monoclonal antibody therapy affords protection in experimental models of septic shock. *J Immunol,* 179**,** 6107-14.

DE CATERINA, R., SICARI, R., GIANNESSI, D., PAGGIARO, P. L., PAOLETTI, P., LAZZERINI, G., BERNINI, W., SOLITO, E. & PARENTE, L. 1993. Macrophage-specific eicosanoid synthesis inhibition and lipocortin-1 induction by glucocorticoids. *J Appl Physiol,* 75**,** 2368-75.

DENG, L., WANG, C., SPENCER, E., YANG, L., BRAUN, A., YOU, J., SLAUGHTER, C., PICKART, C. & CHEN, Z. J. 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell,* 103**,** 351-61.

DEVOSSE, T., GUILLABERT, A., D'HAENE, N., BERTON, A., DE NADAI, P., NOEL, S., BRAIT, M., FRANSSEN, J. D., SOZZANI, S., SALMON, I. & PARMENTIER, M. 2009. Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. *J Immunol,* 182**,** 4974-84.

DIAO, H. & KOHANAWA, M. 2005. Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. *Infect Immun,* 73**,** 3745-8.

DIDIERLAURENT, A., BRISSONI, B., VELIN, D., AEBI, N., TARDIVEL, A., KASLIN, E., SIRARD, J. C., ANGELOV, G., TSCHOPP, J. & BURNS, K. 2006. Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. *Mol Cell Biol,* 26**,** 735-42.

DIEHL, G. E., YUE, H. H., HSIEH, K., KUANG, A. A., HO, M., MORICI, L. A., LENZ, L. L., CADO, D., RILEY, L. W. & WINOTO, A. 2004. TRAIL-R as a negative regulator of innate immune cell responses. *Immunity,* 21**,** 877-89.

DIEHL, L., DEN BOER, A. T., VAN DER VOORT, E. I., MELIEF, C. J., OFFRINGA, R. & TOES, R. E. 2000. The role of CD40 in peripheral T cell tolerance and immunity. *J Mol Med,* 78**,** 363-71.

DING, L., LINSLEY, P. S., HUANG, L. Y., GERMAIN, R. N. & SHEVACH, E. M. 1993. IL-10 inhibits macrophage co-stimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol,* 151**,** 1224-34.

DOYLE, S. L. & O'NEILL, L. A. 2006. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem Pharmacol,* 72**,** 1102-13.

DUNCAN, G. S., PEERS, S. H., CAREY, F., FORDER, R. & FLOWER, R. J. 1993. The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin 1. *Br J Pharmacol,* 108**,** 62-5.

DUNNE, A. & O'NEILL, L. A. 2005. Adaptor usage and Toll-like receptor signalling specificity. *FEBS Lett,* 579**,** 3330-5.

EDWARDS, J. P., ZHANG, X., FRAUWIRTH, K. A. & MOSSER, D. M. 2006. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol,* 80**,** 1298-307.

EHLERS, M. & RAVETCH, J. V. 2007. Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance. *Trends Immunol,* 28**,** 74-9.

EISENBARTH, S. C., PIGGOTT, D. A., HULEATT, J. W., VISINTIN, I., HERRICK, C. A. & BOTTOMLY, K. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med,* 196**,** 1645-51.

EL KASMI, K. C., QUALLS, J. E., PESCE, J. T., SMITH, A. M., THOMPSON, R. W., HENAO-TAMAYO, M., BASARABA, R. J., KONIG, T., SCHLEICHER, U., KOO, M. S., KAPLAN, G., FITZGERALD, K. A., TUOMANEN, E. I., ORME, I. M., KANNEGANTI, T. D., BOGDAN, C., WYNN, T. A. & MURRAY, P. J. 2008. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol,* 9**,** 1399-406.

ERRIDGE, C., STEWART, J. & POXTON, I. R. 2003. Monocytes heterozygous for the Asp299Gly and Thr399Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signalling. *J Exp Med,* 197**,** 1787-91.

ERWIG, L. P. & HENSON, P. M. 2007. Immunological consequences of apoptotic cell phagocytosis. *Am J Pathol,* 171**,** 2-8.

FANG, H., PENGAL, R. A., CAO, X., GANESAN, L. P., WEWERS, M. D., MARSH, C. B. & TRIDANDAPANI, S. 2004. Lipopolysaccharide-induced macrophage inflammatory response is regulated by SHIP. *J Immunol,* 173**,** 360-6.

FANUCCHI, M. V., SCHELEGLE, E. S., BAKER, G. L., EVANS, M. J., MCDONALD, R. J., GERSHWIN, L. J., RAZ, E., HYDE, D. M., PLOPPER, C. G. & MILLER, L. A. 2004. Immunostimulatory oligonucleotides attenuate

airways remodeling in allergic monkeys. *Am J Respir Crit Care Med,* 170**,** 1153-7.

FAURE-ANDRE, G., VARGAS, P., YSEF, MI., HEUZE, M., DIAZ, J., LANKAR, D. 2008. Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. *Science*, 322, 1705-1710

FERLAZZO, V., D'AGOSTINO, P., MILANO, S., CARUSO, R., FEO, S., CILLARI, E. & PARENTE, L. 2003. Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis. *Int Immunopharmacol,* 3**,** 1363-9.

FETEROWSKI, C., EMMANUILIDIS, K., MIETHKE, T., GERAUER, K., RUMP, M., ULM, K., HOLZMANN, B. & WEIGHARDT, H. 2003. Effects of functional Toll-like receptor-4 mutations on the immune response to human and experimental sepsis. *Immunology,* 109**,** 426-31.

FEUILLET, V., MEDJANE, S., MONDOR, I., DEMARIA, O., PAGNI, P. P., GALAN, J. E., FLAVELL, R. A. & ALEXOPOULOU, L. 2006. Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. *Proc Natl Acad Sci U S A,* 103**,** 12487-92.

FILIPENKO, N. R., MACLEOD, T. J., YOON, C. S. & WAISMAN, D. M. 2004. Annexin A2 is a novel RNA-binding protein. *J Biol Chem,* 279**,** 8723-31.

FILIPENKO, N. R. & WAISMAN, D. M. 2001. The C terminus of annexin II mediates binding to F-actin. *J Biol Chem,* 276**,** 5310-5.

FISCHER, E., MARANO, M. A., VAN ZEE, K. J., ROCK, C. S., HAWES, A. S., THOMPSON, W. A., DEFORGE, L., KENNEY, J. S., REMICK, D. G., BLOEDOW, D. C. & ET AL. 1992. Interleukin-1 receptor blockade improves survival and hemodynamic performance in Escherichia coli septic shock,

but fails to alter host responses to sublethal endotoxemia. *J Clin Invest,* 89**,** 1551-7.

FITZGERALD, K. A., MCWHIRTER, S. M., FAIA, K. L., ROWE, D. C., LATZ, E., GOLENBOCK, D. T., COYLE, A. J., LIAO, S. M. & MANIATIS, T. 2003a. IKKepsilon and TBK1 are essential components of the IRF3 signalling pathway. *Nat Immunol,* 4**,** 491-6.

FITZGERALD, K. A., ROWE, D. C., BARNES, B. J., CAFFREY, D. R., VISINTIN, A., LATZ, E., MONKS, B., PITHA, P. M. & GOLENBOCK, D. T. 2003b. LPS-TLR4 signalling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med,* 198**,** 1043-55.

FLIERL, M. A., RITTIRSCH, D., GAO, H., HOESEL, L. M., NADEAU, B. A., DAY, D. E., ZETOUNE, F. S., SARMA, J. V., HUBER-LANG, M. S., FERRARA, J. L. & WARD, P. A. 2008. Adverse functions of IL-17A in experimental sepsis. *Faseb J,* 22**,** 2198-205.

FOELL, D., WITTKOWSKI, H. & ROTH, J. 2007. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol,* 3**,** 382-90.

FRITZ, J. H., GIRARDIN, S. E., FITTING, C., WERTS, C., MENGIN-LECREULX, D., CAROFF, M., CAVAILLON, J. M., PHILPOTT, D. J. & ADIB-CONQUY, M. 2005. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol,* 35**,** 2459-70.

FUJIHARA, M., MUROI, M., TANAMOTO, K., SUZUKI, T., AZUMA, H. & IKEDA, H. 2003. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther,* 100**,** 171-94.

FUJINAMI, R. S. & OLDSTONE, M. B. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science,* 230**,** 1043-5.

FUKAO, T. & KOYASU, S. 2003. PI3K and negative regulation of TLR signalling. *Trends Immunol,* 24**,** 358-63.

GAN, H., LEE, J., REN, F., CHEN, M., KORNFELD, H. & REMOLD, H. G. 2008. Mycobacterium tuberculosis blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. *Nat Immunol,* 9**,** 1189-97.

GAVINS, F. N., LEONI, G. & GETTING, S. J. 2006. Annexin 1 and melanocortin peptide therapy for protection against ischaemic-reperfusion damage in the heart. *ScientificWorldJournal,* 6**,** 1008-23.

GERKE, V. & MOSS, S. E. 2002. Annexins: from structure to function. *Physiol Rev,* 82**,** 331-71.

GHOSH, S., MAY, M. J. & KOPP, E. B. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol,* 16**,** 225-60.

GIRARD, R., PEDRON, T., UEMATSU, S., BALLOY, V., CHIGNARD, M., AKIRA, S. & CHABY, R. 2003. Lipopolysaccharides from Legionella and Rhizobium stimulate mouse bone marrow granulocytes via Toll-like receptor 2. *J Cell Sci,* 116**,** 293-302.

GIRARDIN, E., GRAU, G. E., DAYER, J. M., ROUX-LOMBARD, P. & LAMBERT, P. H. 1988. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N Engl J Med,* 319**,** 397-400.

GORDON, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol,* 3**,** 23-35.

GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol,* 5**,** 953-64.

GOULDING, N. J., GODOLPHIN, J. L., SHARLAND, P. R., PEERS, S. H., SAMPSON, M., MADDISON, P. J. & FLOWER, R. J. 1990. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet,* 335**,** 1416-8.

GREGG, E. O. & DENIS, M. 1991. The effect of macrophage activation state on antigen presenting capability as defined by helper T-cell function. *Int J Immunopharmacol,* 13**,** 217-25.

GREWAL, T., HEEREN, J., MEWAWALA, D., SCHNITGERHANS, T., WENDT, D., SALOMON, G., ENRICH, C., BEISIEGEL, U. & JACKLE, S. 2000. Annexin VI stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. *J Biol Chem,* 275**,** 33806-13.

GUILLOT, L., BALLOY, V., MCCORMACK, F. X., GOLENBOCK, D. T., CHIGNARD, M. & SI-TAHAR, M. 2002. Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol,* 168**,** 5989-92.

GRANUCCI, F., PETRALIA, F., URBANO, M., CITTERIO, S., DI TOTA, F., SANTABROGIO, L. 2003. The scavenger receptor MARCO mediates cytoskeleton rrearrangements in dendritic ccells and microglia. *Blood*, 102, 2940-2947

HACKER, H., MISCHAK, H., MIETHKE, T., LIPTAY, S., SCHMID, R., SPARWASSER, T., HEEG, K., LIPFORD, G. B. & WAGNER, H. 1998.

CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *Embo J,* 17**,** 6230-40.

HACKER, J. & KAPER, J. B. 2000. Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol,* 54**,** 641-79.

HANNON, R., CROXTALL, J. D., GETTING, S. J., ROVIEZZO, F., YONA, S., PAUL-CLARK, M. J., GAVINS, F. N., PERRETTI, M., MORRIS, J. F., BUCKINGHAM, J. C. & FLOWER, R. J. 2003. Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *Faseb J,* 17**,** 253-5.

HARDY, M. P. & O'NEILL, L. A. 2004. The murine IRAK2 gene encodes four alternatively spliced isoforms, two of which are inhibitory. *J Biol Chem,* 279**,** 27699-708.

HARE, J. T. & TAYLOR, J. H. 1985. One role for DNA methylation in vertebrate cells is strand discrimination in mismatch repair. *Proc Natl Acad Sci U S A,* 82**,** 7350-4.

HARTER, L., MICA, L., STOCKER, R., TRENTZ, O. & KEEL, M. 2004. Increased expression of toll-like receptor-2 and -4 on leukocytes from patients with sepsis. *Shock,* 22**,** 403-9.

HASAN, U., CHAFFOIS, C., GAILLARD, C., SAULNIER, V., MERCK, E., TANCREDI, S., GUIET, C., BRIERE, F., VLACH, J., LEBECQUE, S., TRINCHIERI, G. & BATES, E. E. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol,* 174**,** 2942-50.

HAUSCHILDT, S., BASSENGE, E., BESSLER, W., BUSSE, R. & MULSCH, A. 1990. L-arginine-dependent nitric oxide formation and nitrite release in bone

marrow-derived macrophages stimulated with bacterial lipopeptide and lipopolysaccharide. *Immunology,* 70**,** 332-7.

HAWLISCH, H., BELKAID, Y., BAELDER, R., HILDEMAN, D., GERARD, C. & KOHL, J. 2005. C5a negatively regulates toll-like receptor 4-induced immune responses. *Immunity,* 22**,** 415-26.

HAWN, T. R., BERRINGTON, W. R., SMITH, I. A., UEMATSU, S., AKIRA, S., ADEREM, A., SMITH, K. D. & SKERRETT, S. J. 2007. Altered inflammatory responses in TLR5-deficient mice infected with Legionella pneumophila. *J Immunol,* 179**,** 6981-7.

HAWN, T. R., VERBON, A., LETTINGA, K. D., ZHAO, L. P., LI, S. S., LAWS, R. J., SKERRETT, S. J., BEUTLER, B., SCHROEDER, L., NACHMAN, A., OZINSKY, A., SMITH, K. D. & ADEREM, A. 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signalling and is associated with susceptibility to legionnaires' disease. *J Exp Med,* 198**,** 1563-72.

HAYASHI, E. A., AKIRA, S. & NOBREGA, A. 2005. Role of TLR in B cell development: signalling through TLR4 promotes B cell maturation and is inhibited by TLR2. *J Immunol,* 174**,** 6639-47.

HAYASHI, F., MEANS, TK., LUSTER, AD. 2003. Toll-like receptors stimulate human neutrophil function. *Blood*, 102, 2660-2668

HAYASHI, F., SMITH, K. D., OZINSKY, A., HAWN, T. R., YI, E. C., GOODLETT, D. R., ENG, J. K., AKIRA, S., UNDERHILL, D. M. & ADEREM, A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* 410**,** 1099-103.

HAYHOE, R. P., KAMAL, A. M., SOLITO, E., FLOWER, R. J., COOPER, D. & PERRETTI, M. 2006. Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement. *Blood,* 107**,** 2123-30.

HEIL, F., AHMAD-NEJAD, P., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., GELLERT, T., DIETRICH, H., LIPFORD, G., TAKEDA, K., AKIRA, S., WAGNER, H. & BAUER, S. 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur J Immunol,* 33**,** 2987-97.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. & BAUER, S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science,* 303**,** 1526-9.

HEITMEIER, M. R., SCARIM, A. L. & CORBETT, J. A. 1998. Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-kappaB activation. *J Biol Chem,* 273**,** 15301-7.

HEMMI, H., KAISHO, T., TAKEUCHI, O., SATO, S., SANJO, H., HOSHINO, K., HORIUCHI, T., TOMIZAWA, H., TAKEDA, K. & AKIRA, S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signalling pathway. *Nat Immunol,* 3**,** 196-200.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K. & AKIRA, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature,* 408**,** 740-5.

HIRATA, A. & HIRATA, F. 1999. Lipocortin (Annexin) I heterotetramer binds to purine RNA and pyrimidine DNA. *Biochem Biophys Res Commun,* 265**,** 200-4.

HIRSCHFELD, M., KIRSCHNING, C. J., SCHWANDNER, R., WESCHE, H., WEIS, J. H., WOOTEN, R. M. & WEIS, J. J. 1999. Cutting edge: inflammatory signalling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. *J Immunol,* 163**,** 2382-6.

HIRSCHFELD, M., MA, Y., WEIS, J. H., VOGEL, S. N. & WEIS, J. J. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signalling through both human and murine toll-like receptor 2. *J Immunol,* 165**,** 618-22.

HOEBE, K., JANSSEN, E. M., KIM, S. O., ALEXOPOULOU, L., FLAVELL, R. A., HAN, J. & BEUTLER, B. 2003. Upregulation of co-stimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat Immunol,* 4**,** 1223-9.

HOFFMANN, A., LEVCHENKO, A., SCOTT, M. L. & BALTIMORE, D. 2002. The IkappaB-NF-kappaB signalling module: temporal control and selective gene activation. *Science,* 298**,** 1241-5.

HOFFMANN, J. A., KAFATOS, F. C., JANEWAY, C. A. & EZEKOWITZ, R. A. 1999. Phylogenetic perspectives in innate immunity. *Science,* 284**,** 1313-8.

HOLMES, C. L., RUSSELL, J. A. & WALLEY, K. R. 2003. Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy. *Chest,* 124**,** 1103-15.

HONDA, K., YANAI, H., NEGISHI, H., ASAGIRI, M., SATO, M., MIZUTANI, T., SHIMADA, N., OHBA, Y., TAKAOKA, A., YOSHIDA, N. & TANIGUCHI, T.

2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature,* 434**,** 772-7.

HORNUNG, V., ROTHENFUSSER, S., BRITSCH, S., KRUG, A., JAHRSDORFER, B., GIESE, T., ENDRES, S. & HARTMANN, G. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol,* 168**,** 4531-7.

HOSHINO, K., TAKEUCHI, O., KAWAI, T., SANJO, H., OGAWA, T., TAKEDA, Y., TAKEDA, K. & AKIRA, S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol,* 162**,** 3749-52.

HOTCHKISS, R. S., COOPERSMITH, C. M., MCDUNN, J. E. & FERGUSON, T. A. 2009. The sepsis seesaw: tilting toward immunosuppression. *Nat Med,* 15**,** 496-7.

HOU, X. Q., GAO, Y. W., YANG, S. T., WANG, C. Y., MA, Z. Y. & XIA, X. Z. 2009. Role of macrophage migration inhibitory factor in influenza H5N1 virus pneumonia. *Acta Virol,* 53**,** 225-31.

HU, N. J., BRADSHAW, J., LAUTER, H., BUCKINGHAM, J., SOLITO, E. & HOFMANN, A. 2008a. Membrane-induced folding and structure of membrane-bound annexin A1 N-terminal peptides: implications for annexin-induced membrane aggregation. *Biophys J,* 94**,** 1773-81.

HU, X., CHAKRAVARTY, S. D. & IVASHKIV, L. B. 2008b. Regulation of interferon and Toll-like receptor signalling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunol Rev,* 226**,** 41-56.

HUGGINS, A., PASCHALIDIS, N., FLOWER, R. J., PERRETTI, M. & D'ACQUISTO, F. 2009. Annexin-1-deficient dendritic cells acquire a mature phenotype during differentiation. *Faseb J,* 23**,** 985-96.

HUSTON, J. M., GALLOWITSCH-PUERTA, M., OCHANI, M., OCHANI, K., YUAN, R., ROSAS-BALLINA, M., ASHOK, M., GOLDSTEIN, R. S., CHAVAN, S., PAVLOV, V. A., METZ, C. N., YANG, H., CZURA, C. J., WANG, H. & TRACEY, K. J. 2007. Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. *Crit Care Med,* 35**,** 2762-8.

IMAHARA, S. D., JELACIC, S., JUNKER, C. E. & O'KEEFE, G. E. 2005. The TLR4 +896 polymorphism is not associated with lipopolysaccharide hypo-responsiveness in leukocytes. *Genes Immun,* 6**,** 37-43.

IRIE, T., MUTA, T. & TAKESHIGE, K. 2000. TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor-kappaB in lipopolysaccharide-stimulated macrophages. *FEBS Lett,* 467**,** 160-4.

ISHITSUKA, R., KOJIMA, K., UTSUMI, H., OGAWA, H. & MATSUMOTO, I. 1998. Glycosaminoglycan binding properties of annexin IV, V, and VI. *J Biol Chem,* 273**,** 9935-41.

IWASAKI, A. & MEDZHITOV, R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol,* 5**,** 987-95.

JANSSENS, S. & BEYAERT, R. 2003. Role of Toll-like receptors in pathogen recognition. *Clin Microbiol Rev,* 16**,** 637-46.

JONES, B. W., HELDWEIN, K. A., MEANS, T. K., SAUKKONEN, J. J. & FENTON, M. J. 2001a. Differential roles of Toll-like receptors in the elicitation of

proinflammatory responses by macrophages. *Ann Rheum Dis,* 60 Suppl 3**,** iii6-12.

JONES, B. W., MEANS, T. K., HELDWEIN, K. A., KEEN, M. A., HILL, P. J., BELISLE, J. T. & FENTON, M. J. 2001b. Different Toll-like receptor agonists induce distinct macrophage responses. *J Leukoc Biol,* 69**,** 1036-44.

JONES, L. A., ANTHONY, J. P., HENRIQUEZ, F. L., LYONS, R. E., NICKDEL, M. B., CARTER, K. C., ALEXANDER, J. & ROBERTS, C. W. 2008. Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors. *Immunology,* 125**,** 59-69.

JONES, S. A. 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol,* 175**,** 3463-8.

JORRITSMA, P. J., BROGDON, J. L. & BOTTOMLY, K. 2003. Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive CD4+ T cells. *J Immunol,* 170**,** 2427-34.

JOST, M., ZEUSCHNER, D., SEEMANN, J., WEBER, K. & GERKE, V. 1997. Identification and characterization of a novel type of annexin-membrane interaction: Ca2+ is not required for the association of annexin II with early endosomes. *J Cell Sci,* 110 (Pt 2)**,** 221-8.

KAISHO, T. & AKIRA, S. 2006. Toll-like receptor function and signalling. *J Allergy Clin Immunol,* 117**,** 979-87; quiz 988.

KANNABIRAN, C., ZENG, X. & VALES, L. D. 1997. The mammalian transcriptional repressor RBP (CBF1) regulates interleukin-6 gene expression. *Mol Cell Biol,* 17**,** 1-9.

KARIN, M. 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J Biol Chem,* 274**,** 27339-42.

KARIN, M. & BEN-NERIAH, Y. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol,* 18**,** 621-63.

KAWAI, T. & AKIRA, S. 2007. TLR signalling. *Semin Immunol,* 19**,** 24-32.

KAWAI, T., TAKEUCHI, O., FUJITA, T., INOUE, J., MUHLRADT, P. F., SATO, S., HOSHINO, K. & AKIRA, S. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol,* 167**,** 5887-94.

KAYE, P. M. 1995. Costimulation and the regulation of antimicrobial immunity. *Immunol Today,* 16**,** 423-7.

KIECHL, S., LORENZ, E., REINDL, M., WIEDERMANN, C. J., OBERHOLLENZER, F., BONORA, E., WILLEIT, J. & SCHWARTZ, D. A. 2002. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med,* 347**,** 185-92.

KIM, K. D., ZHAO, J., AUH, S., YANG, X., DU, P., TANG, H. & FU, Y. X. 2007. Adaptive immune cells temper initial innate responses. *Nat Med,* 13**,** 1248-52.

KIM, K. M., KIM, D. K., PARK, Y. M., KIM, C. K. & NA, D. S. 1994. Annexin-I inhibits phospholipase A2 by specific interaction, not by substrate depletion. *FEBS Lett,* 343**,** 251-5.

KIM, S. K., RAGUPATHI, G., MUSSELLI, C., CHOI, S. J., PARK, Y. S. & LIVINGSTON, P. O. 1999. Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to

immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine,* 18**,** 597-603.

KIM, S. W., RHEE, H. J., KO, J., KIM, Y. J., KIM, H. G., YANG, J. M., CHOI, E. C. & NA, D. S. 2001. Inhibition of cytosolic phospholipase A2 by annexin I. Specific interaction model and mapping of the interaction site. *J Biol Chem,* 276**,** 15712-9.

KOBAYASHI, K., HERNANDEZ, L. D., GALAN, J. E., JANEWAY, C. A., JR., MEDZHITOV, R. & FLAVELL, R. A. 2002. IRAK-M is a negative regulator of Toll-like receptor signalling. *Cell,* 110**,** 191-202.

KUROWSKA-STOLARSKA, M., STOLARSKI, B., KEWIN, P., MURPHY, G., CORRIGAN, C. J., YING, S., PITMAN, N., MIRCHANDANI, A., RANA, B., VAN ROOIJEN, N., SHEPHERD, M., MCSHARRY, C., MCINNES, I. B., XU, D. & LIEW, F. Y. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol,* 183**,** 6469-77.

KWON, M., MACLEOD, T. J., ZHANG, Y. & WAISMAN, D. M. 2005. S100A10, annexin A2, and annexin a2 heterotetramer as candidate plasminogen receptors. *Front Biosci,* 10**,** 300-25.

LANG, J. D. & MATUTE-BELLO, G. 2009. Lymphocytes, apoptosis and sepsis: making the jump from mice to humans. *Crit Care,* 13**,** 109.

LAUDANNA, C., KIM, J. Y., CONSTANTIN, G. & BUTCHER, E. 2002. Rapid leukocyte integrin activation by chemokines. *Immunol Rev,* 186**,** 37-46.

LE BON, A., SCHIAVONI, G., D'AGOSTINO, G., GRESSER, I., BELARDELLI, F. & TOUGH, D. F. 2001. Type i interferons potently enhance humoral

immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity,* 14**,** 461-70.

LEBOUDER, E., REY-NORES, J. E., RUSHMERE, N. K., GRIGOROV, M., LAWN, S. D., AFFOLTER, M., GRIFFIN, G. E., FERRARA, P., SCHIFFRIN, E. J., MORGAN, B. P. & LABETA, M. O. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signalling are present in human plasma and breast milk. *J Immunol,* 171**,** 6680-9.

LEE, J., CHUANG, T. H., REDECKE, V., SHE, L., PITHA, P. M., CARSON, D. A., RAZ, E. & COTTAM, H. B. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc Natl Acad Sci U S A,* 100**,** 6646-51.

LEMAITRE, B., NICOLAS, E., MICHAUT, L., REICHHART, J. M. & HOFFMANN, J. A. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell,* 86**,** 973-83.

LI, S., STRELOW, A., FONTANA, E. J. & WESCHE, H. 2002. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A,* 99**,** 5567-72.

LI, X. & STARK, G. R. 2002. NFkappaB-dependent signalling pathways. *Exp Hematol,* 30**,** 285-96.

LI, Y., QU, X., YANG, H., KANG, L., XU, Y., BAI, B. & SONG, W. 2005. Bifidobacteria DNA induces murine macrophages activation in vitro. *Cell Mol Immunol,* 2**,** 473-8.

LICHOTA, J. & GRASSER, K. D. 2003. Interaction of maize chromatin-associated HMG proteins with mononucleosomes: role of core and linker histones. *Biol Chem,* 384**,** 1019-27.

LIEW, F. Y., XU, D., BRINT, E. K. & O'NEILL, L. A. 2005. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol,* 5**,** 446-58.

LIM, L. H., SOLITO, E., RUSSO-MARIE, F., FLOWER, R. J. & PERRETTI, M. 1998. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc Natl Acad Sci U S A,* 95**,** 14535-9.

LIN, L. & GHOSH, S. 1996. A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. *Mol Cell Biol,* 16**,** 2248-54.

LINDGREN, C. M., NILSSON, A., ORHO-MELANDER, M., ALMGREN, P. & GROOP, L. C. 2001. Characterization of the annexin I gene and evaluation of its role in type 2 diabetes. *Diabetes,* 50**,** 2402-5.

LING, Q., JACOVINA, A. T., DEORA, A., FEBBRAIO, M., SIMANTOV, R., SILVERSTEIN, R. L., HEMPSTEAD, B., MARK, W. H. & HAJJAR, K. A. 2004. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J Clin Invest,* 113**,** 38-48.

LIPSCOMB, M. F., LYONS, C. R., NUNEZ, G., BALL, E. J., STASTNY, P., VIAL, W., LEM, V., WEISSLER, J. & MILLER, L. M. 1986. Human alveolar macrophages: HLA-DR-positive macrophages that are poor stimulators of a primary mixed leukocyte reaction. *J Immunol,* 136**,** 497-504.

LIU, G., XIA, X. P., GONG, S. L. & ZHAO, Y. 2006. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. *J Cell Physiol,* 209**,** 341-52.

LOCATE, S., COLYER, J., GAWLER, D. J. & WALKER, J. H. 2008. Annexin A6 at the cardiac myocyte sarcolemma--evidence for self-association and binding to actin. *Cell Biol Int,* 32**,** 1388-96.

LONGHI, M. P., TRUMPFHELLER, C., IDOYAGA, J., CASKEY, M., MATOS, I., KLUGER, C., SALAZAR, A. M., COLONNA, M. & STEINMAN, R. M. 2009. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med,* 206**,** 1589-602.

LORENZ, E., MIRA, J. P., CORNISH, K. L., ARBOUR, N. C. & SCHWARTZ, D. A. 2000. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect Immun,* 68**,** 6398-401.

LORENZ, E., MIRA, J. P., FREES, K. L. & SCHWARTZ, D. A. 2002. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med,* 162**,** 1028-32.

LOTZE, M. T. & TRACEY, K. J. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol,* 5**,** 331-42.

LUE, H., KAPURNIOTU, A., FINGERLE-ROWSON, G., ROGER, T., LENG, L., THIELE, M., CALANDRA, T., BUCALA, R. & BERNHAGEN, J. 2006. Rapid and transient activation of the ERK MAPK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CSN5 and Src kinase activity. *Cell Signal,* 18**,** 688-703.

LUMENG, C. N., BODZIN, J. L. & SALTIEL, A. R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest,* 117**,** 175-84.

LUND, J. M., ALEXOPOULOU, L., SATO, A., KAROW, M., ADAMS, N. C., GALE, N. W., IWASAKI, A. & FLAVELL, R. A. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A,* 101**,** 5598-603.

LYNCH, N. R., HAGEL, I., PEREZ, M., DI PRISCO, M. C., LOPEZ, R. & ALVAREZ, N. 1993. Effect of anthelmintic treatment on the allergic reactivity of children in a tropical slum. *J Allergy Clin Immunol,* 92**,** 404-11.

LYONS, C. R., BALL, E. J., TOEWS, G. B., WEISSLER, J. C., STASTNY, P. & LIPSCOMB, M. F. 1986. Inability of human alveolar macrophages to stimulate resting T cells correlates with decreased antigen-specific T cell-macrophage binding. *J Immunol,* 137**,** 1173-80.

MAA, MC., CHANG, MY., LI, J., LI, YY., HSIEH, MY., YANG, CJ., CHEN, YJ., LI, Y., CHEN, HC., CHENG, WE., HSIEH, CH., CHENG, CW., LEU, TH. 2011. The iNOS/Src/FAK axis is critical in Toll-like receptor-mediated cell motility in macrophages. *Biochemica et Biophysica Acta*, 1813, 136-0147

MAHALINGAM, S. & LIDBURY, B. A. 2002. Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NF-kappa B) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. *Proc Natl Acad Sci U S A,* 99**,** 13819-24.

MAILLIARD, W. S., HAIGLER, H. T. & SCHLAEPFER, D. D. 1996. Calcium-dependent binding of S100C to the N-terminal domain of annexin I. *J Biol Chem,* 271**,** 719-25.

MANDAL, P. & HAMILTON, T. 2007. Signalling in lipopolysaccharide-induced stabilization of formyl peptide receptor 1 mRNA in mouse peritoneal macrophages. *J Immunol,* 178**,** 2542-8.

MANDAL, P., NOVOTNY, M. & HAMILTON, T. A. 2005. Lipopolysaccharide induces formyl peptide receptor 1 gene expression in macrophages and neutrophils via transcriptional and posttranscriptional mechanisms. *J Immunol,* 175**,** 6085-91.

MARSHAK-ROTHSTEIN, A. 2006. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol,* 6**,** 823-35.

MARSIK, C., MAYR, F., CARDONA, F., DERHASCHNIG, U., WAGNER, O. F. & JILMA, B. 2003. Endotoxaemia modulates Toll-like receptors on leucocytes in humans. *Br J Haematol,* 121**,** 653-6.

MARTIN, G. S., MANNINO, D. M., EATON, S. & MOSS, M. 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med,* 348**,** 1546-54.

MCARTHUR, S., YAZID, S., CHRISTIAN, H., SIRHA, R., FLOWER, R., BUCKINGHAM, J. & SOLITO, E. 2009. Annexin A1 regulates hormone exocytosis through a mechanism involving actin reorganization. *Faseb J,* 23**,** 4000-10.

MCCARTNEY-FRANCIS, N., JIN, W. & WAHL, S. M. 2004. Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signalling pathway. *J Immunol,* 172**,** 3814-21.

MCKANNA, J. A. 1995. Lipocortin 1 in apoptosis: mammary regression. *Anat Rec,* 242**,** 1-10.

MCLOUGHLIN, R. M., JENKINS, B. J., GRAIL, D., WILLIAMS, A. S., FIELDING, C. A., PARKER, C. R., ERNST, M., TOPLEY, N. & JONES, S. A. 2005. IL-6 trans-signalling via STAT3 directs T cell infiltration in acute inflammation. *Proc Natl Acad Sci U S A,* 102**,** 9589-94.

MCWHIRTER, S. M., FITZGERALD, K. A., ROSAINS, J., ROWE, D. C., GOLENBOCK, D. T. & MANIATIS, T. 2004. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc Natl Acad Sci U S A,* 101**,** 233-8.

MEDZHITOV, R., PRESTON-HURLBURT, P. & JANEWAY, C. A., JR. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature,* 388**,** 394-7.

MEDZHITOV, R., PRESTON-HURLBURT, P., KOPP, E., STADLEN, A., CHEN, C., GHOSH, S. & JANEWAY, C. A., JR. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signalling pathways. *Mol Cell,* 2**,** 253-8.

MENG, G., RUTZ, M., SCHIEMANN, M., METZGER, J., GRABIEC, A., SCHWANDNER, R., LUPPA, P. B., EBEL, F., BUSCH, D. H., BAUER, S., WAGNER, H. & KIRSCHNING, C. J. 2004. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J Clin Invest,* 113**,** 1473-81.

MENKE, M., GERKE, V. & STEINEM, C. 2005. Phosphatidylserine membrane domain clustering induced by annexin A2/S100A10 heterotetramer. *Biochemistry,* 44**,** 15296-303.

MERRELL, D. S. & FALKOW, S. 2004. Frontal and stealth attack strategies in microbial pathogenesis. *Nature,* 430**,** 250-6.

MEYLAN, E., BURNS, K., HOFMANN, K., BLANCHETEAU, V., MARTINON, F., KELLIHER, M. & TSCHOPP, J. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol,* 5**,** 503-7.

MICHEL, O. 2003. Role of lipopolysaccharide (LPS) in asthma and other pulmonary conditions. *J Endotoxin Res,* 9**,** 293-300.

MILLER, R. L., GERSTER, J. F., OWENS, M. L., SLADE, H. B. & TOMAI, M. A. 1999. Imiquimod applied topically: a novel immune response modifier and new class of drug. *Int J Immunopharmacol,* 21**,** 1-14.

MILLER, S. I., ERNST, R. K. & BADER, M. W. 2005. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol,* 3**,** 36-46.

MINGHETTI, L., NICOLINI, A., POLAZZI, E., GRECO, A., PERRETTI, M., PARENTE, L. & LEVI, G. 1999. Down-regulation of microglial cyclo-oxygenase-2 and inducible nitric oxide synthase expression by lipocortin 1. *Br J Pharmacol,* 126**,** 1307-14.

MITCHELL, R. A., METZ, C. N., PENG, T. & BUCALA, R. 1999. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J Biol Chem,* 274**,** 18100-6.

MIZEL, S. B., HONKO, A. N., MOORS, M. A., SMITH, P. S. & WEST, A. P. 2003. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signalling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. *J Immunol,* 170**,** 6217-23.

MODOLELL, M., CORRALIZA, I. M., LINK, F., SOLER, G. & EICHMANN, K. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in

mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol,* 25**,** 1101-4.

MONDINO, A. & JENKINS, M. K. 1994. Surface proteins involved in T cell costimulation. *J Leukoc Biol,* 55**,** 805-15.

MORAND, E. F., HUTCHINSON, P., HARGREAVES, A., GOULDING, N. J., BOYCE, N. W. & HOLDSWORTH, S. R. 1995. Detection of intracellular lipocortin 1 in human leukocyte subsets. *Clin Immunol Immunopathol,* 76**,** 195-202.

MOREL, E. & GRUENBERG, J. 2009. Annexin A2 binding to endosomes and functions in endosomal transport are regulated by tyrosine 23 phosphorylation. *J Biol Chem,* 284**,** 1604-11.

MORGAN, R. O. & FERNANDEZ, M. P. 1997. Annexin gene structures and molecular evolutionary genetics. *Cell Mol Life Sci,* 53**,** 508-15.

MOSSER, D. M. 2003. The many faces of macrophage activation. *J Leukoc Biol,* 73**,** 209-12.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

MOTOYAMA, M., YAMAZAKI, S., ETO-KIMURA, A., TAKESHIGE, K. & MUTA, T. 2005. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. *J Biol Chem,* 280**,** 7444-51.

MULLA, A., LEROUX, C., SOLITO, E. & BUCKINGHAM, J. C. 2005. Correlation between the antiinflammatory protein annexin 1 (lipocortin 1) and serum cortisol in subjects with normal and dysregulated adrenal function. *J Clin Endocrinol Metab,* 90**,** 557-62.

MULLER, M. R., PFANNES, S. D., AYOUB, M., HOFFMANN, P., BESSLER, W. G. & MITTENBUHLER, K. 2001. Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. *Immunology,* 103**,** 49-60.

MULSCH, A., SCHRAY-UTZ, B., MORDVINTCEV, P. I., HAUSCHILDT, S. & BUSSE, R. 1993. Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett,* 321**,** 215-8.

MUNDER, M., EICHMANN, K., MORAN, J. M., CENTENO, F., SOLER, G. & MODOLELL, M. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol,* 163**,** 3771-7.

MUZIO, M., BOSISIO, D., POLENTARUTTI, N., D'AMICO, G., STOPPACCIARO, A., MANCINELLI, R., VAN'T VEER, C., PENTON-ROL, G., RUCO, L. P., ALLAVENA, P. & MANTOVANI, A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol,* 164**,** 5998-6004.

MYLONAS, K. J., NAIR, M. G., PRIETO-LAFUENTE, L., PAAPE, D. & ALLEN, J. E. 2009. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J Immunol,* 182**,** 3084-94.

NAIDU, D. G., RAHA, A., CHEN, X. L., SPITZER, A. R. & CHANDER, A. 2005. Partial truncation of the NH2-terminus affects physical characteristics and membrane binding, aggregation, and fusion properties of annexin A7. *Biochim Biophys Acta,* 1734**,** 152-68.

NAIR, S., HANDE, M. P. & LIM, L. H. Annexin-1 protects MCF7 breast cancer cells against heat-induced growth arrest and DNA damage. *Cancer Lett.*

NAITO, M., UMEDA, S., YAMAMOTO, T., MORIYAMA, H., UMEZU, H., HASEGAWA, G., USUDA, H., SHULTZ, L. D. & TAKAHASHI, K. 1996. Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *J Leukoc Biol,* 59**,** 133-8.

NELSON, D. E., IHEKWABA, A. E., ELLIOTT, M., JOHNSON, J. R., GIBNEY, C. A., FOREMAN, B. E., NELSON, G., SEE, V., HORTON, C. A., SPILLER, D. G., EDWARDS, S. W., MCDOWELL, H. P., UNITT, J. F., SULLIVAN, E., GRIMLEY, R., BENSON, N., BROOMHEAD, D., KELL, D. B. & WHITE, M. R. 2004. Oscillations in NF-kappaB signalling control the dynamics of gene expression. *Science,* 306**,** 704-8.

NEMETH, K., LEELAHAVANICHKUL, A., YUEN, P. S., MAYER, B., PARMELEE, A., DOI, K., ROBEY, P. G., LEELAHAVANICHKUL, K., KOLLER, B. H., BROWN, J. M., HU, X., JELINEK, I., STAR, R. A. & MEZEY, E. 2009. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med,* 15**,** 42-9.

NESS, L., EWING, JL., HOGABOAM, CM., KUNKEL, SL. 2006. CCR4 is a key modulator of innate immune responses. *J Immunol*, 177, 7531-7538.

NOLAN, A., KOBAYASHI, H., NAVEED, B., KELLY, A., HOSHINO, Y., HOSHINO, S., KARULF, M. R., ROM, W. N., WEIDEN, M. D. & GOLD, J. A. 2009. Differential role for CD80 and CD86 in the regulation of the innate immune response in murine polymicrobial sepsis. *PLoS One,* 4**,** e6600.

NOMURA, F., AKASHI, S., SAKAO, Y., SATO, S., KAWAI, T., MATSUMOTO, M., NAKANISHI, K., KIMOTO, M., MIYAKE, K., TAKEDA, K. & AKIRA, S. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages

correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol,* 164**,** 3476-9.

O'NEILL, L. A. & BOWIE, A. G. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol,* 7**,** 353-64.

OGUS, A. C., YOLDAS, B., OZDEMIR, T., UGUZ, A., OLCEN, S., KESER, I., COSKUN, M., CILLI, A. & YEGIN, O. 2004. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J,* 23**,** 219-23.

OHASHI, K., BURKART, V., FLOHE, S. & KOLB, H. 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol,* 164**,** 558-61.

OHL, L., MOHAUPT, M., CZELOTH, N., HINTZEN, G., KIAFARD, Z., ZWIRNER, J. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity*, 21, 279-288

OHLSSON, K., BJORK, P., BERGENFELDT, M., HAGEMAN, R. & THOMPSON, R. C. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature,* 348**,** 550-2.

OHNISHI, T., MUROI, M. & TANAMOTO, K. 2001. N-linked glycosylations at Asn(26) and Asn(114) of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. *J Immunol,* 167**,** 3354-9.

OKAMURA, Y., WATARI, M., JERUD, E. S., YOUNG, D. W., ISHIZAKA, S. T., ROSE, J., CHOW, J. C. & STRAUSS, J. F., 3RD 2001. The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem,* 276**,** 10229-33.

OPAL, S. M., GARBER, G. E., LAROSA, S. P., MAKI, D. G., FREEBAIRN, R. C., KINASEWITZ, G. T., DHAINAUT, J. F., YAN, S. B., WILLIAMS, M. D., GRAHAM, D. E., NELSON, D. R., LEVY, H. & BERNARD, G. R. 2003. Systemic host responses in severe sepsis analyzed by causative microorganism and treatment effects of drotrecogin alfa (activated). *Clin Infect Dis,* 37**,** 50-8.

OSWALD, I. P., WYNN, T. A., SHER, A. & JAMES, S. L. 1992. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a co-stimulatory factor for interferon gamma-induced activation. *Proc Natl Acad Sci U S A,* 89**,** 8676-80.

OUDE NIJHUIS, MM., PASTERKAMP, G., SLUIS, NI., DE KLEIJN, DPV., LAMAN, JD., ULFMAN, LH. 2007. Peptidoglycan increases firm adhesion of monocytes under flow conditions and primes monocyte chemotaxis. *Journal of Vascular Research*, 44, 214-222

PALAGA, T., BURANARUK, C., RENGPIPAT, S., FAUQ, A. H., GOLDE, T. E., KAUFMANN, S. H. & OSBORNE, B. A. 2008. Notch signalling is activated by TLR stimulation and regulates macrophage functions. *Eur J Immunol,* 38**,** 174-83.

PANARO, M. A., ACQUAFREDDA, A., SISTO, M., LISI, S., MAFFIONE, A. B. & MITOLO, V. 2006. Biological role of the N-formyl peptide receptors. *Immunopharmacol Immunotoxicol,* 28**,** 103-27.

PARK, J. S., SVETKAUSKAITE, D., HE, Q., KIM, J. Y., STRASSHEIM, D., ISHIZAKA, A. & ABRAHAM, E. 2004. Involvement of toll-like receptors 2

and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem,* 279**,** 7370-79tlr.

PARKER, LC., WHYTE, MKB., VOGEL, SN., DOWER, SK., SABROE, I. 2004. Toll-Like Receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol*, 172, 4977-4986

PASARE, C. & MEDZHITOV, R. 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science,* 299**,** 1033-6.

PAUL-CLARK, M. J., MCMASTER, S. K., BELCHER, E., SORRENTINO, R., ANANDARAJAH, J., FLEET, M., SRISKANDAN, S. & MITCHELL, J. A. 2006. Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. *Br J Pharmacol,* 148**,** 1067-75.

PAULEAU, A. L. & MURRAY, P. J. 2003. Role of nod2 in the response of macrophages to toll-like receptor agonists. *Mol Cell Biol,* 23**,** 7531-9.

PEERS, S. H., SMILLIE, F., ELDERFIELD, A. J. & FLOWER, R. J. 1993. Glucocorticoid-and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes in vivo. *Br J Pharmacol,* 108**,** 66-72.

PERRETTI, M., AHLUWALIA, A., HARRIS, J. G., HARRIS, H. J., WHELLER, S. K. & FLOWER, R. J. 1996a. Acute inflammatory response in the mouse: exacerbation by immunoneutralization of lipocortin 1. *Br J Pharmacol,* 117**,** 1145-54.

PERRETTI, M., CHRISTIAN, H., WHELLER, S. K., AIELLO, I., MUGRIDGE, K. G., MORRIS, J. F., FLOWER, R. J. & GOULDING, N. J. 2000. Annexin I is

stored within gelatinase granules of human neutrophil and mobilized on the cell surface upon adhesion but not phagocytosis. *Cell Biol Int,* 24**,** 163-74.

PERRETTI, M., CROXTALL, J. D., WHELLER, S. K., GOULDING, N. J., HANNON, R. & FLOWER, R. J. 1996b. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nat Med,* 2**,** 1259-62.

PERRETTI, M. & D'ACQUISTO, F. 2009. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol,* 9**,** 62-70.

PERRETTI, M. & FLOWER, R. J. 1993. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol,* 150**,** 992-9.

PERRETTI, M. & FLOWER, R. J. 1994. Cytokines, glucocorticoids and lipocortins in the control of neutrophil migration. *Pharmacol Res,* 30**,** 53-9.

PERRETTI, M. & GAVINS, F. N. 2003. Annexin 1: an endogenous anti-inflammatory protein. *News Physiol Sci,* 18**,** 60-4.

PERRETTI, M., GETTING, S. J., SOLITO, E., MURPHY, P. M. & GAO, J. L. 2001. Involvement of the receptor for formylated peptides in the in vivo anti-migratory actions of annexin 1 and its mimetics. *Am J Pathol,* 158**,** 1969-73.

PERRETTI, M., INGEGNOLI, F., WHELLER, S. K., BLADES, M. C., SOLITO, E. & PITZALIS, C. 2002. Annexin 1 modulates monocyte-endothelial cell interaction in vitro and cell migration in vivo in the human SCID mouse transplantation model. *J Immunol,* 169**,** 2085-92.

PHILIP, J. G., FLOWER, R. J. & BUCKINGHAM, J. C. 1997. Glucocorticoids modulate the cellular disposition of lipocortin 1 in the rat brain in vivo and in vitro. *Neuroreport,* 8**,** 1871-6.

PINDADO, J., BALSINDE, J. & BALBOA, M. A. 2007. TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase A2/cyclooxygenase-2 pathway. *J Immunol,* 179**,** 4821-8.

PLUDDEMANN, A., MUKHOPADHYAY, S. & GORDON, S. 2006. The interaction of macrophage receptors with bacterial ligands. *Expert Rev Mol Med,* 8**,** 1-25.

POLLARD, J. W. 2008. Macrophages define the invasive microenvironment in breast cancer. *J Leukoc Biol,* 84**,** 623-30.

POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M. Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. & BEUTLER, B. 1998. Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science,* 282**,** 2085-8.

POWER, C. P., WANG, J. H., MANNING, B., KELL, M. R., AHERNE, N. J., WU, Q. D. & REDMOND, H. P. 2004. Bacterial lipoprotein delays apoptosis in human neutrophils through inhibition of caspase-3 activity: regulatory roles for CD14 and TLR-2. *J Immunol,* 173**,** 5229-37.

QIAN, C., JIANG, X., AN, H., YU, Y., GUO, Z., LIU, S., XU, H. & CAO, X. 2006. TLR agonists promote ERK-mediated preferential IL-10 production of regulatory dendritic cells (diffDCs), leading to NK-cell activation. *Blood,* 108**,** 2307-15.

QIAN, Y., COMMANE, M., NINOMIYA-TSUJI, J., MATSUMOTO, K. & LI, X. 2001. IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. *J Biol Chem,* 276**,** 41661-7.

RABIET, M. J., HUET, E. & BOULAY, F. 2007. The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. *Biochimie,* 89**,** 1089-106.

RAETZ, C. R. 1990. Biochemistry of endotoxins. *Annu Rev Biochem,* 59**,** 129-70.

RAND, J. H. 1999. "Annexinopathies"--a new class of diseases. *N Engl J Med,* 340**,** 1035-6.

RAO, P., HAYDEN, M. S., LONG, M., SCOTT, M. L., WEST, A. P., ZHANG, D., OECKINGHAUS, A., LYNCH, C., HOFFMANN, A., BALTIMORE, D. & GHOSH, S. IkappaBbeta acts to inhibit and activate gene expression during the inflammatory response. *Nature,* 466**,** 1115-1119.

RAYNAL, P. & POLLARD, H. B. 1994. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta,* 1197**,** 63-93.

RAZIN, S., YOGEV, D. & NAOT, Y. 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev,* 62**,** 1094-156.

REMICK, D. G. 2003. Cytokine therapeutics for the treatment of sepsis: why has nothing worked? *Curr Pharm Des,* 9**,** 75-82.

RESCHER, U. & GERKE, V. 2004. Annexins--unique membrane binding proteins with diverse functions. *J Cell Sci,* 117**,** 2631-9.

RESCHER, U., GOEBELER, V., WILBERS, A. & GERKE, V. 2006. Proteolytic cleavage of annexin 1 by human leukocyte elastase. *Biochim Biophys Acta,* 1763**,** 1320-4.

RIEDEMANN, N. C., GUO, R. F., GAO, H., SUN, L., HOESEL, M., HOLLMANN, T. J., WETSEL, R. A., ZETOUNE, F. S. & WARD, P. A. 2004. Regulatory role

of C5a on macrophage migration inhibitory factor release from neutrophils. *J Immunol,* 173**,** 1355-9.

RITTIRSCH, D., FLIERL, M. A., NADEAU, B. A., DAY, D. E., HUBER-LANG, M., MACKAY, C. R., ZETOUNE, F. S., GERARD, N. P., CIANFLONE, K., KOHL, J., GERARD, C., SARMA, J. V. & WARD, P. A. 2008. Functional roles for C5a receptors in sepsis. *Nat Med,* 14**,** 551-7.

ROCK, F. L., HARDIMAN, G., TIMANS, J. C., KASTELEIN, R. A. & BAZAN, J. F. 1998. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A,* 95**,** 588-93.

ROGER, T., DAVID, J., GLAUSER, M. P. & CALANDRA, T. 2001. MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature,* 414**,** 920-4.

ROGER, T., FROIDEVAUX, C., LE ROY, D., REYMOND, M. K., CHANSON, A. L., MAURI, D., BURNS, K., RIEDERER, B. M., AKIRA, S. & CALANDRA, T. 2009. Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. *Proc Natl Acad Sci U S A,* 106**,** 2348-52.

ROGER, T., FROIDEVAUX, C., MARTIN, C. & CALANDRA, T. 2003. Macrophage migration inhibitory factor (MIF) regulates host responses to endotoxin through modulation of Toll-like receptor 4 (TLR4). *J Endotoxin Res,* 9**,** 119-23.

ROSENGARTH, A. & LUECKE, H. 2003. A calcium-driven conformational switch of the N-terminal and core domains of annexin A1. *J Mol Biol,* 326**,** 1317-25.

ROVIEZZO, F., GETTING, S. J., PAUL-CLARK, M. J., YONA, S., GAVINS, F. N., PERRETTI, M., HANNON, R., CROXTALL, J. D., BUCKINGHAM, J. C. &

FLOWER, R. J. 2002. The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol,* 53**,** 541-53.

ROWLETT, R. M., CHRESTENSEN, C. A., NYCE, M., HARP, M. G., PELO, J. W., COMINELLI, F., ERNST, P. B., PIZARRO, T. T., STURGILL, T. W. & WORTHINGTON, M. T. 2008. MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. *Am J Physiol Gastrointest Liver Physiol,* 294**,** G452-9.

RUSSELL, J. A. 2006. Management of sepsis. *N Engl J Med,* 355**,** 1699-713.

RYAN, J. L., YOHE, W. B. & MORRISON, D. C. 1980. Stimulation of peritoneal cell arginase by bacterial lipopolysaccharides. *Am J Pathol,* 99**,** 451-62.

SAITOH, S., AKASHI, S., YAMADA, T., TANIMURA, N., KOBAYASHI, M., KONNO, K., MATSUMOTO, F., FUKASE, K., KUSUMOTO, S., NAGAI, Y., KUSUMOTO, Y., KOSUGI, A. & MIYAKE, K. 2004. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *Int Immunol,* 16**,** 961-9.

SAKAMOTO, T., REPASKY, W. T., UCHIDA, K., HIRATA, A. & HIRATA, F. 1996. Modulation of cell death pathways to apoptosis and necrosis of H2O2-treated rat thymocytes by lipocortin I. *Biochem Biophys Res Commun,* 220**,** 643-7.

SALOMAO, R., BRUNIALTI, M. K., GOMES, N. E., MENDES, M. E., DIAZ, R. S., KOMNINAKIS, S., MACHADO, F. R., DA SILVA, I. D. & RIGATO, O. 2009. Toll-like receptor pathway signalling is differently regulated in neutrophils and peripheral mononuclear cells of patients with sepsis, severe sepsis, and septic shock. *Crit Care Med,* 37**,** 132-9.

SALOMAO, R., MARTINS, P. S., BRUNIALTI, M. K., FERNANDES MDA, L., MARTOS, L. S., MENDES, M. E., GOMES, N. E. & RIGATO, O. 2008. TLR signalling pathway in patients with sepsis. *Shock,* 30 Suppl 1**,** 73-7.

SAMPEY, A. V., HALL, P. H., MITCHELL, R. A., METZ, C. N. & MORAND, E. F. 2001. Regulation of synoviocyte phospholipase A2 and cyclooxygenase 2 by macrophage migration inhibitory factor. *Arthritis Rheum,* 44**,** 1273-80.

SANDERS, C. J., FRANCHI, L., YAROVINSKY, F., UEMATSU, S., AKIRA, S., NUNEZ, G. & GEWIRTZ, A. T. 2009. Induction of adaptive immunity by flagellin does not require robust activation of innate immunity. *Eur J Immunol,* 39**,** 359-71.

SARENEVA, T., MATIKAINEN, S., KURIMOTO, M. & JULKUNEN, I. 1998. Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J Immunol,* 160**,** 6032-8.

SATO, S., SUGIYAMA, M., YAMAMOTO, M., WATANABE, Y., KAWAI, T., TAKEDA, K. & AKIRA, S. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signalling. *J Immunol,* 171**,** 4304-10.

SAWMYNADEN, P. & PERRETTI, M. 2006. Glucocorticoid upregulation of the annexin-A1 receptor in leukocytes. *Biochem Biophys Res Commun,* 349**,** 1351-5.

SCHAAF, B., LUITJENS, K., GOLDMANN, T., VAN BREMEN, T., SAYK, F., DODT, C., DALHOFF, K. & DROEMANN, D. 2009. Mortality in human

sepsis is associated with downregulation of Toll-like receptor 2 and CD14 expression on blood monocytes. *Diagn Pathol,* 4**,** 12.

SCHEIBNER, K. A., LUTZ, M. A., BOODOO, S., FENTON, M. J., POWELL, J. D. & HORTON, M. R. 2006. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol,* 177**,** 1272-81.

SCHIAVO, G. & VAN DER GOOT, F. G. 2001. The bacterial toxin toolkit. *Nat Rev Mol Cell Biol,* 2**,** 530-7.

SCHMITZ, M. L. & BAEUERLE, P. A. 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J,* 10**,** 3805-17.

SCHNARE, M., BARTON, G. M., HOLT, A. C., TAKEDA, K., AKIRA, S. & MEDZHITOV, R. 2001. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol,* 2**,** 947-50.

SCHOENEMEYER, A., BARNES, B. J., MANCL, M. E., LATZ, E., GOUTAGNY, N., PITHA, P. M., FITZGERALD, K. A. & GOLENBOCK, D. T. 2005. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signalling. *J Biol Chem,* 280**,** 17005-12.

SCHRODER, N. W., DITERICH, I., ZINKE, A., ECKERT, J., DRAING, C., VON BAEHR, V., HASSLER, D., PRIEM, S., HAHN, K., MICHELSEN, K. S., HARTUNG, T., BURMESTER, G. R., GOBEL, U. B., HERMANN, C. & SCHUMANN, R. R. 2005. Heterozygous Arg753Gln polymorphism of human TLR-2 impairs immune activation by Borrelia burgdorferi and protects from late stage Lyme disease. *J Immunol,* 175**,** 2534-40.

SCHUMANN, R. R., LEONG, S. R., FLAGGS, G. W., GRAY, P. W., WRIGHT, S. D., MATHISON, J. C., TOBIAS, P. S. & ULEVITCH, R. J. 1990. Structure and function of lipopolysaccharide binding protein. *Science,* 249**,** 1429-31.

SELVATICI, R., FALZARANO, S., MOLLICA, A. & SPISANI, S. 2006. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. *Eur J Pharmacol,* 534**,** 1-11.

SEN, R. & BALTIMORE, D. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell,* 47**,** 921-8.

SEONG, S. Y. & MATZINGER, P. 2004. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol,* 4**,** 469-78.

SHARMA, M., OWNBEY, R. T. & SHARMA, M. C. Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation. *Exp Mol Pathol.*

SHEN, H., TESAR, B. M., WALKER, W. E. & GOLDSTEIN, D. R. 2008. Dual signalling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. *J Immunol,* 181**,** 1849-58.

SIBBITT, W. L., JR., GIBBS, D. L., KENNY, C., BANKHURST, A. D., SEARLES, R. P. & LEY, K. D. 1985. Relationship between circulating interferon and anti-interferon antibodies and impaired natural killer cell activity in systemic lupus erythematosus. *Arthritis Rheum,* 28**,** 624-9.

SMIRNOVA, I., MANN, N., DOLS, A., DERKX, H. H., HIBBERD, M. L., LEVIN, M. & BEUTLER, B. 2003. Assay of locus-specific genetic load implicates rare

Toll-like receptor 4 mutations in meningococcal susceptibility. *Proc Natl Acad Sci U S A,* 100**,** 6075-80.

SNELL, J. C., CHERNYSHEV, O., GILBERT, D. L. & COLTON, C. A. 1997. Polyribonucleotides induce nitric oxide production by human monocyte-derived macrophages. *J Leukoc Biol,* 62**,** 369-73.

SOLITO, E., DE COUPADE, C., PARENTE, L., FLOWER, R. J. & RUSSO-MARIE, F. 1998. IL-6 stimulates annexin 1 expression and translocation and suggests a new biological role as class II acute phase protein. *Cytokine,* 10**,** 514-21.

SOLITO, E., NUTI, S. & PARENTE, L. 1994. Dexamethasone-induced translocation of lipocortin (annexin) 1 to the cell membrane of U-937 cells. *Br J Pharmacol,* 112**,** 347-8.

SONG, G. Y., CHUNG, C. S., CHAUDRY, I. H. & AYALA, A. 2000. Immune suppression in polymicrobial sepsis: differential regulation of Th1 and Th2 responses by p38 MAPK. *J Surg Res,* 91**,** 141-6.

SONG, J., SHIH IE, M., CHAN, D. W. & ZHANG, Z. 2009. Suppression of annexin A11 in ovarian cancer: implications in chemoresistance. *Neoplasia,* 11**,** 605-14, 1 p following 614.

SONOKI, T., NAGASAKI, A., GOTOH, T., TAKIGUCHI, M., TAKEYA, M., MATSUZAKI, H. & MORI, M. 1997. Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. *J Biol Chem,* 272**,** 3689-93.

STEER, S. A., MORAN, J. M., CHRISTMANN, B. S., MAGGI, L. B., JR. & CORBETT, J. A. 2006. Role of MAPK in the regulation of double-stranded

RNA- and encephalomyocarditis virus-induced cyclooxygenase-2 expression by macrophages. *J Immunol,* 177**,** 3413-20.

STERNBERG, E. M. 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol,* 6**,** 318-28.

STEVENS, J. H., O'HANLEY, P., SHAPIRO, J. M., MIHM, F. G., SATOH, P. S., COLLINS, J. A. & RAFFIN, T. A. 1986. Effects of anti-C5a antibodies on the adult respiratory distress syndrome in septic primates. *J Clin Invest,* 77**,** 1812-6.

STEWART, C. R., STUART, L. M., WILKINSON, K., VAN GILS, J. M., DENG, J., HALLE, A., RAYNER, K. J., BOYER, L., ZHONG, R., FRAZIER, W. A., LACY-HULBERT, A., KHOURY, J. E., GOLENBOCK, D. T. & MOORE, K. J. 2010. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol,* 11**,** 155-61.

STOUT, R. D. & SUTTLES, J. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol,* 76**,** 509-13.

STRACHAN, D. P. 1989. Hay fever, hygiene, and household size. *Bmj,* 299**,** 1259-60.

STREICHER, W. W., LOPEZ, M. M. & MAKHATADZE, G. I. 2009. Annexin I and annexin II N-terminal peptides binding to S100 protein family members: specificity and thermodynamic characterization. *Biochemistry,* 48**,** 2788-98.

SUDLOW, A. W., CAREY, F., FORDER, R. & ROTHWELL, N. J. 1996. The role of lipocortin-1 in dexamethasone-induced suppression of PGE2 and TNF alpha release from human peripheral blood mononuclear cells. *Br J Pharmacol,* 117**,** 1449-56.

SUNDEN-CULLBERG, J., NORRBY-TEGLUND, A., ROUHIAINEN, A., RAUVALA, H., HERMAN, G., TRACEY, K. J., LEE, M. L., ANDERSSON, J., TOKICS, L. & TREUTIGER, C. J. 2005. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. *Crit Care Med,* 33**,** 564-73.

SWISHER, J. F., BURTON, N., BACOT, S. M., VOGEL, S. N. & FELDMAN, G. M. 2010. Annexin A2 tetramer activates human and murine macrophages through TLR4. *Blood,* 115**,** 549-58.

SWISHER, J. F., KHATRI, U. & FELDMAN, G. M. 2007. Annexin A2 is a soluble mediator of macrophage activation. *J Leukoc Biol,* 82**,** 1174-84.

TADA, H., AIBA, S., SHIBATA, K., OHTEKI, T. & TAKADA, H. 2005. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun,* 73**,** 7967-76.

TAKEDA, K. & AKIRA, S. 2005. Toll-like receptors in innate immunity. *Int Immunol,* 17**,** 1-14.

TAKEUCHI, O., KAWAI, T., MUHLRADT, P. F., MORR, M., RADOLF, J. D., ZYCHLINSKY, A., TAKEDA, K. & AKIRA, S. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol,* 13**,** 933-40.

TEAGUE, T. K., MARRACK, P., KAPPLER, J. W. & VELLA, A. T. 1997. IL-6 rescues resting mouse T cells from apoptosis. *J Immunol,* 158**,** 5791-6.

THOMAS, K. E., GALLIGAN, C. L., NEWMAN, R. D., FISH, E. N. & VOGEL, S. N. 2006. Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. *J Biol Chem,* 281**,** 31119-30.

TIAN, B., NOWAK, D. E. & BRASIER, A. R. 2005a. A TNF-induced gene expression program under oscillatory NF-kappaB control. *BMC Genomics,* 6**,** 137.

TIAN, B., NOWAK, D. E., JAMALUDDIN, M., WANG, S. & BRASIER, A. R. 2005b. Identification of direct genomic targets downstream of the nuclear factor-kappaB transcription factor mediating tumor necrosis factor signalling. *J Biol Chem,* 280**,** 17435-48.

TING, A. Y. & ENDY, D. 2002. Signal transduction. Decoding NF-kappaB signalling. *Science,* 298**,** 1189-90.

TOROSYAN, Y., SIMAKOVA, O., NAGA, S., MEZHEVAYA, K., LEIGHTON, X., DIAZ, J., HUANG, W., POLLARD, H. & SRIVASTAVA, M. 2009. Annexin-A7 protects normal prostate cells and induces distinct patterns of RB-associated cytotoxicity in androgen-sensitive and -resistant prostate cancer cells. *Int J Cancer,* 125**,** 2528-39.

TOSHCHAKOV, V., JONES, B. W., LENTSCHAT, A., SILVA, A., PERERA, P. Y., THOMAS, K., CODY, M. J., ZHANG, S., WILLIAMS, B. R., MAJOR, J., HAMILTON, T. A., FENTON, M. J. & VOGEL, S. N. 2003. TLR2 and TLR4 agonists stimulate unique repertoires of host resistance genes in murine macrophages: interferon-beta-dependent signalling in TLR4-mediated responses. *J Endotoxin Res,* 9**,** 169-75.

TOSHCHAKOV, V., JONES, B. W., PERERA, P. Y., THOMAS, K., CODY, M. J., ZHANG, S., WILLIAMS, B. R., MAJOR, J., HAMILTON, T. A., FENTON, M. J. & VOGEL, S. N. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol,* 3**,** 392-8.

TOUGH, D. F., SUN, S., ZHANG, X. & SPRENT, J. 1999. Stimulation of naive and memory T cells by cytokines. *Immunol Rev,* 170**,** 39-47.

TRACEY, K. J. 2002. The inflammatory reflex. *Nature,* 420**,** 853-9.

TRACEY, K. J., BEUTLER, B., LOWRY, S. F., MERRYWEATHER, J., WOLPE, S., MILSARK, I. W., HARIRI, R. J., FAHEY, T. J., 3RD, ZENTELLA, A., ALBERT, J. D. & ET AL. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science,* 234**,** 470-4.

TRACEY, K. J. & CERAMI, A. 1994. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med,* 45**,** 491-503.

TRACEY, K. J., FONG, Y., HESSE, D. G., MANOGUE, K. R., LEE, A. T., KUO, G. C., LOWRY, S. F. & CERAMI, A. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature,* 330**,** 662-4.

TREFFKORN, L., SCHEIBE, R., MARUYAMA, T. & DIETER, P. 2004. PGE2 exerts its effect on the LPS-induced release of TNF-alpha, ET-1, IL-1alpha, IL-6 and IL-10 via the EP2 and EP4 receptor in rat liver macrophages. *Prostaglandins Other Lipid Mediat,* 74**,** 113-23.

TRINCHIERI, G. & SHER, A. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol,* 7**,** 179-90.

TSUJIMOTO, H., ONO, S., MAJIMA, T., EFRON, P. A., KINOSHITA, M., HIRAIDE, H., MOLDAWER, L. L. & MOCHIZUKI, H. 2006. Differential toll-like receptor expression after ex vivo lipopolysaccharide exposure in patients with sepsis and following surgical stress. *Clin Immunol,* 119**,** 180-7.

TSUJIMOTO, H., ONO, S., MAJIMA, T., KAWARABAYASHI, N., TAKAYAMA, E., KINOSHITA, M., SEKI, S., HIRAIDE, H., MOLDAWER, L. L. & MOCHIZUKI,

H. 2005. Neutrophil elastase, MIP-2, and TLR-4 expression during human and experimental sepsis. *Shock,* 23**,** 39-44.

UEMATSU, S., JANG, M. H., CHEVRIER, N., GUO, Z., KUMAGAI, Y., YAMAMOTO, M., KATO, H., SOUGAWA, N., MATSUI, H., KUWATA, H., HEMMI, H., COBAN, C., KAWAI, T., ISHII, K. J., TAKEUCHI, O., MIYASAKA, M., TAKEDA, K. & AKIRA, S. 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat Immunol,* 7**,** 868-74.

UEMATSU, S., MATSUMOTO, M., TAKEDA, K. & AKIRA, S. 2002. Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *J Immunol,* 168**,** 5811-6.

UNDERHILL, D. M., OZINSKY, A., HAJJAR, A. M., STEVENS, A., WILSON, C. B., BASSETTI, M. & ADEREM, A. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature,* 401**,** 811-5.

VABULAS, R. M., AHMAD-NEJAD, P., DA COSTA, C., MIETHKE, T., KIRSCHNING, C. J., HACKER, H. & WAGNER, H. 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signalling pathway in innate immune cells. *J Biol Chem,* 276**,** 31332-9.

VAN DER POLL, T. & OPAL, S. M. 2008. Host-pathogen interactions in sepsis. *Lancet Infect Dis,* 8**,** 32-43.

VAN HEEL, D. A., GHOSH, S., BUTLER, M., HUNT, K., FOXWELL, B. M., MENGIN-LECREULX, D. & PLAYFORD, R. J. 2005. Synergistic

enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol,* 35**,** 2471-6.

VAN ZOELEN, M. A., YANG, H., FLORQUIN, S., MEIJERS, J. C., AKIRA, S., ARNOLD, B., NAWROTH, P. P., BIERHAUS, A., TRACEY, K. J. & VAN DER POLL, T. 2009. Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. *Shock,* 31**,** 280-4.

VIEMANN, D., DUBBEL, G., SCHLEIFENBAUM, S., HARMS, E., SORG, C. & ROTH, J. 2005. Expression of toll-like receptors in neonatal sepsis. *Pediatr Res,* 58**,** 654-9.

VIGLIANTI, G. A., LAU, C. M., HANLEY, T. M., MIKO, B. A., SHLOMCHIK, M. J. & MARSHAK-ROTHSTEIN, A. 2003. Activation of autoreactive B cells by CpG dsDNA. *Immunity,* 19**,** 837-47.

VISINTIN, A., MAZZONI, A., SPITZER, J. H., WYLLIE, D. H., DOWER, S. K. & SEGAL, D. M. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol,* 166**,** 249-55.

VONG, L., D'ACQUISTO, F., PEDERZOLI-RIBEIL, M., LAVAGNO, L., FLOWER, R. J., WITKO-SARSAT, V. & PERRETTI, M. 2007. Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *J Biol Chem,* 282**,** 29998-30004.

WALD, D., QIN, J., ZHAO, Z., QIAN, Y., NARAMURA, M., TIAN, L., TOWNE, J., SIMS, J. E., STARK, G. R. & LI, X. 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signalling. *Nat Immunol,* 4**,** 920-7.

WALLNER, B. P., MATTALIANO, R. J., HESSION, C., CATE, R. L., TIZARD, R., SINCLAIR, L. K., FOELLER, C., CHOW, E. P., BROWING, J. L.,

RAMACHANDRAN, K. L. & ET AL. 1986. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. *Nature,* 320**,** 77-81.

WALTHER, A., RIEHEMANN, K. & GERKE, V. 2000. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol Cell,* 5**,** 831-40.

WANG, C., DENG, L., HONG, M., AKKARAJU, G. R., INOUE, J. & CHEN, Z. J. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature,* 412**,** 346-51.

WANG, H., BLOOM, O., ZHANG, M., VISHNUBHAKAT, J. M., OMBRELLINO, M., CHE, J., FRAZIER, A., YANG, H., IVANOVA, S., BOROVIKOVA, L., MANOGUE, K. R., FAIST, E., ABRAHAM, E., ANDERSSON, J., ANDERSSON, U., MOLINA, P. E., ABUMRAD, N. N., SAMA, A. & TRACEY, K. J. 1999. HMG-1 as a late mediator of endotoxin lethality in mice. *Science,* 285**,** 248-51.

WANG, T., TOWN, T., ALEXOPOULOU, L., ANDERSON, J. F., FIKRIG, E. & FLAVELL, R. A. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med,* 10**,** 1366-73.

WANG, Z., LIU, J., SUDOM, A., AYRES, M., LI, S., WESCHE, H., POWERS, J. P. & WALKER, N. P. 2006. Crystal structures of IRAK-4 kinase in complex with inhibitors: a serine/threonine kinase with tyrosine as a gatekeeper. *Structure,* 14**,** 1835-44.

WARD, P. A. 2004. The dark side of C5a in sepsis. *Nat Rev Immunol,* 4**,** 133-42.

WARREN, B. L., EID, A., SINGER, P., PILLAY, S. S., CARL, P., NOVAK, I., CHALUPA, P., ATHERSTONE, A., PENZES, I., KUBLER, A., KNAUB, S.,

KEINECKE, H. O., HEINRICHS, H., SCHINDEL, F., JUERS, M., BONE, R. C. & OPAL, S. M. 2001. Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. *JAMA,* 286**,** 1869-78.

WATANABE, H., NUMATA, K., ITO, T., TAKAGI, K. & MATSUKAWA, A. 2004. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock,* 22**,** 460-6.

WATTERS, T. M., KENNY, E. F. & O'NEILL, L. A. 2007. Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. *Immunol Cell Biol,* 85**,** 411-9.

WEIGHARDT, H., KAISER-MOORE, S., VABULAS, R. M., KIRSCHNING, C. J., WAGNER, H. & HOLZMANN, B. 2002. Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. *J Immunol,* 169**,** 2823-7.

WEIN, S., FAUROUX, M., LAFFITTE, J., DE NADAI, P., GUAINI, C., PONS, F. & COMERA, C. 2004. Mediation of annexin 1 secretion by a probenecid-sensitive ABC-transporter in rat inflamed mucosa. *Biochem Pharmacol,* 67**,** 1195-202.

WERTS, C., TAPPING, R. I., MATHISON, J. C., CHUANG, T. H., KRAVCHENKO, V., SAINT GIRONS, I., HAAKE, D. A., GODOWSKI, P. J., HAYASHI, F., OZINSKY, A., UNDERHILL, D. M., KIRSCHNING, C. J., WAGNER, H., ADEREM, A., TOBIAS, P. S. & ULEVITCH, R. J. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol,* 2**,** 346-52.

WHITMORE, M. M., DEVEER, M. J., EDLING, A., OATES, R. K., SIMONS, B., LINDNER, D. & WILLIAMS, B. R. 2004. Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer Res,* 64**,** 5850-60.

WIERSINGA, W. J., WIELAND, C. W., DESSING, M. C., CHANTRATITA, N., CHENG, A. C., LIMMATHUROTSAKUL, D., CHIERAKUL, W., LEENDERTSE, M., FLORQUIN, S., DE VOS, A. F., WHITE, N., DONDORP, A. M., DAY, N. P., PEACOCK, S. J. & VAN DER POLL, T. 2007. Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by Burkholderia pseudomallei (Melioidosis). *PLoS Med,* 4**,** e248.

WILLIAMS, D. L., HA, T., LI, C., KALBFLEISCH, J. H., SCHWEITZER, J., VOGT, W. & BROWDER, I. W. 2003. Modulation of tissue Toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. *Crit Care Med,* 31**,** 1808-18.

WILLIAMS, J. A., PONTZER, C. H. & SHACTER, E. 2000. Regulation of macrophage interleukin-6 (IL-6) and IL-10 expression by prostaglandin E2: the role of p38 mitogen-activated protein kinase. *J Interferon Cytokine Res,* 20**,** 291-8.

WU, C. C., CROXTALL, J. D., PERRETTI, M., BRYANT, C. E., THIEMERMANN, C., FLOWER, R. J. & VANE, J. R. 1995. Lipocortin 1 mediates the inhibition by dexamethasone of the induction by endotoxin of nitric oxide synthase in the rat. *Proc Natl Acad Sci U S A,* 92**,** 3473-7.

WURFEL, M. M., GORDON, A. C., HOLDEN, T. D., RADELLA, F., STROUT, J., KAJIKAWA, O., RUZINSKI, J. T., RONA, G., BLACK, R. A., STRATTON, S., JARVIK, G. P., HAJJAR, A. M., NICKERSON, D. A., RIEDER, M.,

SEVRANSKY, J., MALONEY, J. P., MOSS, M., MARTIN, G., SHANHOLTZ, C., GARCIA, J. G., GAO, L., BROWER, R., BARNES, K. C., WALLEY, K. R., RUSSELL, J. A. & MARTIN, T. R. 2008. Toll-like receptor 1 polymorphisms affect innate immune responses and outcomes in sepsis. *Am J Respir Crit Care Med,* 178**,** 710-20.

XING, Z., GAULDIE, J., COX, G., BAUMANN, H., JORDANA, M., LEI, X. F. & ACHONG, M. K. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest,* 101**,** 311-20.

YAMAMOTO, M., SATO, S., HEMMI, H., HOSHINO, K., KAISHO, T., SANJO, H., TAKEUCHI, O., SUGIYAMA, M., OKABE, M., TAKEDA, K. & AKIRA, S. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signalling pathway. *Science,* 301**,** 640-3.

YAMAMOTO, M., SATO, S., MORI, K., HOSHINO, K., TAKEUCHI, O., TAKEDA, K. & AKIRA, S. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signalling. *J Immunol,* 169**,** 6668-72.

YAN, X., YIN, J., YAO, H., MAO, N., YANG, Y. & PAN, L. Increased expression of annexin a3 is a mechanism of platinum resistance in ovarian cancer. *Cancer Res,* 70**,** 1616-24.

YANG, H., OCHANI, M., LI, J., QIANG, X., TANOVIC, M., HARRIS, H. E., SUSARLA, S. M., ULLOA, L., WANG, H., DIRAIMO, R., CZURA, C. J., ROTH, J., WARREN, H. S., FINK, M. P., FENTON, M. J., ANDERSSON, U. & TRACEY, K. J. 2004. Reversing established sepsis with antagonists of

endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A,* 101**,** 296-301.

YANG, Y. H., AEBERLI, D., DACUMOS, A., XUE, J. R. & MORAND, E. F. 2009. Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *J Immunol,* 183**,** 1435-45.

YANG, Y. H., HUTCHINSON, P., SANTOS, L. L. & MORAND, E. F. 1998. Glucocorticoid inhibition of adjuvant arthritis synovial macrophage nitric oxide production: role of lipocortin 1. *Clin Exp Immunol,* 111**,** 117-22.

YANG, Y. H., TOH, M. L., CLYNE, C. D., LEECH, M., AEBERLI, D., XUE, J., DACUMOS, A., SHARMA, L. & MORAND, E. F. 2006. Annexin 1 negatively regulates IL-6 expression via effects on p38 MAPK and MAPK phosphatase-1. *J Immunol,* 177**,** 8148-53.

YASUKAWA, H., SASAKI, A. & YOSHIMURA, A. 2000. Negative regulation of cytokine signalling pathways. *Annu Rev Immunol,* 18**,** 143-64.

YEO, S. J., GRAVIS, D., YOON, J. G. & YI, A. K. 2003. Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: role of NF-kappaB and p38. *J Biol Chem,* 278**,** 22563-73.

YONA, S., HEINSBROEK, S. E., PEISER, L., GORDON, S., PERRETTI, M. & FLOWER, R. J. 2006. Impaired phagocytic mechanism in annexin 1 null macrophages. *Br J Pharmacol,* 148**,** 469-77.

YOUN, H. S., LEE, J. Y., FITZGERALD, K. A., YOUNG, H. A., AKIRA, S. & HWANG, D. H. 2005. Specific inhibition of MyD88-independent signalling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J Immunol,* 175**,** 3339-46.

YOUN, H. S., LIM, H. J., CHOI, Y. J., LEE, J. Y., LEE, M. Y. & RYU, J. H. 2008. Selenium suppresses the activation of transcription factor NF-kappa B and IRF3 induced by TLR3 or TLR4 agonists. *Int Immunopharmacol,* 8**,** 495-501.

ZACHARCHUK, C. M., DRYSDALE, B. E., MAYER, M. M. & SHIN, H. S. 1983. Macrophage-mediated cytotoxicity: role of a soluble macrophage cytotoxic factor similar to lymphotoxin and tumor necrosis factor. *Proc Natl Acad Sci U S A,* 80**,** 6341-5.

ZHANG, D., ZHANG, G., HAYDEN, M. S., GREENBLATT, M. B., BUSSEY, C., FLAVELL, R. A. & GHOSH, S. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science,* 303**,** 1522-6.

ZHANG, Q., RAOOF, M., CHEN, Y., SUMI, Y., SURSAL, T., JUNGER, W., BROHI, K., ITAGAKI, K. & HAUSER, C. J. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature,* 464**,** 104-7.

ZHU, H., FAN, X., YU, Z., LIU, J., MURATA, Y., LU, J., ZHAO, S., HAJJAR, K. A., LO, E. H. & WANG, X. Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke. *J Cereb Blood Flow Metab.*

ZOUKI, C., OUELLET, S. & FILEP, J. G. 2000. The anti-inflammatory peptides, antiflammins, regulate the expression of adhesion molecules on human leukocytes and prevent neutrophil adhesion to endothelial cells. *Faseb J,* 14**,** 572-80.

ZUNT, S. L., BURTON, L. V., GOLDBLATT, L. I., DOBBINS, E. E. & SRINIVASAN, M. 2009. Soluble forms of Toll-like receptor 4 are present in

human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells. *Clin Exp Immunol,* 156**,** 285-93.