Investigation of SIGIRR and Other TIR Domain Containing Molecules in Primary Human Myeloid Cells and Rheumatoid Arthritis

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"…the savage lives within himself, while social man lives constantly outside himself, and only knows how to live in the opinion of others, so that he seems to receive the consciousness of his own existence merely from the judgment of others concerning him."

Jean-Jacques Rousseau

This thesis is dedicated to my wife Marie-Christine and my son Jacob for their unconditional love and for being there for me no matter what, where or why.

ABSTRACT

Key components of the innate immune response to infections are the Toll-like receptors (TLRs), which are able to detect invading pathogens and subsequently generate inflammatory responses. Many details of the signalling pathways of TLRs have emerged from gene targeted mice or inhibition studies in transformed cell lines. However, the signalling pathways activated in primary human cells and disease tissues are less well understood. Previous studies identified differences in TLR signalling between human cells of myeloid- and non-myeloid origin. While over-expression of a dominant negative construct of the TLR adaptor molecule MyD88 inhibited TLR4 signalling in HUVECs it had no effect on TLR4 signalling in macrophages. Based on this observation, this thesis examined the function of MyD88 in primary human monocyte derived dendritic cells (DCs). Unexpectedly, over-expression of MyD88 dn resulted in the activation of DCs. Subsequent experiments, provided evidence for a DC specific inhibitory mechanism, which depends on endogenous MyD88 and is disrupted by TIR domain over-expression. To further investigate the mechanism, the function of SIGIRR in human DCs was examined. SIGIRR is a member of the TIR domain containing receptor family that has been shown to be expressed in murine and human DCs but not macrophages. However, results in this thesis show that SIGIRR is expressed by human DCs as well as macrophages. While SIGIRR has been studied in murine models, nothing is known about its function in primary human cells. Therefore, an adenoviral construct encoding wild-type SIGIRR was generated and over-expressed in DCs and macrophages, which impaired TLR2, TLR3, TLR4, TLR5, TLR7/8 and IL-1R but not TNFα signalling. In accordance with these results, siRNA knock down of SIGIRR in macrophages led to an increase of TLR3, TLR4, TLR7/8 and IL-1R but not TNFα induced cytokine production. Therefore, SIGIRR seems to be an inhibitor of TIR domain dependent signalling, affecting the MyD88 dependent as well as the TRIF dependent signalling pathway. Furthermore, immunoprecipitation studies of SIGIRR with MyD88 suggest, that SIGIRR interacts with MyD88 constitutively through TIR domain interaction, indicating that SIGIRR inhibits TLR/IL-1R signalling by sequestering MyD88. Given the potency of SIGIRR to inhibit TLR/IL-1R signalling in human DCs and macrophages, this thesis further investigated its role in rheumatoid arthritis disease models. Over-expression of SIGIRR wt in human RA synovial membrane cultures inhibited the spontaneous secretion of cytokines by those cells. In contrast, SIGIRR deficient mice were resistant to collagen induced arthritis (CIA). SIGIRR null mice immunised with CIA showed a loss in IgG2a anti-collagen antibody production as well as reduced Th1 and Th17 immune responses but increased Th2 immunity. Subsequent results indicated that SIGIRR is able to regulate $CD4^+$ T cell development through the inhibition of ST2/IL-33 signalling. Therefore, while SIGIRR inhibits pro-inflammatory cytokine release during the progression stages of RA, it may also regulate the development of Th2 development, thereby reducing CIA incidence. These studies highlight the importance of investigating signalling pathways in physiologically relevant cells in order to fully understand the roles TLRs and specific signalling molecules play in the human immune system and human disease process.

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DECLARATION

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Abbreviations

AEBSF	4-(2-Aminoethyl)benzenesulphonyl fluoride
AP-1	Activator protein-1
APC	Antigen presenting cell
ARE	Adenosine- and uridine-rich elements
ASC	Apoptosis-associated speck-like protein
ASK-1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
ATP	Adenosinetriphosphate
Bcl-3	B cell CLL/lymphoma-3
BCR	B cell receptor
BDCA	Blood DC antigen
BIR	Baculoviral IAP Repeats
Blys	B lymphocyte stimulator
BMDC	Bone marrow derived DC
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CBP	CREB-binding protein
CD40L	CD40 ligand
CFA	Complete Freund´s adjuvant
CIA	Collagen induced arthritis
CIITA	Class II transactivator
CLR	C-type lectin receptor
CMV	Cytomegalovirus
CPE	Cytopathic effect
CpG	Deoxycytidylate-phosphate-deoxyguanylate motifs
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DAB	Diaminobenzidine tetrahydrochloride
DAI	DNA-dependent activator of IFN-regulatory factors
DC	Dendritic cell
DC-ASPGR	DC-asialoglycoprotein receptor
Dcir	DC-immuno-receptor

DC-SIGN	Dendritic cell specific ICAM-3-grabbing nonintegrin
DD	Death domain
Dectin-1	Dendritic cell-associated C-type lectin-1
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethylsulfoxide
dn	Dominant negative
dsRNA	Double stranded RNA
DSS	Dextran sulfate sodium
DTT	Dithiothreitol
EC	Endothelial cell
ECM	Extracellular matrix
ECSIT	Evolutionarily conserved signalling intermediate in Toll pathways
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbent sandwich assay
ERK	Extracellular-regulated kinase
FCS	Foetal calf serum
FLT-3L	Fms-related tyrosine kinase 3 ligand
Foxp-3	Forkhead box protein-3
GAPDH	Glyceraldehyde-3-phosphate
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	Glycosylphophatidylinositol
h	Hour
HA	Hemaglutinin
HBSS	Hank´s buffered salt solution
HEK	Human embryonic kidney
HI	Heat inactivated
His	Histidine
HIV	Human immunodeficiency virus
HMGB-1	High mobility group box-1
HRP	Horse radish peroxidase
HSP	Heat shock protein
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon

Ig	Immunoglobulin
IKK	IκB kinase
IKKi	Inducible IKK
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IP	Immunoprecipitation
IP10	Interferon inducible protein 10
IPAF	Ice protease-activating factor
IPS-1	IFNβ promoter stimulator 1
IRAK	IL-1R associated kinase
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISRE	IFN-stimulated response elements
IκB	Inhibitor κB
JNK	c-Jun-N-terminal kinase
KIR	Kennedy Institute of Rheumatology
KO	Knock out
LAL	Limulus amebocyte assay
Langerin	Langerhans cell specific C-type lectin
LBP	LPS binding protein
LCMV	Lymphocytic choriomeningitis virus
LD	Levels of detection
LOX1	Lectin-like oxidised LDL-receptor
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LT	Leukotriene
LTβ	Lymphotoxin β
LTA	Lipoteichoic acid
m.o.i.	Multiplicity of infection
Mal	MyD88 adaptor like protein
MALP-2	Macrophage activating lipopeptide-2
ManLAM	Mannose-capped lipoarabinomannan
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose binding lectin

MCMV	Murine cytomegalovirus
MCP1	Monocyte chemotactic protein 1
MCS	Multiple cloning site
M-CSF	Macrophage colony stimulating factor
MDA5	Melanoma differentiation associated gene 5
mDC	Myeloid dendritic cell
MDP	Muramyl dipeptide
MEF	Mouse embryonic fibroblast
MEKK	Mitogen-activated protein kinase kinase kinase 3
MHC	Major histocompatibility complex
min	Minutes
MIP1/2	Macrophage inflammatory protein 1/2
MK	MAPK activated protein
MLK	Mixed lineage kinase
MMP	Matrix metalloproteinase
MO	Monocyte
mRNA	Messenger RNA
MR	Mannose receptor
MSK1	Mitogen- and stress-activated protein kinase 1
MTT	3-[4,5-Dimethylthiazol-2yl]-2,5-diphyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
MØ	Macrophage
NACHT	Domain present in NAIP, CIITA, HET-E, TP-1
NAIP	NLR family, apoptosis inhibitory protein
NALP	NACHT, leucine rich repeat and PYD (pyrin domain) containing
NAP1	Nucleosome assembly protein 1
NDV	Newcastle disease virus
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
NK	Natural killer cell
NLR	NOD-like receptor
NLS	Nucleo localisation signal
NO	Nitric oxide
NOD	Nucleotide binding and oligomerisation domain
NOS2	Nitric oxide synthase 2
PAGE	Polyacrylamide gel electrophoresis

Pam_3Cys	am_3Cys-Ser-$(Lys)_4$-trihydrochloride
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
pfu	Plaque forming unit
PG	Prostaglandine
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PMN	Polymorphonuclear cell
Poly I:C	Polyinoxinic-polycytidylic acid
Pro	Proline
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
PtdIns(4.5)P2	Phophatidylinositol-4,5-bisphophate
PTGS	Post-transcriptional gene silencing
PTV	Punta toro virus
PVDF	polyvinylidene difluoride
PYD	Pyrine effector domain
R848	Resiquimod 848
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RANTES	Regulated on activation, normal T expressed and secreted
Ras	Rat sarcinoma
RHD	Rel homology domain
RIG-I	Retinoid inducible gene 1
RIHM	RIP interacting homology motif
RIP	Receptor Interacting Protein
RIP-1	Receptor interacting protein-1
RISC	RNA-induced silencing complex
RLU	Relative luciferase units
RNAi	RNA interference
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates

RP105	Radioprotective 105
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute 1640 medium
SARM	TIR domain-sterile alpha and HEAT/Armadillo motif
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SF	Synovial fibroblast like cells
SIGIRR	Single immunoglobulin IL-1R related protein
siRNA	Short interfering RNA
SLE	Systemic lupus erythematosus
SP	Surfactant protein
SR	Scavenger receptor
SRA	Scavenger receptor A
ssRNA	Single stranded RNA
ST2	Suppressor of tumoigenicity 2
STAT	Signal transduction and activator of transcription
TAB2/3	TAK1 binding protein
TAD	Transcriptional activation domain
TAK1	TGF-activated kinase 1
TBK-1	TRAF family member associated NF-κB activator binding kinase-1
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	N´N´N´N´-Tetraethyl methylene diamine
TGFβ	Transforming growth factor β
Th	Helper T cell
TICAM	Toll-like receptor adaptor molecule
TIR	Toll/IL-1R homology domain
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tollip	Toll interacting protein
Tpl-2	Tumour progression locus-2
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β

TSLP	Thymic stromal lipopoetin
TX	Thromboxane
Ubc13	Ubiquitin-conjugating enzyme E2 13
Uev1A	Ubiquitin-conjugating enzyme E2 variant 1 isoform A
UTR	Untranslated region
UV	Ultraviolet
v	Volume
VCAM-1	Vascular cell adhesion molecule-1
VSV	Vesicular stomatitis virus
w	Weight
WB	Western Blot
wt	Wild type

CHAPTER 1

INTRODUCTION

1.1 Immunity and inflammation

The immune system evolved as a mechanism to protect organisms from infections with pathogenic organisms and other harmful substances. In general the immune system is capable of recognising invading pathogens and their products as well as endogenous danger signals (Gallucci and Matzinger, 2001; Janeway, 2001). This recognition results in the initiation of an immune response, which will, under normal circumstances, eliminate the insult without further damage to the host. However, it is now well recognised that defects in regulating inflammation can lead to an excessive response to infectious agents e.g. sepsis or autoinflammatory diseases including, rheumatoid arthritis (RA). The molecular pathways leading to inflammation as well as the mechanisms underlying its regulation are part of this study.

Primitive versions of an immune system can already be observed in non-vertebrates, such as the horseshoe crab *Limulus polyphemus*, whose blood cells contain a clotting agent that attaches to endotoxins produced by gram-negative bacteria. Moreover, the fruit fly *Drosophila melanogaster* was, and still is, extensively used as a model organism to investigate mechanisms of the immune system (Agaisse, 2007). In mammals the immune system is more highly developed and can be subdivided into an innate and adaptive immune system.

The innate and the adaptive immune system, although different in their mechanisms of action, are interlinked and can influence each other. The innate immune system is an immediate and, compared to the adaptive immune response, a rather unspecific response to an infection or cell damage. Upon infection or injury, cells of the innate immune system recognise pathogens or their products, also termed pathogen associated molecular patterns (PAMPs), using germ line-encoded receptors e.g. pattern recognition receptors (PRR). These receptors are able to recognise a broad range of different pathogens and molecule families, thereby giving rise to the "inflammatory response" and the subsequent elimination of the pathogen. Furthermore, the activation of the innate immune response induces the adaptive immune system through the presentation of characteristic parts of the pathogen, or antigen, to cells of the adaptive immune system. In contrast to the innate immune system the adaptive immune response is highly specific to a certain pathogen. On the other hand it is delayed in its onset and can take days after an initial infection to develop. At the same time the adaptive immune response gives rise to an immunological memory, which results in a faster response to future infections with similar pathogens. Subsequently, the host is immune to repeated infections with the same pathogen.

1.1.1 The inflammatory response

When the physical barrier, e.g. the skin or the mesoderm, is breached by invading microbes, a local infection is established leading to tissue damage. A major aspect of the immune response to infection or injury is the initiation of the inflammatory reaction (Figure 1.1). This is necessary in order to restrict tissue damage, neutralise and destroy foreign material, to alert the adaptive immune response and to induce healing of the injured tissue. As a result of inflammation, serum proteins e.g. complement proteins and leukocytes, are able to migrate from the blood to the infected extra vascular tissue. This is achieved by the sequential release of vasoactive and chemotactic mediators, which as a consequence contribute to the cardinal signs of inflammation, e.g. heat, redness, swelling and pain.

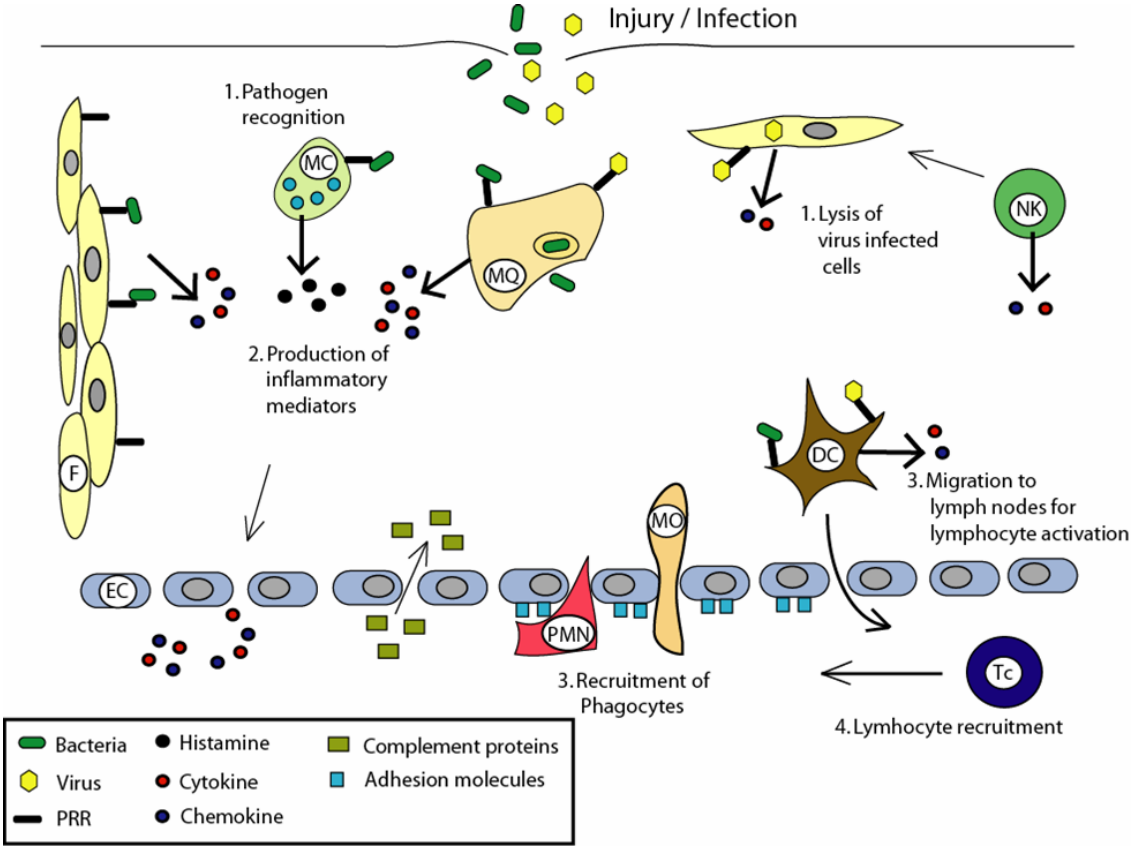


Figure 1.1: Simplified overview of mediators involved in the inflammatory response to injury and infection. See text for details. Abbreviations: DC (dendritic cell), EC (endothelial cell), F (Fibroblast), MC (mast cell), MO (monocyte), MØ (macrophage), NK (natural killer cell), PMN (neutrophil), Tc (T cell), PRR (pattern recognition receptor).

Tissue resident phagocytes, such as macrophages, are the first cells to encounter an invading pathogen. These cells use manifold receptors, termed PRRs, to constantly monitor their surrounding environment. Ultimately the pathogen is recognised by those receptors and its elimination initialised. Beside the ability of the PRRs to recognise non-self molecules, they can

also bind degradation products of the extracellular matrix and endogenous cell contents secreted upon cell necrosis. Since the release of this family of molecules is a direct result of infection or tissue injury they are termed danger signals or DAMPs (Danger associated molecular patterns). The activation of cells through PRRs not only results in the phagocytosis of the microbe but also the release of inflammatory mediators, which subsequently activate neighbouring cells as well as induce the recruitment of additional effector cells to the site of infection. As a consequence of their activation, endothelial cells upregulate their expression of adhesion molecules and the release of chemotactic factors. This in turn facilitates the adherence of circulating cells to the vascular endothelium and their migration into the affected area.

The immediate inflammatory response is characterised by the initial recruitment of neutrophils followed by monocytes, which subsequently differentiate into macrophages at the site of inflammation. The later stages of inflammation involve the adaptive immune system through the recruitment of lymphocytes. These are activated in regional lymph nodes either through antigen presentation by dendritic cells (DCs) that have migrated from the site of inflammation or by antigens drained from the infected area via the lymphatics. The chemotactic gradient, established during the initial stages of inflammation, leads to the recruitment of B cells as well as T cells. Once at the site of inflammation they contribute to the clearance of the pathogen by antibody-mediated phagocytosis, T cell-dependent macrophage activation or lysis by cytotoxic T cells.

The specific characteristics of a pathogen define the mechanisms employed to initiate an appropriate response for elimination of the microbe. Extracellular pathogens and their products, e.g. most bacteria and toxins, are phagocytosed by macrophages and neutrophils or opsonised by antibodies and complement proteins, which coat the pathogenic products making them more readily phagocytosed. Intracellular pathogens, on the other hand, e.g. viruses and mycobacteria, are cleared by increased intracellular destruction by phagocytes stimulated by T helper cells or lysis of the infected cell by cytotoxic effector cells, such as natural killer (NK) cells and cytotoxic T cells.

1.1.2 Cellular components of inflammation

The effectiveness of an immune response depends on two mechanisms: the recognition of a pathogen and/or danger signal, and the elimination of it. Macrophages and DCs are specialised in uptake and recognition of a pathogen through PRRs, which results in antigen presentation and the expression of inflammatory mediators, which direct and regulate the subsequent adaptive immune response. While T cells become activated through antigen presenting DCs in the lymph node, nearly all B cell responses depend on the support of effector T cells and occur after antigen presentation. In addition, “non-professional” immune cells, e.g. endothelial cells

and fibroblasts, can influence the movement and the direction of the migration of immune cells and are therefore considered as gate keepers and amplifiers of an inflammatory reaction.

1.1.2.1 Cells of the innate immune system

1.1.2.1.1 Macrophage activation and effector function

Circulating monocytes, which originate in the bone marrow from the common myeloid progenitor and are released into the peripheral blood, give rise to tissue resident macrophages. Macrophages display a great degree of heterogeneity and display distinct properties depending on the tissue they reside in (Gordon and Taylor, 2005). *In vitro,* macrophages can be differentiated from monocytes using macrophage colony stimulating factor (M-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF) or interferon (IFN)γ (Barreda et al., 2004; Zicari et al., 1992). Macrophages common feature, however, is their capability to monitor their environment for pathogens, to phagocytose microbes and to produce large amounts of inflammatory mediators to direct the inflammatory response.

Macrophage activation is achieved by multiple mechanisms, including phagocytosis and the recognition of foreign particles but not apoptotic cells. Following the encounter with a pathogen PRRs expressed by the macrophage recognise PAMPs. This leads to the production of pro-inflammatory cytokines, including tumour necrosis factor (TNF)α, which on their own are able to activate macrophages to produce inflammatory mediators. An alternative mechanism to activate macrophages is through cytokines produced by antigen-primed T cells, which results in increased endocytic activity and increased tissue repair (Mosser, 2003). The heterogeneity of activated macrophages and their ability to produce pro- as well as anti-inflammatory cytokines allows for a diverse and pathogen adjusted immune response.

1.1.2.1.2 Dendritic cell function and maturation

Similar to macrophages, DCs are derived from myeloid progenitor cells and migrate from the bone marrow to the peripheral tissue where they are present in almost every organ (Figure 1.2). Consequently these resident DCs are classified according to the tissue they are found in, e.g. Langerhans cells located in the epidermis, interstitial DCs in the dermis, splenic marginal zone DCs, T-zone interdigitating DCs, germinal centre DCs, thymic DCs, liver DCs and blood DCs (Shortman and Naik, 2007).

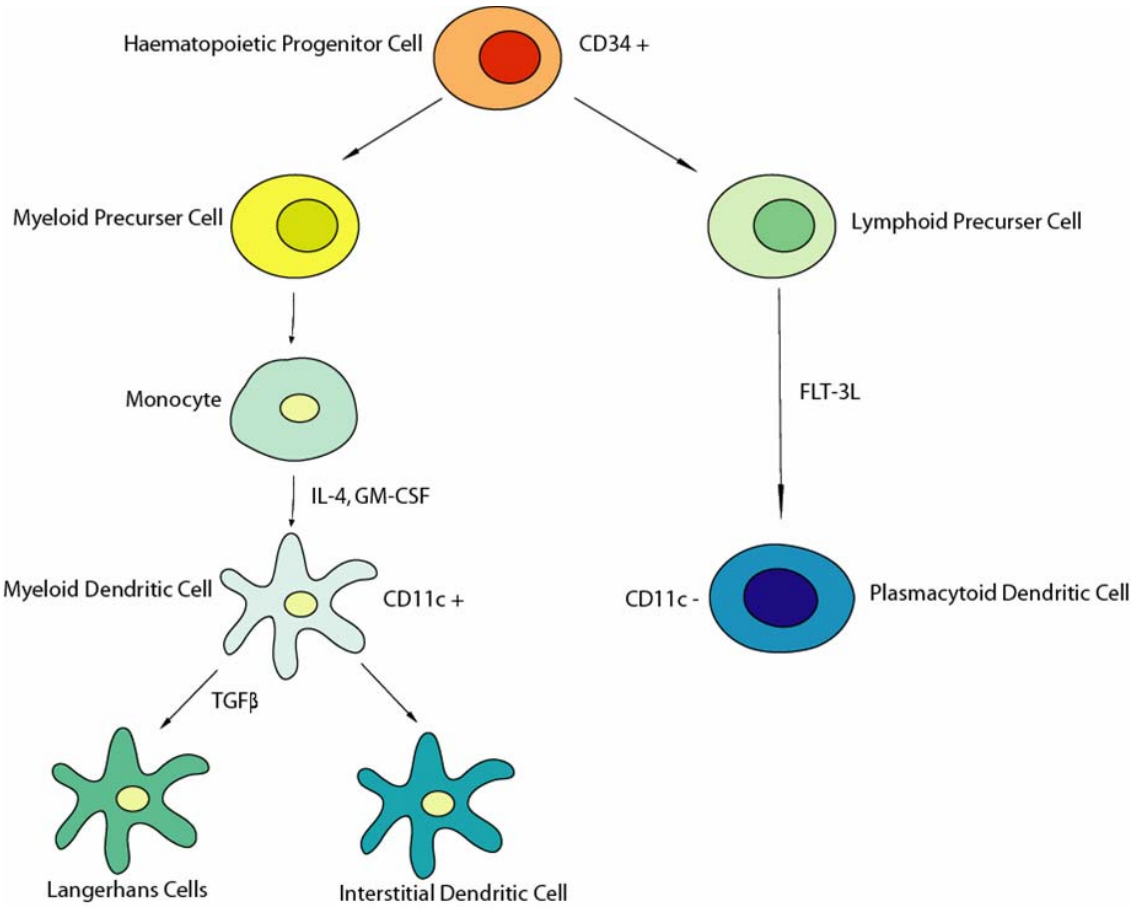


Figure 1.2: Differentiation of DCs from haematopoietic progenitor cells. See text for details. Abbreviations: TGFβ (transforming growth factor β), GM-CSF (granulocyte macrophage colony stimulating factor), FLT-3L (Fms-related tyrosine kinase 3 ligand).

DCs act as the major bridge between the innate and adaptive immune system and are therefore essential for the induction of an antigen specific immune response (Steinman, 1991). The initiation of a T cell response poses several challenges: the frequency of microbe-specific T cells is very low, infected cells express only very few peptide-major histocompatibility complex (MHC) complexes recognised by specific T cells and infected cells do not express co-stimulatory molecules required to induce T cell clonal expansion. These tasks are accomplished by DCs, which undergo distinct developmental stages. In their immature state, DCs act as sentinels monitoring their environment, which is achieved by a high endocytic/phagocytic activity. DCs become activated by numerous agents including PAMPs, danger signals and mediators secreted from innate as well as adaptive immune cells (Ueno et al., 2007). Following their activation DCs undergo a complex process of maturation from an antigen-capturing cell to an antigen presenting cell (APC). These changes include the loss of endocytic/phagocytic activity, change of morphology, production of chemokines and cytokines and the up-regulation of co-stimulatory molecules and peptide-MHC II complexes on their surface (Adams et al., 2005; Dieu-Nosjean et al., 1999; Ueno et al., 2007) (Figure 1.3). As a consequence of their

maturation DCs down-regulate the chemokine receptor CCR5 and up-regulate CCR7, which enables them to migrate to the lymph node. Once there, they provide the three signals necessary for T cell clonal expansion: the antigen, co-stimulatory molecules and the cytokine environment (Adams et al., 2005; Banchereau et al., 2000) (Figure 1.3). While macrophages and B cells have been shown to function as APCs, DCs are considered the most potent APC as they are able to stimulate naïve T cells (Adams et al., 2005).

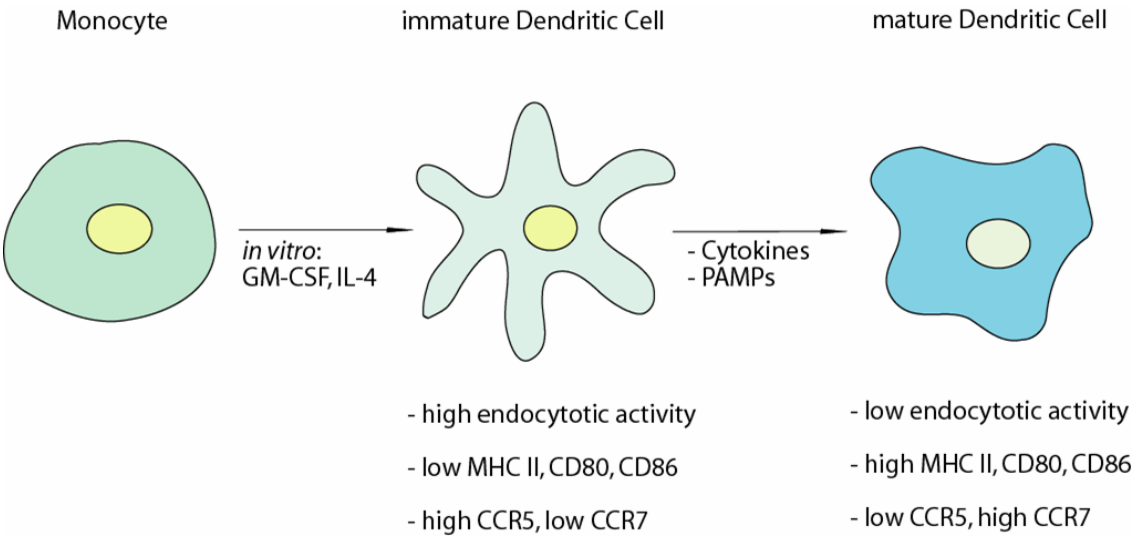


Figure 1.3: Differentiation and maturation of DCs from peripheral blood derived monocytes. See text for details. Abbreviations: GM-CSF (granulocyte macrophage colony stimulating factor), PAMP (pathogen associated molecular pattern), MHC II (major histocompatibility complex II).

Beside their role in initiating and directing a T cell response DCs also appear to be essential in maintaining immunological tolerance in the thymus as well as in the periphery (Steinman et al., 2003). In the thymus, high affinity auto-reactive T cells are eliminated upon encountering self MHC peptide. Evidence suggests that thymic epithelial cells as well as mature DCs in the thymus are involved in this process (Hengartner et al., 1988). Because this mechanism of central tolerance is imperfect, peripheral immature DCs were shown to continuously present self-antigen to auto-reactive T cells in the absence of co-stimulation, thereby inducing anergy or deletion of those T cells (Steinman et al., 2000). Beside this direct influence on potentially harmful T cells, mature DCs are also able to expand functional regulatory T cells (Treg), which might represent a mechanism of controlling the extent of inflammation (Battaglia et al., 2004; Sakaguchi, 2005). The break down of one or more of these mechanisms might contribute to the break of tolerance and thereby to the pathogenesis of autoimmune diseases. Therefore, increasing interest exists to elucidate the role of DCs in autoimmune diseases, such as systemic lupus erythmatosis (SLE) (Ueno et al., 2007). Furthermore, the highly potent antigen presenting

function of DCs is utilised in therapies for cancer as well as vaccination procedures (Banchereau and Palucka, 2005).

More recently it became apparent that two distinct subsets of DCs exist; the conventional myeloid (m)DCs and the plasmacytoid (p)DCs. *In vivo* both subsets are derived from $CD34^+$ haematopoietic progenitor cells but through distinct pathways (McKenna et al., 2005; Shortman and Naik, 2007) (Figure 1.2). These subtypes can be differentiated *in vitro* using GM-CSF and interleukin (IL)-4 or TNFα to obtain mDCs and Flt3L to obtain myeloid as well as plasmacytoid DCs (Leon et al., 2005; McKenna et al., 2005; Sallusto and Lanzavecchia, 1994). Conventional mDCs exist in at least three compartments: peripheral-tissue-resident DCs, secondary lymphoid-organ-resident DCs and circulating blood mDCs (Valladeau and Saeland, 2005). In contrast, plasmacytoid DCs are found in the blood (Ueno et al., 2007). They are named after their characteristic plasma cell-like morphology and are able to produce large amounts of type I IFN in response to viral and bacterial stimuli. Activated pDCs have the ability to influence the activation of numerous cell types, including myeloid DCs, B cells, NK cells and T cells (Ueno et al., 2007). However, while pDCs are able to act as APCs *in vitro*, their role in priming T cell responses *in vivo* is still not fully established.

1.1.2.1.3 Non-professional immune cells

Although being "non-professional" immune cells, endothelial cells and fibroblasts play a major role during an immune response. Endothelial cells are the barrier between migrating immune cells and the infected tissue. Therefore, they are the gate keepers and are responsible for an effective movement of immune cells into the tissue (Cook-Mills and Deem, 2005; Pober and Sessa, 2007). They achieve this by expressing adhesion molecules, e.g. intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), to which leukocytes attach by integrins. This results in a rolling of the leukocytes along the endothelium and subsequent arrest on the endothelial wall. The combination of adhesion molecules expressed by endothelial cells determines the homing of specific leucocytes to the tissue. Endothelial cells in peripheral lymph nodes constitutively express adhesion molecules required for leukocyte migration, whereas cells at sites of inflammation require activation to induce or increase adhesion molecule expression. This expression is induced by inflammatory mediators or by binding of microbial products to the TLRs expressed by endothelial cells (Henneke and Golenbock, 2002; Swerlick and Lawley, 1993). In addition, this activation results in the expression of chemokines and cytokines, which further contribute to the recruitment of specific leukocytes to the site of inflammation.

Inside the tissue, fibroblasts are the predominant cell type. They are responsible for the development and maintenance of the extracellular matrix (ECM). The ECM serves as a scaffold

for cells to move through the tissue. During inflammation the ECM is partly degraded in order to allow increased movement through the site of infection (Vaday and Lider, 2000). Resident fibroblasts also express PRRs, which allows them to sense infections (Colonna, 2004). As a result of PRR activation fibroblasts express a broad range of inflammatory mediators including chemokines and pro-inflammatory cytokines. This leads to further recruitment and activation of leukocytes. Therefore, resident fibroblasts play a role in amplifying and perpetuating the immune response.

1.1.2.2 Cells of the adaptive immune system

Unlike the immediate innate immune response the adaptive immune response is highly specific. Whereas, innate immune cells recognise pathogens through germ-line encoded receptors, the B cell- and the T cell receptor (BCR and TCR respectively) are generated through complex somatic gene rearrangement processes. This provides the adaptive immune system with a great variety of receptors, which are all specific for a single antigen. However, since each receptor is only expressed by one cell, clonal expansion is necessary in order to launch an immune response. This process results in a delay until the effector response can be mounted.

The adaptive immune response is further divided into humoral- and cellular responses. The humoral response is mediated by B cell recognition of extracellular antigens through the BCR, a surface expressed immunoglobulin. Antigen recognition results in the production of antibodies, which opsonise the antigen and promote phagocytosis, complement-mediated cell lysis and antibody dependent cellular cytotoxicity. The cellular response on the other hand involves T cells, which recognise intracellular antigens presented to them as peptides/MHC I complexes on APCs via binding to the TCR. This activation of T cells promotes the cytotoxic lysis of infected cells. The adaptive immune responses are initiated in organised lymphoid tissues close to the site of infections (Janeway, 2001).

1.1.2.2.1 B lymphocytes

Immature B cells originate from the haematopoietic stem cell (HSC) in the bone marrow. After becoming IgM^+, B cells migrate from the bone marrow to the spleen where they complete their developmental process. B cells can function as APCs, but unlike other APCs they bind soluble antigens directly with their surface BCR. As a result of BCR cross-linking the antigen becomes internalised and presented on MHC II (Ollila and Vihinen, 2005). In addition to BCR mediated activation of B cells, they can also become activated as a result of PAMP uptake through TLRs and subsequent antigen presentation on their MHC II to antigen specific T cells (Ollila and Vihinen, 2005; Pasare and Medzhitov, 2004). Moreover, T cells produce cytokines necessary to regulate B cell activation. Subsequent to their activation B cells initiate the production of

antibodies, which are identical in specificity to the BCR but can differ in the heavy chain. Antibodies protect the body through activating the complement cascade, opsonisation for phagocytosis and neutralisation of microbial products, e.g. toxins.

1.1.2.2.2 T lymphocyte development, differentiation and effector functions

Similar to the B cell, T cells ultimately originate from HSCs. However, unlike the B cells their actual differentiation occurs from a small fraction of HSCs, which migrate to the thymus. These T cell precursors are negative for the T cell markers CD4 and CD8. As they progress in their development TCR rearrangement occurs and they become CD4, CD8 double positive. Following positive- as well as negative selection mature T cells migrate as single positive cells (either $CD4^+$ or $CD8^+$) from the thymus to the peripheral tissues (Rothenberg et al., 2008).

CD4 and CD8 are co-receptors for the TCR, which is necessary for antigen recognition. They also provide markers for two distinct T cell populations. $CD4^+$ T cells are known as helper T (Th) cells, whereas $CD8^+$ T cells are referred to as cytotoxic T lymphocytes (CTL). CTLs recognise antigens presented in the context of MHC I molecules expressed on virally infected cells. This leads to the cytotoxic lysis of the infected cell. One mechanism of cell lysis is the granule-exocytosis pathway, which utilises perforin to channel the pro-apoptotic protein granzyme into the target cell and induce programmed cell death. Furthermore, CTLs also express Fas ligand that interacts with Fas on the target cell and initiates apoptosis.

Unlike CTLs, $CD4^+$ Th cells recognise extra-cellular antigens that were endocytosed/phagocytosed, processed and presented to them by APCs on MHC II molecules. Activation of a naive Th cell in this manner results in the development of specialised subsets of Th cells (Figure 1.4). The development of these subtypes depends on the cytokine environment provided by the APCs and other inflammatory cells. Furthermore, Th cells are characterised by the signalling pathways utilised and by the production of distinct sets of cytokines (Figure 1.4). Th1 cell development depends on the presence of IL-12 and they characteristically produce IFNγ and IL-2. Th1 cells are involved in promoting cellular immune responses by activating macrophages, NK cells and CTL responses. In contrast, Th2 cells require IL-4 for their development and typically secrete IL-13, IL-10, IL-5 and IL-4. By doing so, Th2 cells contribute to the humoral immune response by promoting B cell activation and antibody production (Santana and Rosenstein, 2003). More recently, a third distinct Th subtype has been described, which is characterised by its production of IL-17 (Harrington et al., 2005; Langrish et al., 2005). Therefore, this subtype was termed Th17 and although the exact conditions for Th17 development are not yet defined, IL-6, IL-23 and transforming growth factor (TGF)β seem to be essential (Bettelli et al., 2006; Langowski et al., 2006; Mangan et al., 2006). After their initial characterisation Th17 cells have been strongly implicated in autoimmune diseases

(Langrish et al., 2005; Park et al., 2005). Their natural function, however, seems to be in the immune response against extracellular bacteria and fungal infections, where IL-17 was shown to be preferentially induced (Mangan et al., 2006). While the initial cytokine environment produced by the APCs is detrimental in directing the development of the Th cells, the cytokines subsequently produced by differentiated Th cells further determine Th differentiation. In this context, it has been shown that the Th1 and Th2 cytokines IFNγ and IL-4 respectively negatively regulate Th17 development while IL-4 also inhibits Th1 development and the Th1 cytokines IL-12 similarly inhibits Th2 differentiation (Bettelli et al., 2006; Mangan et al., 2006).

The function of the Th cells described above are regulated by yet another $CD4^+$ T cell, termed regulatory T cell (Treg). As a result of the IL-10 and TGFβ production by Tregs an inhibitory signal is transmitted, thereby controlling the extent of the inflammatory response (Tang and Bluestone, 2008) (Figure 1.4).

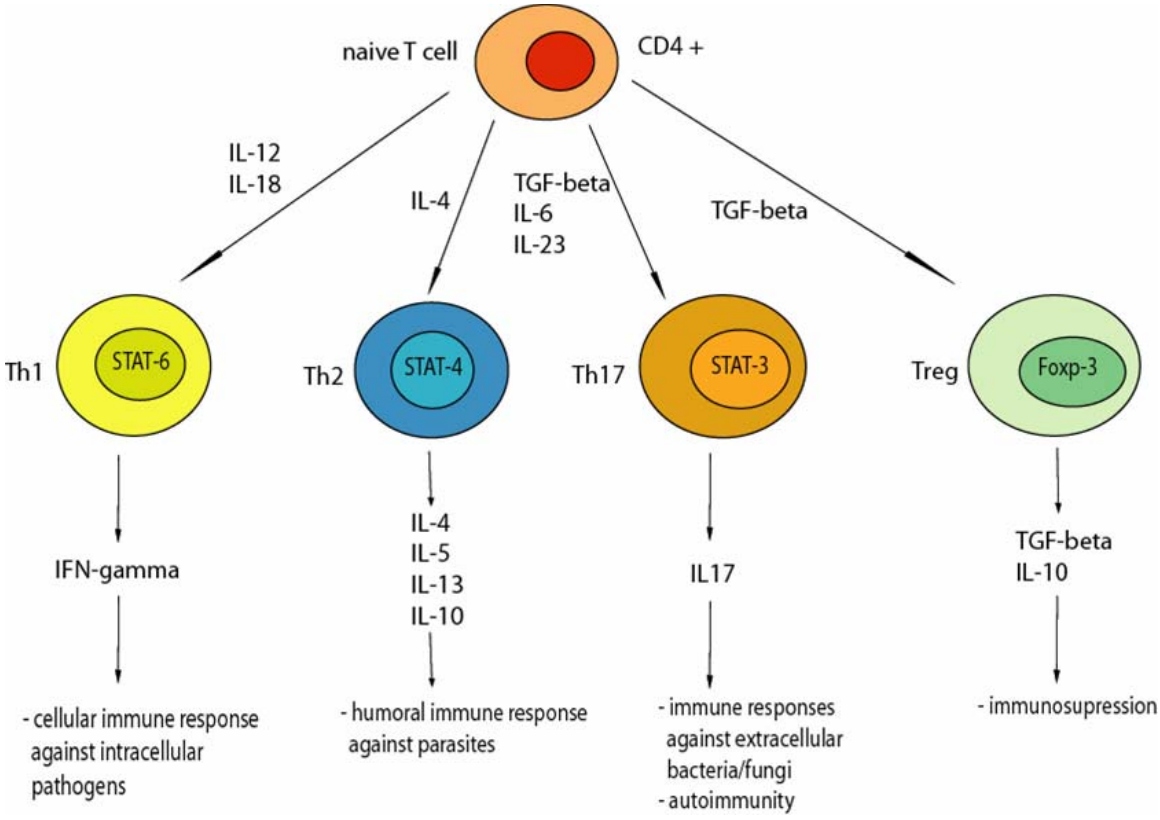


Figure 1.4: Differentiation of $CD4^+$ T cells. See text for details. Abbreviations: Th (Helper T cell), TGFβ (Transforming growth factor β), IL (Interleukin), STAT (Signal transduction and activator of transcription), IFN (Interferon), Foxp-3 (Forkhead box protein-3).

As already mentioned above (see section 1.1.2.1.2), in order to stimulate a T cell response three signals are necessary: the antigen, co-stimulation by surface molecules and cytokine stimulation. The first two are delivered through the so called immunological synapse where

APC and T cell interact. While the antigen is presented on the MHC complex to the TCR, co-stimulation occurs through interaction with CD80 and CD86 on the APC surface with CD28 expressed on the T cell. T cells, which bind antigen but do not encounter co-stimulation become anergic and cannot further respond to antigen even if co-stimulation is received. Once activated, T cells begin to express further surface molecules that either enhance co-stimulation or dampen it. CD40 ligand (CD40L) is expressed early by T cells in their activation process and sends an additional activation signal to the T cell by binding to CD40 on the APC. Furthermore, binding of CD40L to CD40 further promotes activation of APCs themselves (Xu and Song, 2004). Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is structurally similar to CD28 and is able to bind CD80 and CD86 on the APC (Rudd, 2008). Unlike CD28, however, it results in the inactivation of the T cell. These combinations of activation and inactivation signalling mechanisms are necessary to keep the powerful immune response under control, which otherwise would result in tissue destruction.

1.1.3 Molecular mediators of inflammation

During the process of inflammation cells communicate with each other using soluble mediators (Table 1.1). The immediate responses are mediated by lipid molecules such as arachidonic acid metabolites, prostaglandins and leukotrienes, as well as nitric oxide intermediates (Fang, 2004; Yoshikai, 2001). These molecules are also partially responsible for the cardinal signs of inflammation, e.g. fever, pain, swelling and redness.

This initial response is followed by the secretion of cytokines at the site of infection as well as systemically. Cytokines exert their broad effects through paracrine/autocrine and in some cases endocrine mechanisms. This family of signalling molecules encompass a large group of low molecular weight peptides, proteins and glycoproteins, which are characterised by their cellular source or function (Oppenheim and Feldmann, 2001a). Cytokines are further subdivided into interleukins (act as communicators between leukocytes), interferons (with primarily anti-viral functions) and chemokines (responsible for establishing a chemotactic gradient). Once secreted, cytokines bind to their specific cell surface receptor and induce signalling pathways, which ultimately lead to cell-activation, -proliferation and –differentiation (Oppenheim and Feldmann, 2001b). Due to their specific properties, cytokines are classified as pro- or anti-inflammatory. Furthermore, cytokines are involved in negative feedback loops thereby regulating their own expression as well as those of other cytokines. As a consequence a coordinated cytokine response during an infection results in the appropriate degree of inflammation. However, a failure in regulating the cytokine response can lead to delayed wound healing, failure to eliminate an infection and in the worst cases, chronic inflammation, organ failure and death (Brennan and Feldmann, 1996; Feldmann et al., 1996).

Table 1.1: Properties of selected mediators of inflammation

Mediator	Source	Effects
Pro-inflammatory cytokines		
IL-1α	MO, MØ, DC	Induces production of prostaglandin, MMPs, pro-inflammatory cytokines and acute phase proteins, expression of adhesion molecules, lymphocyte activation and regulates hormone function.
TNFα	MØ, DC, PMN	Same as above, also up-regulates effector functions of MØ and PMN, tumour cytotoxicity and induces MHC I expression
IL-6	MØ, DC, EC, fibroblast, T cells, B cells	Induces B cell growth, antibody production, secretion of acute phase proteins, NK activation and IFN production
IL-12/IL-18	MO, MØ, DC	Initiates Th1 responses, production of IFNγ by T cells and NK cells
IFNγ	Th1, NK	Induces activation of MØ, NK cells, PMN, expression of MHC I and II, CTL differentiation, blocks Th2/TH17 activity, inhibits B cell proliferation and IgE production
IFNα/β	Ubiquitous	Produced by all virus infected cells, provides early innate immune response against viruses, induces expression of MHC I, blocks viral protein synthesis and replication through autocrine or paracrine stimulation, enhances activities of CTL, MØ, DC, NK cells and B cells
Anti-inflammatory cytokines		
IL-10	MØ, Th2, B cell	Inhibits activation of MØ and DC, inhibits expression of co-stimulatory molecules and MHC II, inhibits production of IL-12, TNF, IL-6 and IL-1
TGFβ	Ubiquitous	Inhibits proliferation and effector function of T cells, proliferation of B cells, macrophage function, promotes tissue repair
IL-4	Th2, Mast cell	Major stimulus for production of IgE and the development of Th2 cells, antagonises the effects of IFNγ and inhibits TNFα production
IL-13	Th2, Mast cell	Inhibits production of IgE by B cells, inhibits expression of Th1 polarising cytokines by MØ, increases mucus production
Chemokines		
IL-8	Ubiquitous	Induces chemotaxis of neutrophils, basophils, eosinophils, and T cells, proliferation of keratinocytes, activation of neutrophils
IP10	Ubiquitous	Induces chemotaxis of MO, NK cells and Th1 cells
MCP-1	MO, MØ, DC, fibroblast	Induces chemotaxis and activation of MO and basophils
MIP-1α/β	MO, MØ, DC, fibroblast	Induces chemotaxis of MO, T cells and B cells
RANTES	MO, MØ, DC, fibroblast	Induces chemotaxis of MO
Arachidonic acid metabolites (eicosanoids)		
PGE_2, $PGF_{2\alpha}$ PGD_2	MØ	Induces vasodilatation, edema and pain
TXA_2	MØ	Induces vasoconstriction
LTB_4	MØ	Induces chemotaxis
LTC_4	MØ	Induces vasoconstriction and increased vascular permeability
Nitrogen and oxygen intermediates		
NO	MØ, PMN	Induces smooth muscle relaxation, vasodilatation, neurotransmission, tissue homeostasis, inhibition of platelet aggregation
RNI, ROI	MØ, PMN	Released in response to bacteria, viruses, fungi and parasites, oxidation and nitration of proteins, lipids and nucleic acids, regulation of inflammation, viral mutation, cytotoxicity, tissue damage, inflammation

Abbreviations: dendritic cells (DC), endothelial cells (EC), interleukin (IL), leukotriene (LT), macrophage (MØ), macrophage inflammatory protein (MIP), monocytes (MO), monocyte chemotactic protein (MCP), matrix metalloproteinases (MMP), nitric oxide (NO), prostaglandin (PG), polymorphonuclear cells (PMN), reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), T helper lymphocyte (Th), thromboxane (TX).

1.2 Pattern recognition receptors and the detection of infection

The innate immune system is responsible for the early detection and destruction of invading microbes. In doing so, it relies on a set of limited germ-line encoded PRRs for detection (Table 1.2). To initiate an immune response PRRs recognise PAMPs, which are present in pathogens but not the host. Subsequently, PRRs induce several extracellular activation cascades such as the complement pathways and various intracellular signalling pathways, leading to inflammatory responses. In numerous cases several PRRs recognize a given pathogen simultaneously or sequentially, activating distinct and shared signalling pathways. This enables cross-talk between the pathways as well as with other immunomodulatory signalling events generated by a particular inflammatory environment. This interplay between signalling pathways eventually determines the specific immune response directed at clearing the pathogen. PRRs are present in three different compartments: body fluids, cell membranes and cytoplasm. The PRRs in the body fluids play a major role in PAMP opsonisation and the activation of complement pathways. PRRs in the cell membrane have diverse functions, such as the presentation of PAMPs to other PRRs, the promotion of microbial uptake by phagocytosis and the initiation of signalling pathways. Cytoplasmic PRRs can be grouped into two classes: The IFN inducible PRRs including the CARD (caspase recruitment domain) family, which are involved in antiviral defence and the NLRs (NOD-like receptor), which are mainly involved in anti-bacterial immune responses (Lee and Kim, 2007).

Table 1.2: Selected, non-TLR pattern recognition receptors implicated in microbial recognition

Receptor	Type	Expression	Ligands
C-type lectins			
MR	Type I	DC, MØ, EC	Mediates antigen uptake of mannose-, fucose-, glucose-, GlcNAc-containing carbohydrates. Recognises L-selecting to mediate leukocyte rolling
DEC205	Type I	DC, EC	Ligand unknown
DC-SIGN	Type II	DC	Mediates antigen uptake of mannose-, fucose-containing carbohydrates, viral envelope glycoproteins, glycosylated bacterial endotoxin, parasite and fungus. Role in DC migration (ICAM-2) and T cell interaction (ICAM-3)
BDCA-2	Type II	DC, MØ, PMN	Ligand unknown, ligation of receptor leads to internalisation
Dectin-1	Type II	DC, MØ	Recognises zymosan, fungal wall derived β- glucan, live yeast
Langerin	Type II	LC specific	Recognises mannose-containing carbohydrates. Formation of birbeck granules
DC-ASGPR	Type II	DC	Recognises galactose-containing carbohydrates
SP-A/D	Collectin	n/a	Surfactant protein. Bind terminal non-reducing carbohydrates
MBL	Collectin	n/a	Obsonising mannose-containing carbohydrates and apoptotic cells. Binds to complement receptor CR1

table continued on next page…

CARD helicases			
RIG-I		ubiquitous (IFN inducible)	Recognises dsRNA, RNA from RNA viruses
MDA5		ubiquitous (IFN inducible)	Recognises poly I:C, RNA from EMCV
NOD like receptors			
NOD1		DC, MØ, EC	Recognises iE-DAP
NOD2		DC, MØ	Recognises MDP
NALP1		MØ, PBL	
NALP1b		MØ	-
NALP2		ubiquitous	Ligand unknown
NALP3		MØ, PBL	Recognises bacterial mRNA, extracellular ATP, uric-acid crystals, R848, *Listeria monocytogenes, Staphylococcus aureus*
NALP12		MØ, eosinophil	Ligand unknown
NAIP5		MØ	Recognises *Legionella* flagellin
IPAF		MØ	Recognises *Salmonella* flagellin
Scavenger receptors			
SR-AI/II	Class A	DC, MØ, EC	Recognise LTA, lipid A, CpG-DNA, acytylated and oxidised LDL, clearance of apoptotic cells
MARCO	Class A	MØ	Binds bacteria
CD36	Class B	MØ, EC	Recognises oxidised LDL, diacylglycerides, involved in clearance of apoptotic cells, collagen and parasitized erythrocytes
LOX-1	Class D	MØ, EC, SMC	Binds bacteria, involved in clearance of apoptotic cells
Complement receptors			
CR3 (Mac-1, CD18/CD11b)	cell surface	DC, MØ, NK, PMN	Promotes phagocytosis of targets opsonised by iC3b and non-opsonising phagocytosis of zymosan, LPS, fibrinogen, heparin sulfate and apoptotic cells. Binds ICAM-1/2
CR4 (CD18/CD11c)	cell surface	MØ	Promotes phagocytosis of targets opsonised by iC3b and non opsonising phagocytosis of LPS, fibrinogen. Binds ICAM-1
CR1	cell surface	MØ, B cell, PMN	Promotes phagocytosis of targets opsonised by C1q and MBL. Binds C3b and C4b
C1q	soluble	n/a	Soluble complement component. Opsonise immune complexes, DNA and apoptotic cells
Fc receptors			
FcγR I-III	γ	DC, MØ, B cell, NK, PMN	Mediate uptake of IgG-, CRP- and SAP-opsonised particles
FcεR I-III	ε	DC, MØ, B cell, NK, PMN	Mediate uptake of IgE-opsonised particles
FcαR I	α	DC, MØ, B cell, NK, PMN	Mediate uptake of IgA-opsonised particles

Abbreviations: blood DC antigen (BDCA), complement receptor (CR), C-reactive protein (CRP), dendritic cell specific ICAM-3-grabbing nonintegrin (DC-SIGN), Dendritic cell-associated C-type lectin-1 (Dectin-1), DC-asialoglycoprotein receptor (DC-ASGPR), N-acetylglucosamine (GlcNAc), Langerhans-cell-specific C-type lectin (Langerin), lectin-like oxidised LDL-receptor (LOX-1), macrophage Ag-1 (Mac-1), macrophage receptor with collagenous structure (MARCO), mannose binding lectin (MBL), mannose receptor (MR), serum amyloid P (SAP), smooth muscle cells (SMC), surfactant protein (SP), scavenger receptor (SR), nucleotide binding and oligomerisation domain (NOD), NACHT (domain present in NAIP, CIITA, HET-E, TP-1), leucine rich repeat and PYD containing (NALP), retinoid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5), caspase recruitment domain (CARD).

1.2.1 Phagocytic, endocytic and scavenger receptors

The main function of phagocytic PRRs is to internalise self-antigens or microbial components resulting in clearance of blood lipoproteins, pathogen elimination as well as enhanced processing and presentation by APCs. This group comprises several receptor families, including C-type lectins (CLRs), scavenger receptors, Fc receptors and opsonic receptors (Table 1.2). In contrast to CARD Helicases, NLRs or TLRs, ligand binding to phagocytic PRRs generally does not induce the expression of proinflammatory cytokines.

CLRs are either expressed as membrane bound receptors or soluble proteins, also termed collectins, which are secreted. While the membrane bound CLRs capture pathogens leading to their intracellular destruction and antigen loading on MHC molecules, soluble collectins opsonise microbes (Haagsman et al., 2008). Following microbe binding, collectins associate with cell surface receptors, thereby enhancing phagocytosis of the pathogen by APCs (Agramonte-Hevia et al., 2002). In general, CLRs recognise a broad range of carbohydrates, which are part of the microbial cell wall as well as being present on endogenous glycoproteins (Robinson et al., 2006).

Scavenger receptors are a structurally unrelated group of receptors. Their common feature is to bind and internalise lipoproteins, which allows them to recognise microbes as well as to clear apoptotic cells (Peiser et al., 2002).

The Fc- and complement-receptors (CR) bind antibody- and complement-coated pathogens and apoptotic cells, which leads to enhanced uptake and destruction (Gordon, 2002; Underhill and Ozinsky, 2002). In addition, several members of the CR family were shown to bind directly to carbohydrates on pathogen surfaces and host glycoproteins (Agramonte-Hevia et al., 2002; Gasque, 2004). The FcR- and CR-families of receptors also show a certain degree of cross-talk to facilitate phagocytosis (Agramonte-Hevia et al., 2002).

1.2.2 CARD helicases

Numerous viruses, such as Newcastle disease virus (NDV), enter the cytoplasm of cells directly and dsRNA, synthesized during viral replication, is present in the cytoplasm. In order to detect this kind of virus cytoplasmic PRRs are essential. The CARD-helicase family consists of RIG-I (Retinoid inducible gene I) and MDA5 (Melanoma differentiation associated gene 5) (Andrejeva et al., 2004; Yoneyama et al., 2004), which detect viral RNA with the DExD/H amino acid sequence (where x can be any amino acid) in their RNA helicase domain. Unlike the PRRs described in section 1.2.1 CARD-helicases actively induce a signalling pathway. This is achieved through the recruitment of IPS-1 (IFNβ promoter stimulator-1) through CARD domains. The localisation of IPS-1 at the mitochondria is essential for downstream signalling

and ultimately leads to the induction of pro-inflammatory mediators and type I interferons by activation of NF-κB and IRF-3 respectively (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

LGP2 is a homolog of RIG-I and MDA5. It is, however, lacking the signal transducing CARD domains and is suggested to function as inhibitor of CARD-helicases (Rothenfusser et al., 2005; Yoneyama et al., 2005).

1.2.3 NOD-like receptor family

NLRs are a family of cytoplasmic PRRs, which recognise bacterial PAMPs and danger signals and subsequently initiate signalling pathways to induce an immune response (Fritz et al., 2006; Lee and Kim, 2007; Ting et al., 2006). NLRs consist of a C-terminal leucine rich repeat (LRR) domain, which mediates ligand binding, a centrally located NACHT (domain present in NAIP, CIITA, HET-E, TP-1) domain, which mediates oligomerisation and activation of the NLRs, and an N-terminal effector domain, which initiates downstream signalling. On the basis of the effector domains and the phylogenetic history of the NACHT domain, NLRs have been grouped into the subfamilies NODs (nucleotide binding and oligomerisation domain), NALPs (NACHT, leucine rich repeat and PYD (pyrin domain) containing), CIITA (Class II transactivator), IPAF (Ice protease-activating factor) and NAIPs (NLR family, apoptosis inhibitory protein). NODs and IPAF contain CARD effector domains, whereas NALPs and NAIPs contain PYD and three BIR (Baculoviral IAP Repeats) domains, respectively (Lee and Kim, 2007).

NOD1 and NOD2 were the first mammalian NLRs to be described to sense cytoplasmic PAMPs. NOD1 specifically recognises the peptide γ-D-glutamyl-*meso*-diaminopimelic acid and acts as a sensor for gram-negative bacteria, whereas NOD2 binds muramyl dipeptide (MDP), a degradation product of peptidoglycan (PGN), and acts as a general sensor for most bacteria (Inohara et al., 2005; Strober et al., 2006). The activation of NODs leads to the recruitment of the CARD-containing kinase RIP2 (receptor interacting protein 2) to the receptor through CARD-CARD interactions, which ultimately leads to the activation of NF-κB and MAPKs (Girardin et al., 2001; Inohara et al., 2005; Kobayashi et al., 2005; Strober et al., 2006). The physiological importance of NODs is demonstrated by the increased susceptibility of NOD1 or NOD2 deficient mice to gastrointestinal bacterial infections (Strober et al., 2006). In humans NOD2 mutations are linked to Crohn´s disease, however, the pathogenic mechanism underlying this association is still unclear (Eckmann and Karin, 2005; Fritz et al., 2006; Strober et al., 2006).

The NALP family consists of 14 members, which are characterised by their PYD effector domain (Tschopp et al., 2003). Although the ligands and function of most of the NALPs is still unknown, their critical role in forming an inflammasome upon activation and subsequently inducing the production of mature IL-1β and IL-18 has been shown in the case of NALP1 and NALP3. Activated NALPs recruit ASC (Apoptosis-associated speck-like protein) via their PYD domain, which leads to the recruitment of Caspase-1 and Caspase-5 through CARD-CARD interactions. The close proximity of the caspases in the inflammasome promotes cross-activation, thus leading to the maturation of IL-1β and IL-18 (Martinon and Tschopp, 2004). The physiological stimulus for NALP1 is unknown. However, cell rupture has been shown to be sufficient for inflammasome assembly (Martinon et al., 2002). Numerous stimuli for NALP3 have recently been described, which include exogenous PAMPs, such as bacterial RNA, DNA and the bacteria *Staphylococcus aureus* and *Listeria monocytogenes* as well as endogenous danger signals including uric acid crystals and extracellular ATP (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Muruve et al., 2008). Given this great variety of ligands, the hypothesis was formed that NALP3, instead of binding these ligands directly, recognises common intracellular changes generated from the multiple inflammasome-activating pathways such as lower intracellular K^+ concentrations (Mariathasan et al., 2006). It has also been shown that TLR stimuli as well as inflammasome activating stimuli interplay for efficient IL-1β induction. The importance of NALP3 is demonstrated by the association of NALP3 mutations with cold-induced autoinflammatory syndrome (Fritz et al., 2006; Ting et al., 2006).

IPAF has a similar structure to NOD1 and has been shown to be critical for inducing IL-1β secretion in response to *Salmonella* infection (Mariathasan et al., 2004). Flagellin from *Salmonella,* introduced into the cytosol, has been demonstrated to be the key PAMP of IPAF (Franchi et al., 2006; Miao et al., 2006). Although, IPAF activation by *Salmonella* flagellin is independent of TLR5 (which is the extracellular PRR for flagellin, see below), it is still unclear if IPAF binds flagellin directly. Similar to IPAF, NAIP5 also recognises flagellin, but only if derived from *Legionella* and introduced into the cytosol (Molofsky et al., 2006; Ren et al., 2006). Therefore, IPAF and NAIP5 seem to control intracellular bacterial growth directly through Caspase-1 activation and IL-1β production, whereas TLR5 recognises extracellular flagellin to induce an inflammatory response.

1.2.4 Toll-like receptor family

TLRs are the most intensively studied PRRs and similar to CARD-Helicases and NLRs, their major function is to induce signalling pathways, which lead to an inflammatory response (Akira and Takeda, 2004; Lee and Kim, 2007). Unlike NLRs or CARD-Helicases, however, TLRs

recognise extracellular PAMPs either directly on the cell surface or in the early endosome following endocytoisis/phagocytosis.

The Toll receptor was discovered in *Drosophila melanogaster*, where it has originally been described to be involved in dorsal/ventral development of the fly (Anderson et al., 1985). Only later it was discovered that Toll deficient flies were highly susceptible to fungal infections, which suggested an involvement of Toll signalling in the immune response of the fly (Lemaitre et al., 1996). Subsequently the discovery and functional analysis of its human homologue, TLR4, as well as its ligand, lipopolysaccharide (LPS), demonstrated that it was capable of inducing the expression of inflammatory cytokines and co-stimulatory molecules in human cell lines (Medzhitov et al., 1997; Poltorak et al., 1998). This observation for the first time provided an explanation of how the innate immune system is able to recognise a broad range of microbes and induce an immediate inflammatory response. To date, 13 mammalian TLRs have been described based on their sequence homology to the *Drosophila* Toll, and have been shown to recognise a wide variety of conserved molecular features of bacteria, fungi and viruses. 10 of these TLRs were shown to be expressed in humans.

1.2.4.1 TLR structure and expression profile

All TLRs are type I integral membrane glycoproteins that belong to the TLR/IL-1R super family, which also includes the receptors for the pro-inflammatory cytokines IL-1 and IL-18 (Figure 1.5). This super family is characterised by its cytoplasmic TLR/IL-1R homology (TIR) domain, which is essential for signal transduction. Despite the similarities in the cytoplasmic region, the extracellular portions of the TIR domain receptor family are unrelated to each other. While the IL-1R family recognises its ligands using multiple Ig(immunoglobulin)-like domains, TLRs contain LRR domains, similar to *Drosophila* Toll and NODs, to bind their ligands.

TLRs can be further subdivided by their distribution within the cell. TLR3, TLR7, TLR8 and TLR9, which all recognise nucleotide structures, belong to a functional subfamily, which is mainly expressed in intracellular compartments and detect PAMPs following endosomal/lysosomal acidification (Heil et al., 2003; Latz et al., 2004; Matsumoto et al., 2003). TLR1, TLR2, TLR4, TLR5 and TLR6, on the other hand, are located mainly on the cell surface and primarily recognise structurally conserved peptides and proteins derived from bacteria and fungi. Most TLRs bind their ligands as homodimers, whereas TLR2 heterodimerises with either TLR1 or TLR6 or other PRRs, such as Dectin-1, for specific ligand recognition (Lee and Kim, 2007).

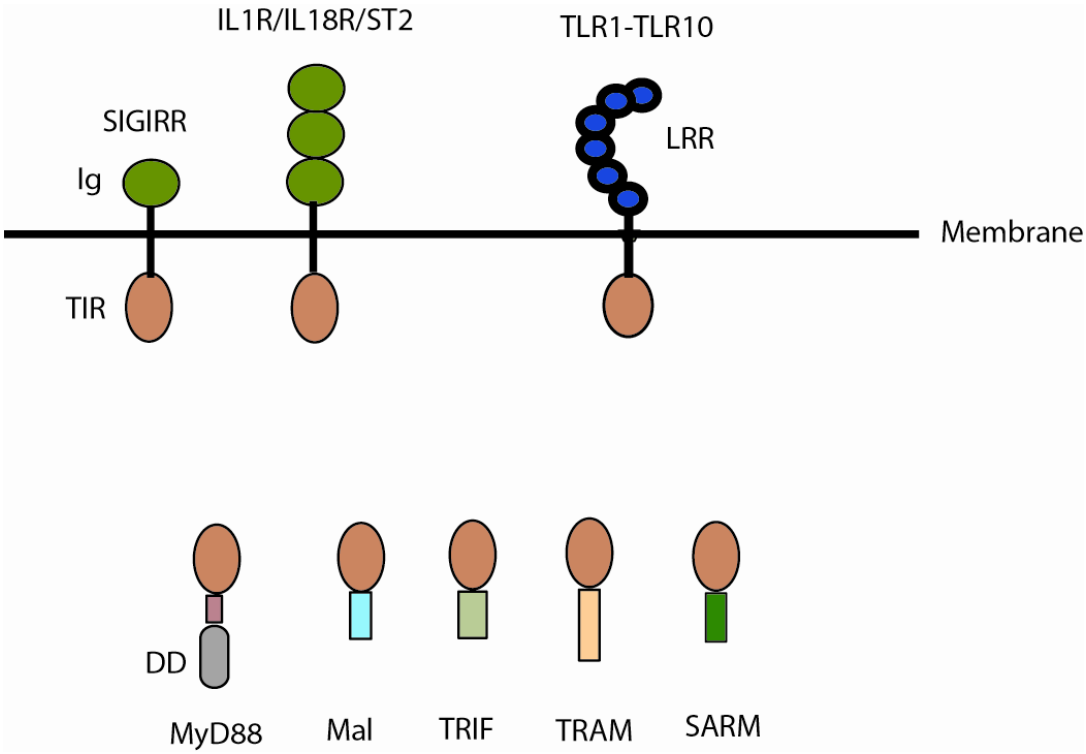


Figure 1.5: TLR/IL-1R super family. The members of the TLR/IL-1R family are defined by the presence of the cytoplasmic TIR domain and can be divided into two subgroups based on their extracellular domains. The TLRs contain leucine-rich repeats (LRRs), whereas the IL-1R family has Ig-like domains. This group include the IL-1R and the IL-18R and their accessory proteins (IL-1RacP and IL-18RacP), as well as ST2 and the orphan receptor SIGIRR. In addition, the super family also includes the TIR domain containing TLR/IL-1R adaptors MyD88, Mal, TRIF, TRAM and SARM.

1.2.4.2 TLR recognition of pathogenic compounds

1.2.4.2.1 TLR4

Since TLR4 was described to be the receptor for LPS and the first mammalian TLR to be identified in 1997 (Medzhitov et al., 1997) it is also the most extensively studied. Further investigations, using two strains of LPS-hyporesponsive mice, which exhibit mutations in the *TLR4* gene, revealed that TLR4 is a signal transducer for the gram-negative bacterial cell wall component LPS. C3H/Hej mice contain a point mutation (P712H) in its TIR domain, whereas C57BL/10ScCr mice appear to be null for the TLR4 locus (Poltorak et al., 1998). The role of TLR4 as a signal transducer for LPS has been confirmed using TLR4 deficient mice as well as *in vitro* studies in the human embryonic kidney (HEK) cell line HEK293 transfected with human TLR4 (Chow et al., 1999; Hoshino et al., 1999). In humans, the TLR4 polymorphism Asp299Gly, which alters the LRR domain, results in impaired efficacy of LPS signalling and impaired capacity to elicit inflammation (Arbour et al., 2000).

Although, TLR4 has been established as the essential receptor recognising LPS, several additional co-receptors have been shown to be involved in LPS binding. LPS is opsonised by the serum protein LPS-binding protein (LBP), which enhances LPS binding to CD14 on the cell

surface (Wright et al., 1990). CD14, on the other hand, is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that lacks transmembrane- as well as cytoplasmic domains and is therefore incapable of signal transduction (Lee et al., 1993). The involvement of CD14 in TLR4 signalling depends on the LPS chemotype as has been shown in mice containing dysfunctional CD14, called the Heedless mutation (Jiang et al., 2005b). LPS consists of a lipid A moiety, a core polysaccharide and an *O*-polysaccharide of variable length. Microbial variants with long *O*-polysaccharide chains form smooth colonies, whereas those that lack an *O*-polysaccharide chain form rough colonies, this led to the designations smooth and rough LPS. While CD14 defective macrophages still show NF-κB activation in response to rough LPS via the MyD88 dependent signalling pathway, smooth LPS induced NF-κB activation is impaired (Jiang et al., 2005b). Moreover, the MyD88 independent signalling pathway was impaired in response to both types of LPS. CD14 is also present in the serum as a soluble receptor lacking the GPI-tail (Frey et al., 1992). This enables CD14 negative cells, such as endothelial cells, to respond to LPS in the absence of membrane bound CD14 (Pugin et al., 1993). In addition to LBP and CD14, the secreted molecule MD-2 associates with the LRR domain of TLR4 and thereby enhances LPS responsiveness (Akashi et al., 2000; Shimazu et al., 1999; Viriyakosol et al., 2001).

Beside the specificity of CD14 in regard to smooth and rough LPS, species specific differences have also been demonstrated, depending on the acetylation state of LPS. *Pseudomonas aeruginosa* hexa-acetylates its LPS during adaptation to the cystic fibrosis airway. Whereas the LRR domain of human TLR4 is able to recognise hexa-acetylated LPS from *Pseudomonas aeruginosa,* murine TLR4 does not (Hajjar et al., 2002). Moreover, an analogue of lipid A was shown to act agonistically on murine TLR4 but not on the human receptor (Akashi et al., 2001). This discrimination is attributed to species specific differences in the structure of MD-2. In addition, the anti-tumour agent Taxol has been shown to be recognised by TLR4 in murine but not human cells (Byrd-Leifer et al., 2001; Kawasaki et al., 2000) (Figure 1.6).

1.2.4.2.2 TLR1, TLR2 and TLR6

TLR2 is a unique TLR since it is able to recognise a great variety of bacterial, fungal and even virally derived ligands (Figure 1.6). This is achieved by its heterodimerisation with TLR1 or TLR6, which are closely related to TLR2, as well as other PRRs such as Dectin-1 (Lee and Kim, 2007; Takeuchi et al., 1999). The usage of additional TLRs to recognise ligands enables the discrimination of subtle differences in the acylation of lipopeptides derived from microbial pathogens. In association with TLR1, TLR2 binds to triacyl lipopeptides (Takeuchi et al., 2002), whereas TLR2 responds to diacyl lipopeptides in combination with TLR6 (Takeuchi et al., 2001).

The triacylated lipopeptides recognised by TLR2/1 include the 19 kDa mycobacterial lipoprotein (Takeuchi et al., 2002), *Borrelia burgdorferi* outer membrane lipoprotein A (Alexopoulou et al., 2002), the mycobacterial aralipoarabinomannan (Sandor et al., 2003) and the synthetic lipoprotein structure Pam_3Cys (Pam_3Cys-Ser-$(Lys)_4$-trihydrochloride) (Takeuchi et al., 2001). TLR2/6 heterodimers, on the other hand, recognise the synthetic diacylated lipopeptide macrophage activating lipopeptide-2 (MALP-2) (Takeuchi et al., 2001), phenol-soluble modulin (PSM) from *Staphylococcus epidermidis* (Hajjar et al., 2001) and group B streptococcal soluble factor (Henneke et al., 2001). A number of ligands appear to be recognised by TLR2 homodimers such as *Porphyromonas gingivelis* LPS (Hirschfeld et al., 2001) and chlamydial HSP (heat shock protein) 60 (Vabulas et al., 2001). Other ligands seem to require the association with the co-receptor CD14 as has been shown in the case of gram-positive lipoteichoic acid (LTA) (Cleveland et al., 1996; Schwandner et al., 1999) and mycobacterial lipoarabinomannan (Means et al., 1999). Furthermore, CD36 has been shown to further enable discrimination of TLR2/6 ligands as it contributes to the responses to MALP-2 but not zymosan (Hoebe et al., 2005) (Figure 1.6).

The recognition of the fungal cell wall component zymosan occurs in concert with Dectin-1 (Brown, 2006; Gantner et al., 2003). Moreover, the cooperation of TLR2 with Dectin-1, results in a distinct cytokine profile compared to TLR2/6 signalling on its own (Brown, 2006). In contrast, TLR2 association with TLR1 or TLR6 increases the spectrum of ligands being recognised but it does not seem to lead to differential signalling events.

In addition to bacterial structures, TLR2 has also been reported to interact with different viruses, including hemagglutinin (HA) protein from measles virus and envelope glycoproteins of the human cytomegalovirus (CMV) (Bieback et al., 2002). TLR2 has also been described to be involved in signalling responses to herpes simplex virus-1 (HSV-1) (Ahmad et al., 2008; Kurt-Jones et al., 2004) and lymphocytic choriomeningitis virus (LCMV) (Zhou et al., 2008).

1.2.4.2.3 TLR3

Double-stranded (ds) RNA is a product of the replication cycle of numerous viruses inside the cell. It was the first viral product to be implicated in antiviral immunity by its ability to induce virus-like symptoms (Field et al., 1971). In 2001, dsRNA was described to be the ligand for TLR3, which forms homodimers in order to bind its ligand (Alexopoulou et al., 2001; Matsumoto et al., 2002). Purified genomic reovirus dsRNA and its synthetic analogue polyinosinic-polycytidylic acid (poly I:C) stimulate cells such as myeloid DCs to express type I IFNs and inflammatory cytokines. This expression is reduced but not abolished in cells from TLR3 deficient mice due to the presence of other dsRNA binding PRRs such as the CARD-Helicases described above (Section 1.2.2) (Alexopoulou et al., 2001).

Although the physiological importance of TLR3 in anti-viral responses has been demonstrated, its specific role is still unclear. The effector responses elicited by poly I:C treatment inhibits murine γ herpesvirus 68 replication in murine macrophages (Doyle et al., 2002). Similarly, it has been shown, that the administration of the synthetic dsRNA poly (I:C12U) protects mice against punta toro virus (PTV) infection in a TLR3 dependent manner (Gowen et al., 2007). In support of this, the viral load in TLR3 deficient mice inoculated with murine cytomegalovirus (MCMV) have been shown to be substantially augmented (Tabeta et al., 2004). However, conflicting data shows that the adaptive antiviral immune response to both RNA and DNA viruses is equivalent in $TLR3^{-/-}$ and wild type mice (Edelmann et al., 2004). A report by Schulz *et al.* describes a more specific *in vivo* function of TLR3, which is to promote cross-priming for the initiation of a $CD8^+$ T cell response against viruses that do not directly infect DCs (Schulz et al., 2005). Moreover, TLR3 has been shown to be expressed by human $CD8^+$ T cells and stimulation of these cells using poly I:C led to an increase in IFNγ production (Hervas-Stubbs et al., 2007; Tabiasco et al., 2006). In addition, murine TLR3 recognises dsRNA from eggs of the parasite *Schistosoma mansoni*, indicating that TLR3 might also be involved in non-viral- as well as viral immune responses (Aksoy et al., 2005) (Figure 1.6).

1.2.4.2.4 TLR5

The ligand for TLR5 has been determined to be monomeric flagellin, a structural component of flagella, which enables bacteria to be mobile (Hayashi et al., 2001; Smith et al., 2003). TLR5 is suggested to be important for microbial recognition at the mucosal surface, since flagellin activates intestinal epithelial cells (Gewirtz et al., 2001), human intestinal microvascular endothelial cells (Maaser et al., 2004), and lung epithelial cells (Smith et al., 2003). Furthermore, stimulation with flagellin has also been reported to lead to maturation of human DCs (Means et al., 2003) (Figure 1.6).

These *in vitro* results have been confirmed in individuals with a TLR5 polymorphism ($TLR5^{392STOP}$) resulting in a stop codon in the extracellular ligand-binding domain of TLR5 (Hawn et al., 2003). This mutation leads to an impaired ability to respond to flagellin and an increased risk of developing pneumonia due to the flagellated bacteria *Legionella pneumophila* (Hawn et al., 2003). In mice, the role of TLR5 is still unclear. While TLR5 deficiency leads to increased susceptibility to *Escherichia coli* urinary tract infections, it also results in the development of spontaneous colitis, suggesting a role for TLR5 in recognising infections as well as protective functions in the gut (Andersen-Nissen et al., 2007; Carvalho et al., 2008; Vijay-Kumar et al., 2007).

1.2.4.2.5 TLR7 and TLR8

The common ligand for TLR7 and the structurally related TLR8 is single stranded (ss)RNA derived from ssRNA viruses such as vesicular stomatitis virus (VSV) (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). However, there appear to be species specific differences in its recognition. Murine, but not human, TLR7 binds ssRNA from VSV and influenza virus (Diebold et al., 2004; Lund et al., 2004), as well as guanosine- and uridine (GU)-rich ssRNA from human immunodeficiency virus-1 (HIV-1) U5 region (Heil et al., 2004). In contrast, the response to GU-rich ssRNA is mediated by TLR8 in humans, whereas this could not be observed with murine TLR8 (Heil et al., 2004). TLR7 and TLR8 have also been shown to recognise the synthetic anti-viral compounds imidazoquiolines, which are structurally related to nucleic acids. Again, these results vary between the human and murine forms of the receptors. Both human and murine TLR7 recognise the imidazoquiolines resiquimod (R848), whereas R848 only signals from human but not murine TLR8 (Hemmi et al., 2002; Jurk et al., 2002) (Figure 1.6).

TLR7, but not TLR8, is highly expressed by human blood plasmacytoid DCs, which is one reason for the secretion of large amounts of type I IFNs following TLR7 stimulation, characteristic to those cells (Kawai and Akira, 2006; Liu, 2005).

1.2.4.2.6 TLR9

Bacterial DNA exhibits immunostimulatory activity through the presence of the abundantly expressed unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs, which rarely occur in mammalian DNA (Bauer et al., 2001a). The activity of these motifs can be mimicked by synthetic DNA oligonucleotides (CpG-DNA or CpG-ODN). Stimulation of TLR9 deficient mice with CpG-DNA indicated TLR9 as its receptor (Hemmi et al., 2000). This is supported by data in primary human cells, where expression of TLR9 correlates with CpG-DNA responsiveness (Bauer et al., 2001b). The function of TLR9 also seems to be species specific, since human and murine TLR9 require distinct CpG motifs for signal initiation (Bauer et al., 2001b). However, a recent report challenges the view, that CpG motifs are the defining features recognised by TLR9 (Haas et al., 2008; Yasuda et al., 2006). Haas *et. al* have shown that the DNA sugar backbone can activate TLR9 in a sequence independent manner (Haas et al., 2008) (Figure 1.6).

In addition to bacterial DNA, TLR9 has also been shown to recognise viral-derived CpG-DNA from genomic HSV-1 and -2 as well as CMV (Krug et al., 2004; Lund et al., 2003; Tabeta et al., 2004). Plasmacytoid DCs from TLR9 mutant mice are unable to produce type I IFNs in response to treatment with viral DNA, implying a role for TLR9 in anti-viral responses.

However, studies also implicated TLR9 independent responses to DNA from HSV-1, providing evidence for other PRRs involved in this process (Krug et al., 2004). A recent report identified DAI (DNA-dependent activator of IFN-regulatory factors) as a cytosolic PRR recognising DNA and subsequently inducing the production of type I IFNs (Takaoka et al., 2007). Therefore, DAI represents a new family of cytosolic PRRs, which, similar to RNA recognition by CARD-Helicases, provides a mechanism to recognise DNA in the cytosol and inhibit bacterial/viral replication.

Similar to TLR7, TLR9 is highly expressed on pDCs but not on conventional mDCs, therefore providing an explanation for the specific role of pDCs in producing large amounts of type I IFNs (Kawai and Akira, 2006; Liu, 2005).

1.2.4.2.7 TLR10-TLR13

The most recently identified Toll homologues are TLR10 and TLR11, however, their role in anti-microbial responses is not yet defined (Chuang and Ulevitch, 2001; Zhang et al., 2004). In humans, TLR10 is expressed on B cells and pDCs and although a ligand is still elusive, a constitutively active version of TLR10 lead to the expression of inflammatory genes, which was dependent on MyD88 (Bourke et al., 2003; Hasan et al., 2005; Hasan et al., 2004; Hornung et al., 2002). Although the *TLR10* gene is present in mice it is non-functional, due to a partial genomic sequence disruption by a retroviral insertion (Hasan et al., 2005). TLR10 is closely related to TLR1 and TLR6, the co-receptors of TLR2, and it has been demonstrated, that TLR10 dimerises with TLR1 as well as TLR2 (Hasan et al., 2005). Therefore, the hypothesis has been formed that TLR10 might cooperate with these receptors for ligand recognition.

Unlike TLR10, TLR11 has been found to be expressed in murine epithelial cells, whereas no TLR11 expression could be observed in humans, due to the presence of a stop codon in the genomic sequence (Zhang et al., 2004). Profilin-like molecule from the protozoan parasite *Toxoplasma gondii* has been described as the ligand for murine TLR11 and it has been demonstrated that TLR11 is critical for optimal resistance to an infection with this parasite (Yarovinsky et al., 2005). Moreover, TLR11 transfected cells specifically respond to uropathogenic bacterial lysates and TLR11 deficient mice fail to recognise and respond to uropathogenic *Escherichia coli* (Zhang et al., 2004).

The sequences of two novel murine TLRs have been identified, based on homology searches to the TIR homology domain. These are termed TLR12 and TLR13 but they lack human orthologs and their function and ligands are still elusive (Tabeta et al., 2004).

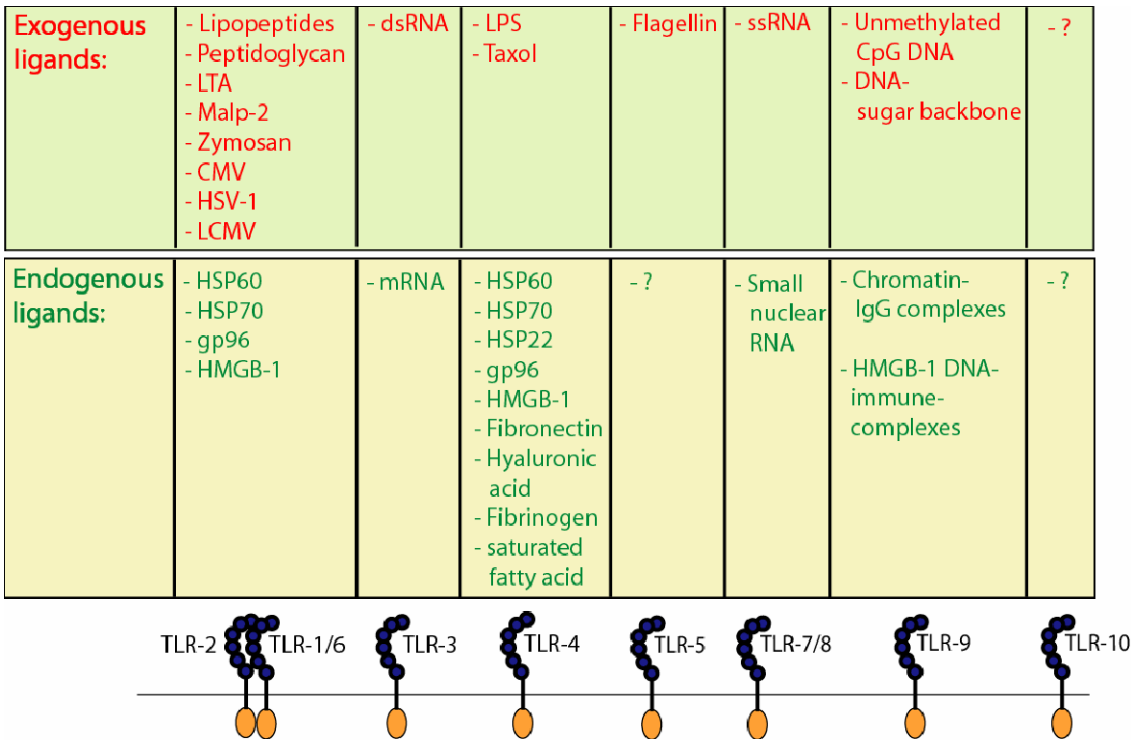


Figure 1.6: Selected exogenous and endogenous TLR ligands. See text for details. Abbreviations: HSP (heat-shock protein), Malp-2 (macrophage activating lipopeptide-2), CMV (cytomegalovirus), HSV (herpes simplex virus), HMGB-1(high mobility group box-1), LCMV (Lymphocytic choriomeningitis virus), LTA (lipoteichoic acid), LPS (lipopolysaccharide), TLR (toll-like receptor).

1.2.5 Cross-talk between TLRs with other pattern recognition receptors

TLR induced gene expression together with antigen uptake by phagocytic PRRs is an essential part of how innate immunity fights infections. Numerous studies have tried to examine whether these mechanisms influence each other. The phagocytic receptors Dectin-1 (Dendritic cell-associated C-type lectin-1), DC-SIGN (Dendritic cell specific ICAM-3-grabbing nonintegrin) and the mannose receptor have all been described to influence TLR signalling (Underhill and Gantner, 2004). LPS induced maturation of DCs is blocked by simultaneous stimulation of DC-SIGN with lipoarabinomannan from *Mycobacterium tuberculosis* (Geijtenbeek et al., 2003). Similarly, cross-linking of the mannose receptor as well as stimulation of it, using Mannose-capped lipoarabinomannan (ManLAM) from *Mycobacterium tuberculosis,* has been shown to inhibit LPS-induced IL-12 production (Chieppa et al., 2003; Nigou et al., 2001). In contrast, Dectin-1 has been reported to collaborate with TLR2 following Zymosan stimulation to produce pro-inflammatory cytokines in macrophage cell lines and BMDCs (Brown et al., 2003; Gantner et al., 2003; Gross et al., 2006) (Figure 1.7).

The innate immune system utilises numerous signal inducing PRRs providing the immune system with TLR independent mechanisms to induce inflammatory mediators (Figure 1.7).

However, TLRs have been shown to interact with them and influence their signalling. This is important, since it leads to potential amplification of the inflammatory response.

NOD1 or NOD2 induced pro-inflammatory cytokine production, was shown to be markedly enhanced by stimulation with TLR ligands and mice treated with the NOD2 ligand MDP are more susceptible to LPS induced septic shock (Kobayashi et al., 2005; Netea et al., 2005; van Heel et al., 2005; Wolfert et al., 2002). This indicates the existence of a synergistic mechanism between NOD2 and TLRs. The molecular basis for this mechanism is still unclear. However, it has been described, that TLR4 stimulation upregulates NOD2 expression and similarly that NOD2 stimulation induces MyD88 expression (Inohara et al., 2005). Furthermore, MDP stimulated macrophages induce the transcription of TNFα mRNA, which is not translated into protein. Subsequent LPS stimulation, leads to TNFα protein synthesis and its secretion (Wolfert et al., 2002).

Although the CARD-helicase family has only recently been characterised, reports of cross-talk between them and TLRs have already been published. The stimulation of TLR3, TLR4, TLR7, TLR8 and TLR9 induces the expression of type I IFNs, which consequently results in the expression of RIG-I and MDA5, as these are IFN inducible genes, leading to a potentially amplified anti-viral response (Kang et al., 2002). In contrast, the LPS induced negative regulator A20 has been shown to reduce RIG-I mediated IRF and NF-κB activation (Lin et al., 2006).

These results demonstrate that TLR signalling communicates with other PRRs in an amplifying as well as inhibitory manner. These mechanisms are part of the regulatory mechanisms, which are essential to conduct a successful immune response.

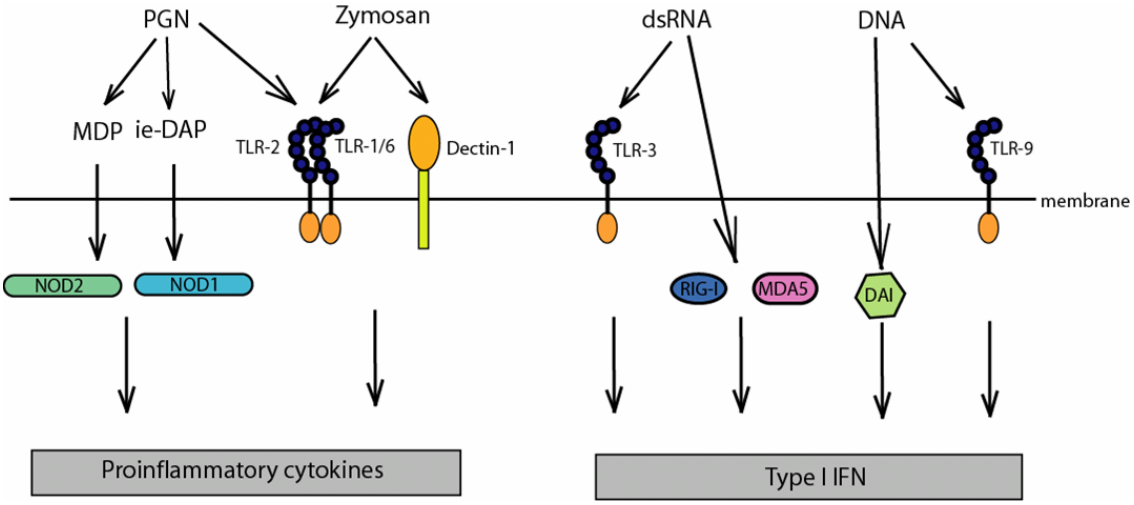


Figure 1.7: Recognition of PGN, dsRNA and DNA by TLR-dependent and independent pathways. See text for details. Abbreviations: PGN (peptidoglycan), MDP (muramyl dipeptide), ie-DAP (diaminopimelic acid containing dipeptide), NOD (nucleotide-binding oligomerisation domain), RIG-I (retinoid inducible gene-I), MDA5 (melanoma differentiation associated gene 5), DAI (DNA dependent activator of IFN regulatory factors).

1.3 Activation of TLRs by danger signals and their role in chronic inflammation

The main role of TLRs is considered to be the recognition and response to microbial pathogens but they have also been reported to recognise endogenous ligands. Moreover, the evolutionary conserved Toll pathway in *Drosophila melanogaster* is induced by binding of the endogenous ligand Spätzle to Toll (Lemaitre et al., 1996), suggesting that Toll receptors are able to bind endogenous ligands. This is an area of TLR research that has recently gained momentum with increasing numbers of publications about endogenous TLR ligands (reviewed in (Brentano et al., 2005a; Drexler et al., 2006; Marshak-Rothstein, 2006; Marshak-Rothstein and Rifkin, 2007; Rifkin et al., 2005; Roelofs et al., 2008; Tsan and Gao, 2004).

Endogenous ligands are thought to be released during necrotic cell death induced by tissue damage, stress factors or infection, which result in the release of cell components that initiate an inflammatory response (Searle et al., 1982). Subsequently, these endogenous molecules were termed "danger signals" (Gallucci and Matzinger, 2001). The release of these danger signals as a result of injury and the consequent stimulation of TLR signalling provides a mechanism to alarm the immune system as well as to induce tissue repair. An involvement of TLRs in tissue repair and homeostasis has been suggested by the observations in MyD88- and TLR4 deficient mice, which show decreased proliferation of epithelial cells in the gut following injury (Fukata et al., 2006; Fukata et al., 2005; Rakoff-Nahoum et al., 2004). In accordance with these results is the observation that epithelial cells from mice deficient of the TLR inhibitor SIGIRR hyperproliferate (Xiao et al., 2007).

1.3.1 Endogenous ligands for TLR2 and TLR4

One of the first reported groups of endogenous TLR ligands were the heat shock proteins (HSP)s. HSP60 (Vabulas et al., 2001), HSP70 (Vabulas et al., 2002a) and gp96 (glycoprotein 96) (Vabulas et al., 2002b). Recent studies also suggest HSP22 as a TLR4 ligand, which is able to activate monocyte-derived DCs (Roelofs et al., 2006). HSP60, -70 and gp96 have been reported to signal through TLR2 and TLR4 in a MyD88- and TRAF6- dependent manner in murine DCs and macrophages (Vabulas et al., 2001; Vabulas et al., 2002a; Vabulas et al., 2002b).

However, several studies have raised doubts about the ability of HSPs to act as endogenous TLR ligands. Highly purified murine liver HSP70 does not induce TNFα release from murine macrophages even at high concentrations (Wallin et al., 2002) leading to suggestions that the TNFα-inducing activity previously attributed to HSP70 (Vabulas et al., 2002a) has been due to endotoxin contamination (Gao and Tsan, 2003). Similarly, other studies using LPS-free

preparations of HSP70, HSP60 and gp96 have all suggested that the TLR activity previously reported may have been due to LPS contamination (Bausinger et al., 2002; Gao and Tsan, 2003; Reed et al., 2003). Other studies using expression of gp96 or addition of gp96 secreting fibroblasts into mice have avoided potential contamination. Activation of APCs has been observed in these studies but it is unclear if this has been due to a direct effect gp96 (Baker-LePain et al., 2004; Liu et al., 2003).

High mobility group box (HMGB)-1 is a molecule released during cell necrosis (Andersson et al., 2000). HMGB-1 has been suggested to signal through TLR4 as it shares a similar signalling pathway to LPS when stimulating human endothelial cells and neutrophils (Fiuza et al., 2003; Park et al., 2003). However, neutrophils lacking TLR4 still respond to HMGB-1 (Park et al., 2004). A recent study demonstrated a role for TLR2, TLR4 and TLR9 in recognising HMGB-1 and the subsequent activation of NF-κB, suggesting multiple mechanisms of HMGB-1 recognition (Ivanov et al., 2007; Park et al., 2006; Tian et al., 2007).

Another source of endogenous TLR2 and TLR4 ligands is the remodelling and/or destruction of the ECM due to tissue damage. This process results in the release of fragments from ECM components, which can induce inflammation. Heparan sulphate is associated with the cell membrane as well as part of the ECM (Yanagishita and Hascall, 1992) and is only found at low levels in healthy tissue. Increased levels of heparan sulphate are detected in the extracellular fluid around injuries (Kainulainen et al., 1998), in RA synovium (Lohmander et al., 1989) and in urine from patients with infections (Oragui et al., 2000). Hyaluronic acid, a major component of the ECM, is rapidly degraded at sites of inflammation (Johnson et al., 2002; Weigel et al., 1986). Both heparan sulphate and hyaluronic acid have been shown to signal via TLR4. DCs from the C3H/HeJ mouse, which expresses a non-functional TLR4, do not respond to either stimulus (Johnson et al., 2002; Termeer et al., 2002). Fragments of hyaluronan released during injury can also stimulate TLR4 (Jiang et al., 2005a; Taylor et al., 2004) and biglycan a ubiquitously expressed proteoglycan in the ECM has been reported to signal through TLR2 and TLR4 in macrophages (Schaefer et al., 2005). Fibronectin is released from cells in response to tissue damage (George et al., 2000; Hino et al., 1995; Jarnagin et al., 1994; Saito et al., 1999) and is able to stimulate synovial cells to release pro-inflammatory cytokines and MMPs (Saito et al., 1999). Studies using transfected TLR4 HEK293 cells have shown fibronectin to be a ligand for TLR4 (Okamura et al., 2001).

While HSPs, hyaluronic acid, heparan sulphate and fibronectin are released as a result of cell necrosis or ECM damage, other endogenous TLR ligands are a sign of inflammation or infection. These include fibrinogen and β-defensins. Fibrinogen is an early hallmark of inflammation. It migrates from the vasculature into the extravascular space during

inflammation due to increased permeability of endothelial cells (Dvorak et al., 1985). Data from macrophage cell lines have shown that fibrinogen-induced secretion of macrophage inflammatory protein 1 α (MIP1α), MIP1β and MIP2 requires functional TLR4 (Smiley et al., 2001).

Murine β-defensin, an anti-microbial peptide of the innate immune response, has been shown to stimulate TLR4 on immature DCs, inducing maturation and up-regulation of co-stimulatory molecules (Biragyn et al., 2002). In addition, β-defensin has been shown to act as a chemoattractant of immature DCs (Biragyn et al., 2001; Yang et al., 1999) (Figure 1.6).

1.3.2 Endogenous ligands for TLR3, -7 and -8

The exogenous ligand for TLR3 is dsRNA (Kariko et al., 2004) but not ssRNA, which is instead recognised by TLR7 and TLR8 (Heil et al., 2004). Although cellular RNAs are mainly regarded as ssRNA, they can produce hairpin loop formations that act as sources of endogenous dsRNA (Panet, 1983). Endogenous dsRNA released from necrotic cells stimulates DCs via TLR3 to produce IFNα whereas pre-treatment of necrotic cells with RNase prior to incubation with DCs, prevented this response (Kariko et al., 2004). Single stranded RNA-associated autoantigens have been reported to activate B cells by combined B cell antigen receptor-TLR7 engagement in mice, thereby implicating a role for endogenous RNA recognition by TLR7. (Lau et al., 2005) (Figure 1.6).

1.3.3 Endogenous ligands for TLR9

While non-methylated CpG-motifs in bacterial and viral DNA are recognized by TLR9, endogenous DNA on its own is normally inert (Krieg et al., 1995). However, cells from autoimmune humans and mice show decreased CpG-methylation (Richardson et al., 1990) yet de-methylation of murine DNA does not enable it to stimulate B cells (Sun et al., 1997). Thus, the mechanism of activation by bacterial and endogenous DNA appears to be more complex than simply methylation. Indeed, a recent report by Wagner and colleagues describes that the sugar backbone of DNA is able to activate TLR signalling independent of the base sequence (Haas et al., 2008). Given that TLR9 is capable of recognising self-DNA (IgG-chromatin) complexes in B-cells (Leadbetter et al., 2002), in situations where necrotic cells are abundant the DNA released may form autoantibody immune complexes, which can stimulate TLR9.

1.4 The role of TLRs in inflammatory diseases

TLR activation is a double edged sword as exacerbated TLR signalling can lead to tissue damage and chronic inflammation. TLRs are emerging as major factors in many disease conditions. While enabling TLRs to respond to tissue injury the recognition of danger signals may also have a role in the development of autoimmune disease and chronic inflammatory disorders. In contrast to apoptosis, necrotic cell death is typically associated with inflammation. This is most likely due to the release of the cell contents into the surrounding environment leading to activation of adjacent cells (Edinger and Thompson, 2004). Apoptotic cells retain their membrane integrity and are rapidly cleared by macrophages before lysis (Platt et al., 1998). This has been demonstrated in DCs, which are activated by exposure to necrotic cells but not apoptotic cells (Gallucci et al., 1999; Sauter et al., 2000). The contents released from necrotic cells may activate TLRs, generating further inflammation and thus more necrosis. This cycle of inflammation may explain the chronic inflammatory state found in autoimmune diseases such as RA (Figure 1.8).

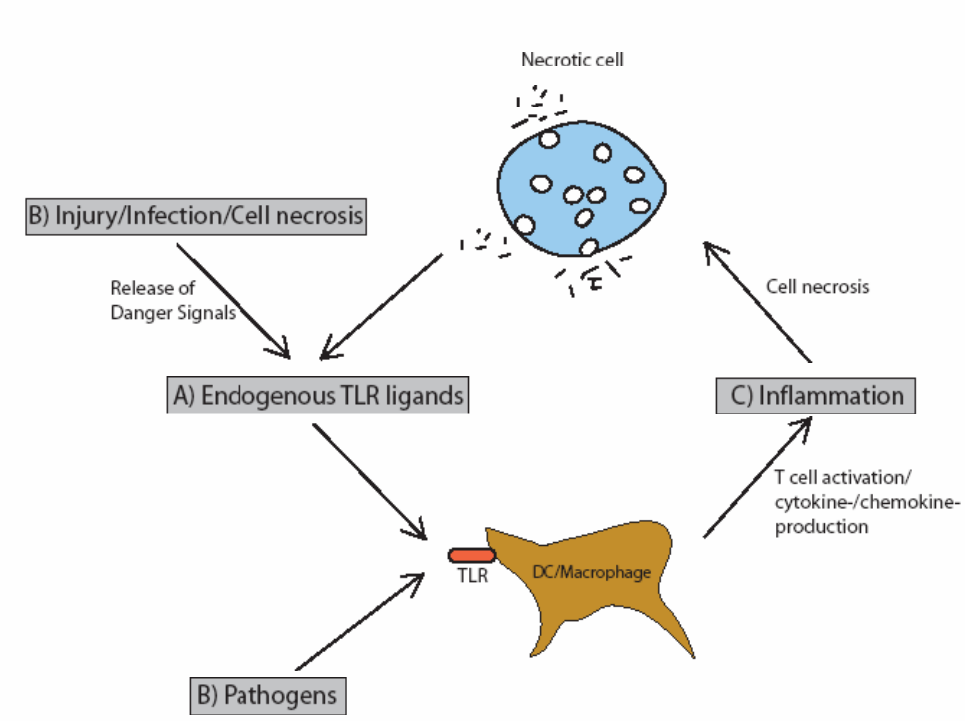


Figure 1.8: Hypothesis for TLR participation in chronic inflammation. A) The release of endogenous TLR ligands (e.g., HMGB-1, RNA) due to cell necrosis, breakdown of extracellular matrix, or infections subsequently leads to the activation of TLRs. As a result proinflammatory cytokines and chemokines, which induce inflammation, are produced. B) Exogenous TLR ligands from bacterial or viral infection may activate TLRs resulting in inflammation. C) The inflammation generated by endogenous and/or exogenous ligands may cause cells to undergoing necrotic cell death and the release of endogenous TLR ligands. Abbreviations: HMGB (high mobility group box)

Several studies emphasize a role for TLRs in the promotion of systemic lupus erythematosus (SLE), asthma, Crohn's disease, multiple sclerosis, type 1 diabetes, and RA (Table 1.3). In this chapter I will concentrate on the existing evidence of an involvement of TLRs in RA due to the research interests of the Kennedy Institute.

Table 1.3: Toll-like receptors were shown to be involved in inflammatory diseases and injury

Disease/Injury	Toll-like receptor	Potential ligands	References
Systemic Lupus Erythematosis (SLE)	- TLR7 - TLR8 - TLR9	- RNA - RNA - Immune complexes/dsDNA - HMGB-1	(Lau et al., 2005; Tian et al., 2007; Vollmer et al., 2005)}
Atherosclerosis	- TLR2 - TLR4	- Fatty acids - Fatty acids	(Lee et al., 2001; Lee et al., 2004; Yamashita et al., 2006)
Diabetes (type I)	- TLR3 - TLR7 - TLR9	- RNA - RNA - DNA	(Lang et al., 2005; Wen et al., 2004; Zipris et al., 2007)
Multiple sclerosis	- TLR4 - TLR9	- ? - ?	(Ichikawa et al., 2002; Kerfoot et al., 2004; Prinz et al., 2006; Waldner et al., 2004)
Inflammatory bowl disease	- TLR2 - TLR5 - TLR9	- Microbial antigens - Microbial antigens - Bacterial DNA	(Fukata et al., 2005; Netea et al., 2004; Rakoff-Nahoum et al., 2004)
Asthma	- TLR2 - TLR4	- Hyaluronan fragments - Hyaluronan fragments	(Jiang et al., 2005a)
Rheumatoid Arthritis (RA)	- TLR2 - TLR3 - TLR4 - TLR9	- HMGB-1 - RNA - HMGB-1 - HSPs - Hyaluronan oligosaccharides - Fibronectin fragments - Bacterial DNA - HMGB-1	(Kawane et al., 2006; Sacre et al., 2007a; Sacre et al., 2007b; Schett et al., 1998; Scott et al., 1994; Taniguchi et al., 2003; Tian et al., 2007; Yu et al., 1997) (Roelofs et al., 2008)

1.4.1 Involvement of TLRs in the pathogenesis of RA

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases. It affects ~0.5-1% of the adult population, with three times more females than males affected. RA is marked by persistent inflammatory polyarticular synovitis mainly affecting the small joints. TLRs were first suggested to have a role in RA in response to a pathogen initiating the disease (van der Heijden et al., 2000). Several animal models use bacterial cell wall components to induce experimental arthritis. The injection of streptococcal cell wall (SCW), containing a toxic peptidoglycan-polysaccharide fragment, into rats leads to a chronic arthritis comparable to human RA (Cromartie et al., 1977). Similar experiments in mice showed that TLR2 knockout or MyD88 knockout mice were protected from SCW induced joint inflammation since these animals did not develop joint swelling (Joosten et al., 2003). In addition, the intra-articular administration of the TLR2 and NOD2 ligand peptidoglycan, also led to the development of destructive arthritis in mice (Liu et al., 2001). As well as peptidoglycan, the administration of LPS together with arthritogenic serum in IL-1 receptor-deficient mice, but not in MyD88-deficient mice, resulted in acute paw swelling (Choe et al., 2003), indicating a role for TLR4 in murine RA. Furthermore, mice deficient of IL-1RA display uncontrolled IL-1 signalling and develop a chronic T cell-driven arthritis (Horai et al., 2000). In contrast, IL-1RA and TLR4 double knock out mice show a markedly reduced arthritis severity, which has not been observed in IL-1RA and TLR2 double knock out mice, suggesting a TLR4 specific function in driving RA pathology in this model (Abdollahi-Roodsaz et al., 2008). In support of this study are results obtained by blocking TLR4 with a naturally occurring antagonist. In mice immunised with collagen induced arthritis (CIA), the administration of TLR4 antagonist led to a reduction in disease severity even when administered post disease onset (Abdollahi-Roodsaz et al., 2007). Moreover, serum transferred arthritis has been shown to be not sustained in TLR4 mutant mice compared with controls (Choe et al., 2003). Thus, animal disease models show a possible role for TLR2 and TLR4 in RA. However, while bacterial TLR ligands have been detected in human RA synovium (van der Heijden et al., 2000), they were also found in synovial tissue of healthy donors without any inflammatory response (Schumacher et al., 1999). Similarly, reports of bacterial DNA in the synovium of RA patients (Gerard et al., 2001; Kempsell et al., 2000; van der Heijden et al., 2000) were called into question by a more sensitive study detecting the bacterial marker muramic acid, which did not identify any bacterial components in RA patients (Chen et al., 2003b). It has been demonstrated, however, that B cells become activated by chromatin-IgG complexes binding to the antigen receptor and TLR9 to produce rheumatoid factor (RF) independently of T cells (Leadbetter et al., 2002). Moreover, DNase $II^{-/-}$ mice develop chronic polyarthritis due to excessive release of DNA following cell death, which might be due to TLR9 activation (Kawane et al., 2006). Therefore, the search for the trigger initiating RA has now turned to the involvement of endogenous TLR

ligands. Cell necrosis due to tissue damage or inflammation are a major source of endogenous danger signals and Hyaluronan oligosaccharides, fibronectin fragments, HSPs, antibody-DNA complexes and high mobility group box (HMGB)-1 have all been identified in the RA joint (Schett et al., 1998; Scott et al., 1981; Taniguchi et al., 2003; Yu et al., 1997).

Expression of TLR2, -3, -4, -7 and -9 has been demonstrated in human synovial tissue from RA patients (Brentano et al., 2005b; Iwahashi et al., 2004; Radstake et al., 2004; Roelofs et al., 2005; Seibl et al., 2003). TLR2 expression has been identified in synovial fibroblasts, $CD16^+$ monocytes and $CD16^+$ macrophages in the synovium (Iwahashi et al., 2004; Kyburz et al., 2003; Seibl et al., 2003). Interestingly, expression of TLR2, -3 and -4 was shown to be increased in the synovial tissue of RA patients compared with healthy donors or OA samples (Brentano et al., 2005b; Radstake et al., 2004). Functional studies have been performed by stimulation of TLR2- and TLR9-expressing RA synovial fibroblasts with peptidoglycan, a TLR2 ligand, which led to the expression of MMPs and secretion of IL-6 and IL-8 while no activation has been observed in response to the TLR9 ligand CpG oligodeoxynucleotides (Kyburz et al., 2003). More recently, RNA released from necrotic synovial fluid cells has been shown to activate synovial fibroblasts via TLR3 (Brentano et al., 2005b). Interestingly, RA synovial fibroblasts were shown to respond to TLR3 stimulation by producing TNFα while primary human skin fibroblasts do not (Lundberg et al., 2007). This shows that TLR3 is functional in the inflamed synovium but also shows a tissue specific mechanism of TLR signalling. In addition, evidence for a TLR4 ligand in the serum and synovial fluid of RA patients has been observed. Serum and synovial fluid from RA patients stimulated TLR4 expressing CHO cells to up regulate CD25 but had no effect on untransfected CHO cells (Roelofs et al., 2005). Moreover, conditioned media from RA synovial membrane cultures has been shown to activate human macrophages in a MyD88 and Mal dependent manner, further strengthening the involvement of an endogenous ligand driving RA pathology (Sacre et al., 2007a; Sacre et al., 2007b). In support of this data, the over-expression of a dominant negative construct of MyD88 or Mal inhibits the spontaneous release of cytokines and MMPs by RA synovial membrane cultures (Sacre et al., 2007a; Sacre et al., 2007b). However, no ligand has so far been defined.

A strong candidate for an endogenous TLR ligand involved in RA pathology is the DNA binding protein HMGB-1. It exhibits cytokine like properties as it is actively released by LPS, TNFα or IL-1 stimulated macrophages as well as passively released during cell necrosis (Andersson et al., 2000; Wang et al., 1999). It has been shown to activate NF-κB in human endothelial cells and human neutrophils to produce pro-inflammatory cytokines (Fiuza et al., 2003; Park et al., 2003). Therefore, it can function as a mediator of inflammatory responses. RA patients as well as Lewis rats with *Mycobacterium tuberculosis*-induced adjuvant arthritis

contain detectable amounts of HMGB-1 in the synovial fluid (Kokkola et al., 2002). Interestingly, HMGB-1 expression is increased in synovial fibroblasts of RA patients compared to osteoarthritis patients, which may result in the release of pro-inflammatory cytokines by RA synovial fibroblasts (Taniguchi et al., 2003). TLR2 and TLR4 have been described to recognise HMGB-1 leading to the subsequent activation of NF-κB (Park et al., 2006). A more recent study demonstrated the activation of TLR9 by HMGB-1 and DNA containing immune complexes, which require the immunoglobulin family member RAGE (Receptor for advanced glycation end products) (Tian et al., 2007).

Naturally occurring polymorphisms can give clues to the relevance of TLR involvement in human RA. However, no association between TLR2 and TLR4 polymorphisms and the susceptibility to RA have been found. The amino acid substitution Asp299Gly in the TLR4 gene results in the disruption of TLR4 signalling and two studies investigating this polymorphism found no correlation with susceptibility to RA (Kilding et al., 2003; Lamb et al., 2004). The polymorphisms Arg677Trp and Arg753Gln in the TLR2 gene and Thr399Ile in the TLR4 gene also showed no association with RA (Sanchez et al., 2004). Investigating the TLR adaptor Mal, no association between the Mal polymorphism Ser180Leu and the susceptibility or severity of RA could be found (Sheedy et al., 2008).

1.5 TLR signalling pathways

The existing evidence of an involvement of TLRs in the pathogenesis of RA and other inflammatory diseases makes it necessary to investigate and understand the molecular basis of the signalling pathways induced by these receptors. Potentially this research could result in the identification of therapeutic targets for the treatment of inflammatory diseases.

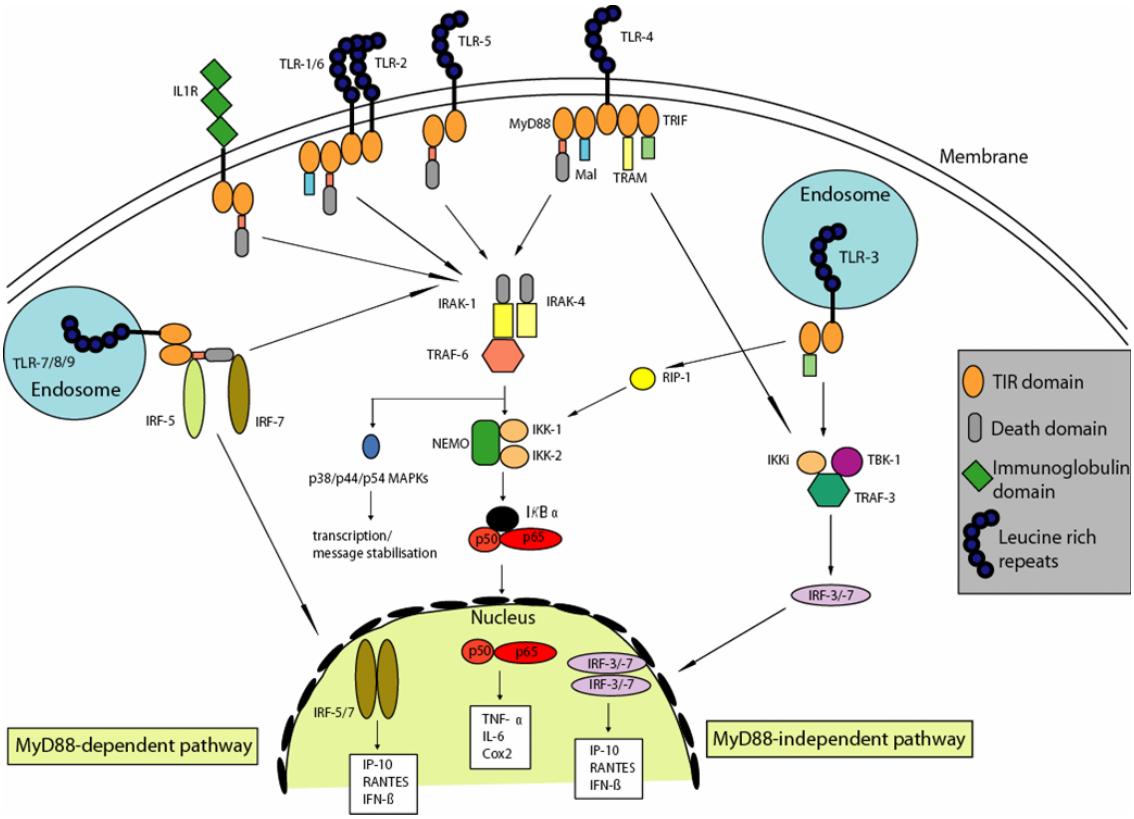


Figure 1.9: TLR signalling pathways. The IL-1R pathway is solely dependent on the adaptor molecule MyD88 and induces NF-κB through IRAK-4, IRAK-1 and TRAF-6. In general TLR signalling is induced following binding of the ligand and the recruitment of the adaptor molecules MyD88 (Myeloid differentiation primary response gene 88), Mal (MyD88 adaptor like protein), TRIF (TIR domain containing adaptor inducing interferon β) and TRAM (TRIF-related adaptor molecule) are recruited to the TIR domain of the TLR. MyD88-dependent pathway: IRAK (IL-1 receptor associated kinase)-4 and IRAK-1 are recruited to the Death Domain (DD) of MyD88, where they become phosphorylated and recruit TRAF (TNF receptor associated factor)-6. IRAK-1 and TRAF-6 dissociate from the receptor and activate the IKK complex, resulting in the translocation of NF-κB to the nucleus and the up regulation of pro-inflammatory genes. Beside the induction of NF-κB, the MyD88-dependent pathway also activates the mitogen activated protein kinases p38 and JNK (c-Jun-N-terminal kinase). This pathway is used by TLR1, -2, -4, -5, -6, -7 and -9. Mal, a second TIR-domain containing adaptor protein, is involved in the MyD88-dependent signalling pathway through TLR2 and TLR4 and acts as a bridging molecule for MyD88 to these receptors. MyD88-independent pathway: By contrast, TLR3 and TLR4 mediated activation of IRF (interferon regulating factor)-3 and the initiation of IFN (interferon)-β are observed in a MyD88-independent manner. The adaptor molecule TRIF is essential in this pathway. TRIF recruits and activates RIP-1, which is responsible for a late induction of NF-κB as well as TBK-1 and IKKi, which form a complex with TRAF-3 activating IRF-3 and IRF-7 and the induction of type I interferons. The fourth adaptor protein TRAM, which is only involved in TLR4 MyD88-independent signalling, serves as a bridge between TLR4 and TRIF.

Due to their structural relationship TLRs share certain signalling pathways with the IL-1R family (Akira and Takeda, 2004) (Figure 1.5). TLR and IL-1R signalling is initiated by ligand induced hetero- or homodimerisation of the receptors or association with accessory proteins (Wesche et al., 1997b). The signal is transduced by the intracellular TIR domain, present in TLRs as well as IL-1Rs, through the recruitment of TIR domain containing adaptor molecules (Wesche et al., 1997a). The TLRs use distinct combinations of these adaptors to turn on the common TLR/IL-1R pathway as well as pathways unique to TLRs, leading to the activation of transcription factors (Figure 1.9).

1.5.1 TLR adaptor proteins

IL-1Rs and all TLRs, with the exception of TLR3, share a common signalling pathway which depends on the adaptor molecule MyD88 (Myeloid differentiation primary response gene 88), the first TLR adaptor to be described (Adachi et al., 1998; Burns et al., 1998; Janssens and Beyaert, 2002; Wesche et al., 1997a). It has originally been identified as a protein induced during myeloid differentiation (Lord et al., 1990) but has since been shown to be recruited to IL-1Rs and most TLRs through its carboxyl-terminal TIR domain (Burns et al., 1998; Wesche et al., 1997a). In addition MyD88 contains an amino-terminal death domain (DD), which is responsible for the recruitment of downstream signalling mediators, including IRAK (IL-1R associated kinase) family members and TRAF6 (TNF receptor associated factor 6), to the receptor complex (Burns et al., 1998; Wesche et al., 1997a). Ultimately, this leads to the activation of MAPKs as well as NF-κB and the transcription of inflammatory mediators (Akira and Takeda, 2004).

The essential role of MyD88 in IL-1R/TLR signal transduction has been demonstrated in MyD88 deficient mice. In response to IL-1R and IL-18R stimulation, MyD88 deficient macrophages show a loss in NF-κB and MAPKs activation, as well as TNFα and IL-6 production (Adachi et al., 1998). This has also been observed for most TLRs, with the exception of TLR3 and TLR4 (Hayashi et al., 2001; Heil et al., 2003; Schnare et al., 2000; Takeuchi et al., 2000). TLR4 stimulation only led to a reduced production of pro-inflammatory cytokines and activation of NF-κB and MAPKs could still be observed with delayed kinetics. In contrast, MyD88 deficiency had no effect on TLR3 induced activation of NF-κB and MAPKs in macrophages. (Alexopoulou et al., 2001; Kawai et al., 1999). In addition, DCs from MyD88 deficient mice still showed functional maturation in response to LPS and dsRNA and retained their capacity to activate IRF3 (Alexopoulou et al., 2001; Doyle et al., 2002; Kaisho et al., 2001; Kawai et al., 2001). Together, these results suggested the presence of additional adaptor molecules essential for MyD88 independent signalling.

TIR domain homology searches led to the discovery of Mal (MyD88 adaptor like protein, also termed TIRAP (TIR domain containing adaptor protein)), which at the time was thought to be the missing link responsible for MyD88 independent signal transduction (Fitzgerald et al., 2001; Horng et al., 2001). Studies using HEK293 cells have shown that Mal is required for NF-κB activation following TLR4 stimulation; while IL-1R, TLR2 and TLR9 induced NF-κB activation was unaffected. However, Mal deficient mice, generated by two groups, showed a reduction in TLR4 and TLR2 induced NF-κB activation, suggesting species specific differences (Horng et al., 2002; Yamamoto et al., 2002). To date Mal is thought to function as a sorting adaptor for TLR2 and TLR4, recruiting MyD88 to the receptor complex in the plasma membrane, through its ability to interact with phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) (Kagan and Medzhitov, 2006). In addition, Mal has been shown to get phosphorylated following TLR2 or TLR4 stimulation, which leads to its interaction with SOCS1 and its subsequent degradation (Mansell et al., 2006). This makes Mal part of a negative feedback loop to restrict TLR2 and TLR4 induced signalling.

Three additional TLR adaptor molecules have been described, so far, through TIR domain homology searches: TRIF (TIR-domain-containing adapter-inducing interferon-β, also termed TICAM-1 (Toll-like receptor adaptor molecule 1)), TRAM (TRIF-related adaptor molecule, also termed TICAM2) and SARM (TIR domain-sterile alpha and HEAT/Armadillo motif) (O'Neill and Bowie, 2007). Stimulation of cells with dsRNA or LPS (TLR3- and TLR4-ligands respectively) results in the recruitment of TRIF, and in case of TLR4 also TRAM (Oshiumi et al., 2003a; Oshiumi et al., 2003b; Yamamoto et al., 2003). The dissociation of TRIF leads to the activation of IRF-3 and IRF-7 subsequently resulting in the expression of IFN inducible genes including IFN-β, IP10 (inducible protein 10) and RANTES (Regulated on activation, normal T expressed and secreted) (Akira and Takeda, 2004; Honda and Taniguchi, 2006; Kawai et al., 2001) (Figure 1.9). Moreover, TRIF recruitment has also been shown to be responsible for the MyD88 independent activation of NF-κB. However, the exact mechanism of NF-κB activation by TRIF is still unclear. Studies show that binding of RIP (receptor interacting protein)-1 to the RIHM (RIP interacting homology motif) domain of TRIF leads to the induction of NF-κB while Covert *et al.* suggest that an autocrine effect of TNFα, initially induced through IRF-3 is responsible for NF-κB activation (Covert et al., 2005; Cusson-Hermance et al., 2005).

TRAM is structurally related to Mal and has therefore been suggested to function as a sorting adaptor, recruiting TRIF to TLR4 (Kagan and Medzhitov, 2006; Oshiumi et al., 2003b). In this context, TRAM has been shown to be recruited to the plasma membrane by myristoylation (Rowe et al., 2006). Moreover, a recent study provides evidence that TRAM recruitment is subsequent to the endocytosis of the TLR4 complex (Kagan et al., 2008). Therefore, TRAM

provides a mechanism, which allows sequential activation of MyD88 dependent signalling while TLR4 is located in the plasma membrane, followed by TRIF dependent signalling after TLR4 internalisation (Kagan et al., 2008). In addition, studies using primary human synovial fibroblasts and HUVECs as well as murine embryonic fibroblasts demonstrate that TRAM is involved in TLR2/6 signal transduction (Sacre et al., 2007c 2859). Furthermore, one group could show a role for TRAM in IL-1R signalling (Bin et al., 2003). These results might indicate a broader and possibly a cell type specific function of TRAM.

SARM is the least investigated TLR adaptor molecule. So far, no activation function could be assigned to it. However, recent data describes SARM as an inhibitor of TRIF dependent signalling (Carty et al., 2006). SARM has been shown to interact with TRIF and expression of SARM in HEK293 cells led to the inhibition of TRIF dependent but not MyD88 dependent NF-κB activation (Carty et al., 2006).

1.5.2 Induction of NF-κB signalling pathways by TLRs

The transcription factor family NF-κB is involved in numerous cell processes including development, apoptosis and inflammation (Karin and Lin, 2002). The NF-κB pathway integrates signals from a wide range of stimuli, including cytokines, growth factors, UV-radiation and PAMPs, and in turn activates numerous cellular responses (Hayden and Ghosh, 2004). The NF-κB family consists of the five subunits c-Rel (also known as Rel), p65 (also known as RelA and NF-κB3), RelB, p50 (also known as NF-κB1) and p52 (also known as NF-κB2), which all contain the characteristic Rel homology domain (RHD). Three NF-κB family members, p65, RelB and c-Rel, contain carboxyl-terminal transcriptional activation domains (TAD), which are crucial for their ability to activate gene expression. The other two members, p50 and p52, are processed from large precursor proteins, p105 and p100 respectively. Because of the lack of a TAD, p50 and p52 are transcriptionally inactive on their own. The most common canonical NF-κB dimer consists of p65/p50, however, there are numerous other combinations, which can induce transcriptional activation (Table 1.4).

Table 1.4: Proposed role of NF-κB dimer

NF-κB dimers	Function
p65/p50	Classical heterodimer of the canonical NF-κB pathway, involved in expression of target genes such as IL-8, IL-6 TNFα and IκBα
p65/p65	No DNA binding activity
RelB/p50 or p52	Classical heterodimers of the non-canonical NF-κB pathway, results in RelB-mediated transcriptional activation
p50 or p52/p65 or c-Rel	p50 and p52 are derived from processing of p105 and p100, respectively. Form transcriptionally active timers with p65 or c-Rel
p50 and p52 homodimers	Are able to bind DNA but cannot induce transcriptional activation on their own. Can function by recruiting co-factors to the promoter of target genes or by repression the induction of their target genes, such as TNFα and IL-6.

In an inactive state, NF-κB dimers are retained in the cytosol through the interaction with IκB (Inhibitor κB) proteins (Hayden and Ghosh, 2004). In order for NF-κB dimers to be able to translocate to the nucleus IκBs need to be degraded, which is achieved by IκB phosphorylation through the IKK (IκB kinases) complex. NF-κB activation is further divided into a canonical- and a non-canonical pathway (Bonizzi and Karin, 2004). The canonical pathway is induced by inflammatory signals including TNFα, IL-1, as well as TLR ligands (Kolls and Linden, 2004; Martinon and Tschopp, 2005; O'Neill, 2006; Osborn et al., 1989) and leads to the expression of inflammatory mediators and the maturation of DCs (Kopp and Medzhitov, 1999). Strong inducers of the non-canonical NF-κB pathway are lymphotoxin (LT), CD40L and B lymphocyte stimulator (Blys). Activation of the alternative NF-κB pathway plays a major role in lymphoid organogenesis and humoral immunity by inducing B cell maturation (Hayden and Ghosh, 2004).

TLR stimulation ultimately results in the translocation of NF-κB transcription factors to the nucleus. This signalling pathway is initiated upon the recruitment of the serine-threonine kinases IRAK4 and IRAK1 (Cao et al., 1996a; Li et al., 2002; Li et al., 1999b; Suzuki et al., 2002) as well as the scaffolding protein TRAF6 (Cao et al., 1996b) to the receptor complex (Figure 1.9). IRAK4 is recruited via DD-DD interactions with MyD88 and facilitates the recruitment of IRAK1. Subsequently, IRAK1 becomes hyperphosphorylated, most likely by IRAK4 (Li et al., 2002; Lye et al., 2004; Qin et al., 2004). In mice, IRAK4 deficiency results in an ablation of cytokine production in response to IL-1 or LPS while IRAK1 deficiency reduces,

but not abolishes, cytokine production (Kanakaraj et al., 1998; Suzuki et al., 2002; Swantek et al., 2000; Thomas et al., 1999). Human patients with IRAK4 deficiency do not respond to IL-1 or IL-18 as well as ligands for TLR2, TLR3, TLR4, TLR5 or TLR9, indicating the crucial role of IRAK4 in IL-1R/TLR signalling in primary human cells (Picard et al., 2003).

Phosphorylated IRAK1 interacts with TRAF6 and dissociates as a complex from the receptor (Jiang et al., 2003). Several models have been proposed for TRAF6 dependent activation of the IKK complex (Hayden and Ghosh, 2004). TRAF6 has been shown to function as an ubiquitine ligase, which, upon oligomerisation, triggers non-classical ubiquitination. This process involves TAB2 (TGF-activated kinase 1 binding protein 2) and TAB3 and is catalysed by the ubiquitination proteins Ubc13 (ubiquitin-conjugating enzyme E2 13) and Uev1A (ubiquitin-conjugating enzyme E2 variant 1 isoform A) (Kishida et al., 2005; Wang et al., 2001). TAB2 and/or TAB3 bind the ubiquitin chains on TRAF6 and recruit the TAK1 (TGF-activated kinase 1) kinase complex (TAK1/TAB1/TAB2 or TAB3) (Kanayama et al., 2004). TAK1 is activated by additional ubiquitination through the TRAF6 complex and is subsequently able to phosphorylate IKKβ (Wang et al., 2001). It is suggested, that TAB2 is responsible for enabling the interaction with the IKK complex through its ability to associate with IKKα and NEMO (Kanayama et al., 2004; Kishida et al., 2005).

Alternative mechanisms bridging TRAF6 dependent IKK complex activation are suggested to involve ECSIT (Evolutionarily conserved signalling intermediate in Toll pathways) or MEKK3 (Mitogen-activated protein kinase kinase kinase 3) (Huang et al., 2004; Kopp et al., 1999; Xiao et al., 2003). Both have been shown to associate with TRAF6 and dominant negative constructs of these proteins have been shown to inhibit TRAF6 induced NF-κB activation. In addition the ability of MEKK3 to phosphorylate IKKβ and its necessity for IKK complex association with IκBα has been described (Schmidt et al., 2003). However, it is still unclear how these signals cooperate with the TAK1 kinase complex in mediating NF-κB activation.

1.5.2.1 The IKK complex

In order to allow the release of NF-κB dimers IκBs need to become phosphorylated by IKKs (Karin and Ben-Neriah, 2000). The IKK complex, inducing the canonical NF-κB pathway, consists of the regulatory subunit NEMO (NF-κB essential modulator) and the catalytic subunits IKKα and IKKβ (Rothwarf et al., 1998). While NEMO and IKKβ appear to be essential for NF-κB activation, cells lacking IKKα still show normal levels of NF-κB DNA binding activity (Chen et al., 2003a; Hu et al., 1999; Hu et al., 2001; Li et al., 1999a; Li et al., 1999c; Makris et al., 2000). Subsequently, IKKα kinase activity was found to be involved in the resolution of inflammation, induced by NF-κB activation, by accelerating the turn-over of NF-κB and its release from the gene promoter (Lawrence et al., 2005). In addition, IKKα was

shown to be an activator of the non-canonical NF-κB pathway (Dejardin et al., 2002; Ghosh and Karin, 2002; Senftleben et al., 2001). IKKα homo-dimer complexes induce the processing of p100 to p52 and consequently the nuclear translocation of p52/RelB hetero-dimers. The two non-canonical IKKs IKKi (also known as IKKε) and TBK-1 (TRAF family member-associated NF-κB activator binding kinase 1) have been shown to be involved in the induction of IRF-3 in response to the TLR4 ligand LPS and the TLR3 ligand dsRNA (Akira and Takeda, 2004; Honda and Taniguchi, 2006). Their role in the activation of NF-κB, however, is still not well understood.

1.5.2.2 IκB proteins

IκBα is the archetypical NF-κB inhibitor in an evolutionary conserved family consisting of IκBβ, IκBε, B cell CLL/lymphoma (Bcl)-3, IκBNS and IκBζ as well as IκBγ, the degradation product of p50 and p52 (Baldwin, 1996; Ghosh et al., 1998; Kuwata et al., 2006; Verma et al., 1995). With the exception of Bcl-3 and IκBζ, these proteins can inhibit the translocation of NF-κB dimers to the nucleus by interacting with RHD and thereby inactivating the nuclear localization signal (NLS). IκBβ resembles IκBα in its primary sequence as well as tertiary structure. However, while IκBα degradation leads to a rapid oscillatory NF-κB activation, degradation IκBβ results in prolonged NF-κB activation (Ghosh et al., 1998; Thompson et al., 1995; Tran et al., 1997). This prolonged activation appears to play a role in numerous pathological conditions including asthma and cystic fibrosis (Bitko and Barik, 1998; Blackwell et al., 2001; DeLuca et al., 1999; Hiscott et al., 2001; Palmer et al., 1997; Stecenko et al., 2001; Thompson et al., 1995). The oscillatory propensity in the NF-κB signalling system, caused by IκBα, is counteracted by a negative feedback mechanism mediated by IκBε, which is delayed and functions in anti-phase to IκBα (Kearns et al., 2006). This indicates that cells have the capacity to modulate NF-κB activity depending on cell type and stimuli. Beside the canonical inhibitors IκBα, IκBβ and IκBε, the IκB family members IκBNS, Bcl-3 and IκBζ seem to have a more multi-facetted role. IκBNS has been shown to be involved in the inhibition of NF-κB induced genes including IL-6 and IL-12p40 following TLR stimulation (Kuwata et al., 2006). Bcl-3 specifically interacts with the intrinsically inactive NF-κB1 and NF-κB2 homodimers in the nucleus and is not degraded following cellular stimulation (Bours et al., 1993; Fujita et al., 1993; Heissmeyer et al., 1999; Nolan et al., 1993). Depending on its phosphorylation it can either act as an inhibitor, dissociating NF-κB1 or NF-κB2 homodimers from DNA, or as a co-activator that is recruited to the gene promoter by NF-κB1 or NF-κB2 homodimers (Bours et al., 1993; Dechend et al., 1999; Franzoso et al., 1992; Fujita et al., 1993; Heissmeyer et al., 1999; Nolan et al., 1993). The more recently described IκBζ is highly homologous to Bcl-3 (Kitamura et al., 2000; Yamazaki et al., 2001). Its transcription is rapidly induced upon stimulation with TLR ligands and IL-1. Cells from IκBζ deficient mice show a severe

impairment of IL-6 production in response to TLR2-, TLR4-, TLR7/8- and TLR9 ligands as well as IL-1 but not in response to TNFα (Yamamoto et al., 2004). Similar results were obtained using NF-κB1 knock-out mice, which might indicate that IκBζ specifically interacts with the NF-κB1 subunit (Yamamoto et al., 2004).

1.5.3 Mitogen-activated protein kinases

TLR/IL-1R stimulation also results in the activation of the MAPK signalling pathways, which are crucial for a wide range of cellular processes including cell cycle regulation, differentiation and inflammation. MAPKs comprise four subfamilies: ERK1/2 (extracellular-signal-regulated kinases 1/2), ERK5, JNKs (c-Jun N-terminal kinase) and p38 MAPK (Ashwell, 2006). ERK1/2, JNK and p38 MAPK kinases are the most well characterised and are generally considered as serine/threonine kinases, although during autophosphorylation they can also transfer phosphates to tyrosine residues (Crews et al., 1991). MAPKs are activated by a wide variety of stimuli, but in general ERK1/2 are preferentially activated in response to mitogens while JNKs and p38 MAPK are more responsive to stress stimuli including cytokines and PAMPs (Ashwell, 2006; Roux and Blenis, 2004).

Activation of MAPKs requires dual phosphorylation on tyrosine and threonine residues within the activation loop of their kinase domain (Ashwell, 2006; Roux and Blenis, 2004). This is achieved by a cascade of numerous kinases, which for simplicity are termed in accordance with their order in the cascade. The first (the most membrane proximal) kinases to be activated by a stimulus are serine/threonine kinases known as MAPK kinase kinase (MAPKKK). The MAPKKKs phosphorylate and activate MAPKKs, which are dual specificity kinases and are able to activate MAPKs. Each MAPK has a different set of initiating MAPKKK and MAPKK, which results in signal specificity. Activated MAPKs are able to translocate to the nucleus where they phosphorylate and regulate numerous substrates including transcription factors and MAPK-activated protein kinases (MKs) (Handley et al., 2007a; Handley et al., 2007b; Yang et al., 2003) (Figure 1.10).

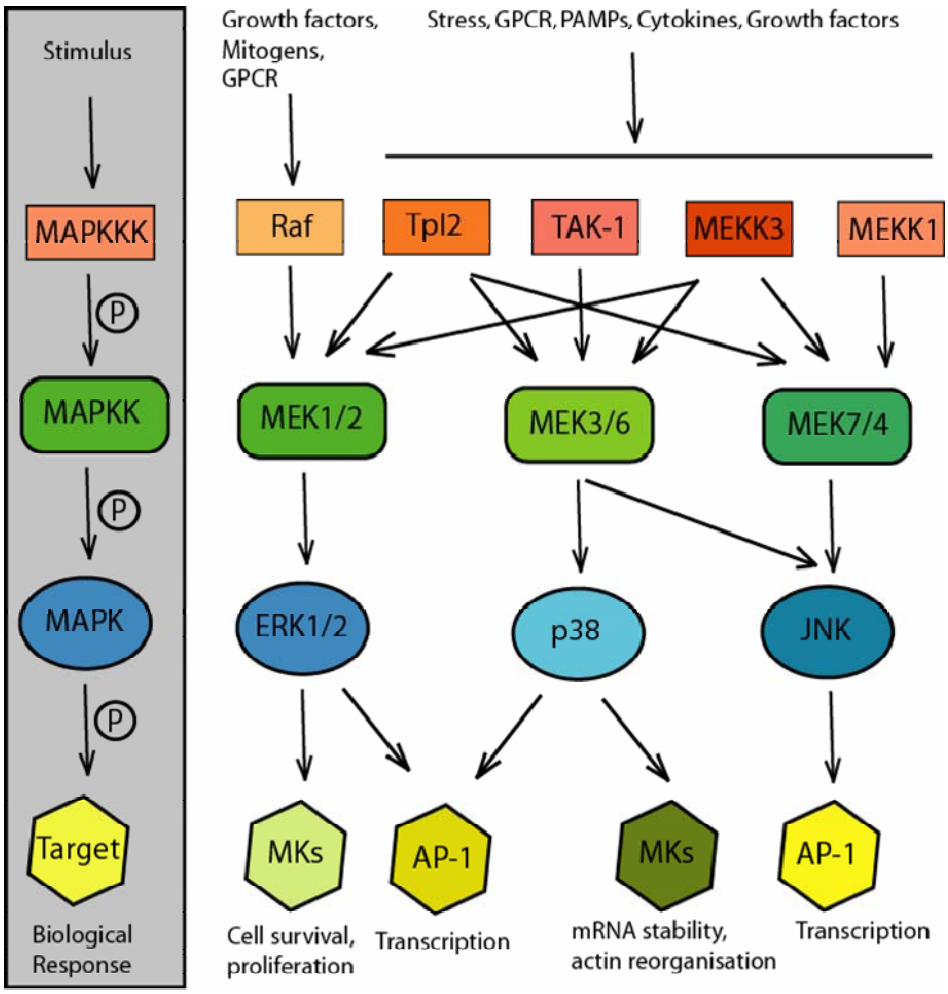


Figure 1.10: Selected members of the MAPK signalling cascades leading to the activation of AP-1 and MKs. The MAPKs are activated via a conserved three-tiered kinase cascade. The MAPKs, ERK1/2, JNK and p38 MAPK are activated by dual phosphorylation on Threonine and Tyrosine motifs caused by members of the MAPKK group of protein kinases. The MAPKKs are in turn activated by phosphorylation mediated by a group of MAPKKKs. The activated MAPKs are then able to phosphorylate target proteins such as the AP-1 subunits and the MKs. The regulation of AP-1 is also mediated through transcriptional induction of the AP-1 subunits. see text for abbreviations.

MAPKs regulate the activation of the ATF (Activating transcription factor) transcription factor family. This family comprises the subunits of AP-1 typically consisting of c-Jun and JunD heterodimers along with members of the fos family. Additional regulation is gained through the substrate specificity of MAPKs for different members of the AP-1 family (Yang et al., 2003).

ERK1/2 (p44/p42) is activated by the upstream kinases MEK1/2 and once phosphorylated is able to activate transcription factors such as Elk-1 and c-Fos (Kyriakis and Avruch, 2001; Roux and Blenis, 2004). JNK is activated by the kinases MEK4/6/7 and has a critical role in the regulation of AP-1 transcription activity through phosphorylation and activation of several AP-1 proteins such as c-Jun and ATF2. The p38 MAPK exists as four isoforms (α, β, γ, δ), from which α and β are the best characterised (Lee et al., 1999). p38α was originally identified as a

38 kDa protein, which is phosphorylated on tyrosine residues in macrophages stimulated with LPS (Han et al., 1994). Moreover, administration of the p38α and β inhibitor SB203580 has been shown to have beneficial effects in animal disease models such as CIA and endotoxin-induced septic shock (Lee et al., 1999). The p38 MAPK pathway is induced by MEK3/6 and controls inflammatory response genes at the transcriptional level through AP-1 and NF-κB regulation as well as at the post-transcriptional level by stabilising mRNA (Briata et al., 2005; Dean et al., 2004; Hitti et al., 2006; Ono and Han, 2000). Post-transcriptional mRNA stabilisation is achieved through the activation of the p38 MAPK substrate MK-2 (Clark et al., 2003). P38 MAPK and its substrate activate several proteins, which bind to adenosine- and uridine-rich elements (ARE), incorporated in the 3´ untranslated region (UTR) of mRNAs, and regulate their degradation. Through this mechanism, p38 is able to control the production of several proinflammatory mediators by reducing or prolonging protein expression.

1.5.3.1 MAPK activation by TLR signalling pathways

Although it has been known for a long time that TLR/IL-1R signalling results in the activation of MAPKs, the signalling mediators involved are not as well characterised as for the NF-κB pathway. The divergence of the TIR signalling pathway, leading to MAPK activation, is still under debate and there are numerous reports describing possible linking proteins.

To date the accepted pathway suggests a linear cascade, with the NF-κB and MAPK pathway bifurcating at the level of the TRAF6/TAK-1 complex (Figure 1.9). Beside being able to phosphorylate IKKβ, TAK-1 has also been shown to act as a MAPKKK by phosphorylating MEK6, which in turn stimulates JNK and p38 MAPK, leading to c-Jun and AP-1 activation (Wang et al., 2001). The RING-finger and zinc-finger domains of TRAF6 have been shown to be essential for MAPKs activation, although it is unclear if this is due to interactions with TAK-1 or other proteins such as ECSIT and MEKK3 (Kobayashi et al., 2001). ECSIT bound to TRAF6 activates MEKK1, which subsequently activates AP-1 (Kopp et al., 1999). The crucial role for MEKK3 in TLR/IL-1R induced JNK and p38 MAPK activation has been demonstrated in MEKK3 deficient mice (Huang et al., 2004). Furthermore, evidence from other studies have revealed the existence of alternative pathways diverging upstream of TAK-1 as IRAK-1 has been shown to be able to activate JNK (Li et al., 2001). More recently, ASK-1 (Apoptosis signal-regulating kinase 1) has been described to specifically induce p38 MAPK activation following LPS stimulation, which is dependent on reactive oxygen species, via association with TRAF6 (Matsuzawa et al., 2005). In addition, small GTPase Ras, activated by IL-1, associates with the IRAK-1/TRAF6/TAK-1 complex and induces phosphorylation of MEK3 and MEK6, the MAPKKs upstream of p38 MAPK (McDermott and O'Neill, 2002). Moreover, it has been demonstrated that mixed lineage kinases (MLKs) function as MAPKKKs in DCs and are

necessary for TLR3 and TLR4 induced JNK and p38 MAPK activation (Handley et al., 2007a; Handley et al., 2007b)

Evidence suggests a high degree of cross-talk between the pathways leading to the activation of NF-κB and MAPKs. Using macrophages from Tpl-2 (tumour progression locus-2) deficient mice, this MAPKKK has been shown to participate in LPS induced TNFα production (Dumitru et al., 2000). Tpl-2 associates with the IκB protein p105, the precursor of p50, in resting cells and is thereby inhibited. Upon LPS stimulation, p105 is proteolysed as a result of IKKβ activation (Heissmeyer et al., 1999). Tpl-2 is subsequently released from its inhibitor to phosphorylate MEK1, which leads to ERK1/2 activation (Beinke et al., 2004).

1.5.4 IRF signalling pathways

Initiation of TLR signalling also results in the expression of type I IFNs as well as IFN inducible genes such as IP10 and RANTES. The expression of these genes is regulated by the IRF transcription factor family. The mammalian IRF family comprises nine members: IRF1 to IRF9. Each IRF is characterised by a highly conserved DNA binding domain, which is located at the amino-terminus (Honda and Taniguchi, 2006). The DNA binding domain recognises IFN-stimulated response elements (ISREs) in the promoter of their target genes. IRFs have distinct roles in the development and function of immune cells, as has been shown by gene-disruption studies (Honda and Taniguchi, 2006). Four IRF family members have been implicated in the induction of type I IFN genes. IRF1 was the first IRF family member that has been described to activate expression of type I IFNs (Miyamoto et al., 1988). However, IRF1- as well as IRF5-deficient mouse embryonic fibroblasts (MEFs) are still able to induce type I IFNs in response to NDV, suggesting redundancy between IRF family members (Matsuyama et al., 1993; Takaoka et al., 2005). The structurally related IRF3 and IRF7, on the other hand, have gained much attention as essential regulators of type I IFN gene expression. IRF3 is constitutively expressed while IRF7 is expressed at low levels in most cells but becomes strongly induced in response to type I IFN mediated signalling (Honda and Taniguchi, 2006). IRF3 and IRF7 are activated by serine phosphorylation in their carboxyl-terminus upon, which they form either homodimers or heterodimers with each other and translocate to the nucleus (Lin et al., 1998; Sato et al., 1998; Yoneyama et al., 1998).

1.5.4.1 IRF activation by TRIF dependent TLR signalling pathways

TLR4 as well as TLR3 utilise the adaptor molecule TRIF, which is essential for the induction of type I IFNs following LPS or poly I:C stimulation, respectively (Akira and Takeda, 2004). The expression of IFNβ has been shown to be reduced in IRF3- or IRF7-deficient mice in response to poly I:C (Honda and Taniguchi, 2006). However, while IRF3 deficiency also leads

to an abrogated IFNβ response following LPS stimulation, IRF7 deficiency has almost no effect suggesting a predominant role for IRF3 in TLR4 induced IFN production (Hoebe et al., 2003; Honda et al., 2005b; Sakaguchi et al., 2003). Interestingly pre-treatment of IRF3 deficient DCs with IFNβ, which leads to the up-regulation of IRF7, restores IFNβ expression in response to LPS (Sakaguchi et al., 2003). This might suggest that IRF7 is able to participate in TLR4 signalling. In addition to IRF3 and IRF7, the induction of nitric oxide synthase (NOS)2 by macrophages in response to LPS has been shown to depend on IRF1 (Kamijo et al., 1994).

Following TLR3 or TLR4 stimulation, TIRF forms a complex with IKKi, TRAF3 and TBK-1, which subsequently leads to the phosphorylation of IRF3 and IRF7 by TBK-1 (Figure 1.9) (Hacker et al., 2006; Hemmi et al., 2004; Oganesyan et al., 2006; Perry et al., 2004; Sasai et al., 2005). TRAF6 has also been described to associate with the amino-terminal region of TRIF but studies using TRAF6 deficient mice indicate that it is dispensable for the TLR4 and TLR3 induced TRIF pathway (Gohda et al., 2004). In addition TRAF1 and TRAF4 have been shown to function as negative regulators of TRIF dependent IFNβ expression, however, the mechanisms are so far unclear (Su et al., 2006; Takeshita et al., 2005).

1.5.4.2 IRF activation by MyD88 dependent TLR signalling pathways

Plasmacytoid DCs are characterised by their ability to produce large amounts of type I IFNs in response to viral infections (McKenna et al., 2005). TLR7 and TLR9, which are localised in the endosome, are the TLRs used by pDCs to detect viruses and induce type I IFNs. Splenic pDCs from IRF7-deficient mice show a defect in type I IFN production following stimulation with the TLR7 or TLR9 ligands ssRNA or CpG-DNA respectively (Diebold et al., 2004; Heil et al., 2004; Klinman, 2004). Since TLR7 and TLR9 do not utilise TRIF, the pathways induced are distinct from TLR3 and TLR4. MyD88 has been shown to specifically interact with IRF7, but not IRF3, at the endosomal compartment through its DD (Honda et al., 2005a; Honda et al., 2004; Kawai et al., 2004). Furthermore, pDCs deficient in MyD88 or IRAK1 or IRAK4 or IKKα do exhibit a defect in IFNα expression following TLR9 stimulation (Honda et al., 2004; Hoshino et al., 2006; Uematsu et al., 2005). These data suggests, that TLR9 and TLR7 utilise a similar pathway to NF-κB activation leading to the phosphorylation of IRF7 in murine pDCs (Figure 1.9).

In addition to IRF7, IRF5 also seems to be utilised by the MyD88 dependent pathway of numerous TLRs. The induction of TNFα, IL-6 and IL12p40 by macrophages and myeloid DCs from IRF5-deficient mice is impaired in response to TLR4, TLR5, TLR7 and TLR9 stimulation (Schoenemeyer et al., 2005; Takaoka et al., 2005). Interaction between IRF5 and MyD88 as well as TRAF6 has been described but unlike IRF7, IRF5 binds the intermediary domain and part of the TIR domain of MyD88 (Negishi et al., 2005; Takaoka et al., 2005). However, the

function of IRF5 in the MyD88 pathway is far from understood and seems to be cell type specific, as B cells from IRF5 deficient mice do show normal pro-inflammatory cytokine production and NF-κB as well as MAPKs in response to TLR stimulation (Takaoka et al., 2005). IRF4 has also been shown to form complexes with MyD88 (Negishi et al., 2005). Similar to IRF5, IRF4 binds the central region of MyD88 and is suggested to compete with IRF5 for binding (Negishi et al., 2005). Indeed, peritoneal macrophages from IRF4 deficient mice show increased cytokine production induced by TLR2, TLR3, TLR4 and TLR9 stimulation (Negishi et al., 2005). Due to its restricted expression pattern, being highly expressed in B- and T cells, IRF4 might provide a cell type specific mechanism for the inhibition of MyD88 dependent gene induction (Lohoff and Mak, 2005).

Beside the induction of the TLR signalling pathways described above, emerging evidence suggest that numerous other signalling pathways are activated following TLR stimulation. These include the phosphoinositide 3-kinase (PI3K) pathway, which is generally thought to inhibit TLR induced pro-inflammatory cytokine expression (Hazeki et al., 2007; Ruse and Knaus, 2006), as well as the tyrosine kinase families Tec and Src, which become activated at the receptor proximal level and have been shown to take part in TLR induced cytokine production (Horwood et al., 2003; Palmer et al., 2008; Smolinska et al., 2008). However, since these pathways are not the subject of this study they will not be discussed in further detail.

1.5.5 Negative regulation of TLR signalling

As described in section 1.5 TLR signalling induces a strong inflammatory response in macrophages and DCs. While inflammation is important for clearing infections and tissue repair, excessive inflammation can lead to chronic inflammatory diseases (see section 1.4). In order to keep an inflammatory response in check the signalling pathways inducing it must be tightly regulated. Regulatory mechanisms, inhibiting TLR signalling, have been described at all levels of signal transduction (Figure 1.11) (Lang and Mansell, 2007; Liew et al., 2005).

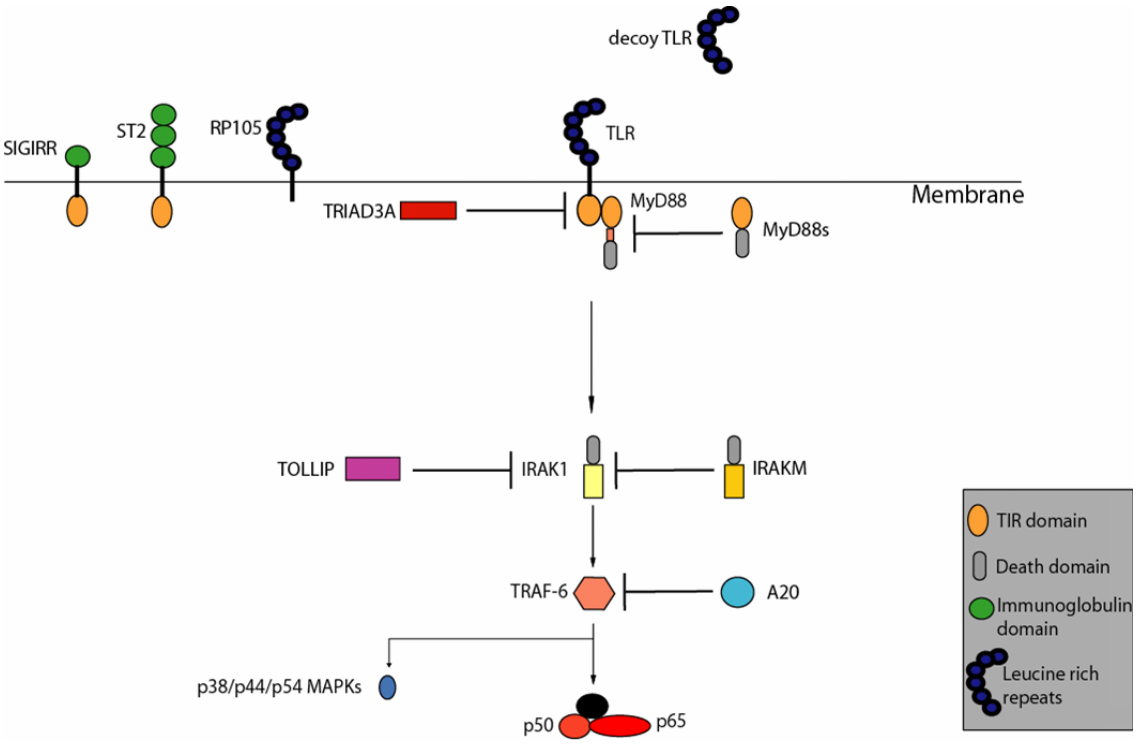


Figure 1.11: Negative regulation of the TLR signalling pathway. See text for details. Abbreviations: SIGIRR (single immunoglobulin IL-1R related), ST2 (suppressor of tumorigenicity 2), RP105 (radioprotective 105), MyD88 (myeloid differentiation primary response gene 88), TOLLIP (toll-interacting protein), IRAK (IL-1R associated kinase), TRAF (TNF-receptor associated factor).

1.5.5.1 Extracellular TLR inhibitors

Soluble decoy receptors, including IL-1RA, are well established negative regulators, which are able to prevent acute inflammatory responses by directly interacting with the ligand and/or receptor (Colotta et al., 1994). In case of TLR signalling soluble decoy receptors have been described for TLR2 and TLR4. Although, the TLR4 gene is present as one copy several mRNA products have been identified in mice and humans, which indicates the existence of TLR4 isoforms (Qureshi et al., 1999). The screening of a mouse macrophage cDNA library described a 122 amino acid long, soluble form of TLR4, which corresponds largely to the extracellular domain of TLR4 (Iwami et al., 2000). This soluble isoform has been shown to inhibit LPS induced activation of NF-κB and the production of TNFα in macrophages (Iwami et al., 2000). While the mechanism is still unclear the same study suggested that soluble TLR4 might block the interaction between TLR4 and co-receptors such as MD-2 and CD14. Similarly, soluble isoforms of TLR2 have been identified in human milk and plasma (LeBouder et al., 2003). It has been demonstrated, that soluble TLR2 directly binds the TLR2 ligand peptidoglycan, thereby inhibiting the production of IL-8 and TNFα from peptidoglycan stimulated monocytes (Iwaki et al., 2002).

1.5.5.2 Transmembrane TLR inhibitors

Negative regulators of TLR signalling located in the transmembrane are generally thought to function by either sequestering ligands and/or signalling transducers or by blocking access to the receptor (Figure 1.11).

Described transmembrane inhibitors include the IL-1R family members SIGIRR (Single immunoglobulin IL-1R related protein) and ST2 (suppressor of tumoigenicity 2) as well as the TLR4 homologue RP105 (radioprotective 105). Unlike SIGIRR and ST2, RP105 does not posses an intracellular TIR domain. Although, this suggests that RP105 is unable to signal, LPS induced phosphorylation of CD19 in murine B cells has been shown to depend on RP105 while being TLR4 independent (Yazawa et al., 2003). Contrary to this report, studies using HEK293 cells reported that RP105 interacts with TLR4 and MD-2 following LPS stimulation, thereby inhibiting TLR4 signalling through blocking access of the ligand to TLR4 (Divanovic et al., 2005b). In addition, RP105 has been shown to require the co-receptor MD-1 in order to be able to associate with the TLR4/MD-2 complex (Divanovic et al., 2005a).

The IL-1R family member ST2 exists in two isoforms, membrane bound ST2L and soluble sST2, which corresponds to the extracellular region of ST2L (Bergers et al., 1994; Kropf et al., 2003; Tominaga, 1989). ST2L has been originally described as an orphan receptor specifically expressed on Th2 but not Th1 or Th17 cells and has been shown to be essential for Th2 development and function (Coyle et al., 1999; Lohning et al., 1999; Lohning et al., 1998; Nakae et al., 2007; Townsend et al., 2000; Xu et al., 1998). In addition, ST2L has been described to specifically inhibit the MyD88 dependent TLR signalling pathway in murine macrophages and HEK293 cells by sequestering MyD88 and Mal through its TIR domain (Brint et al., 2004). Subsequently, its ligand, IL-33, has been discovered, which has the capability to drive Th2 associated cytokine expression *in vivo* as well as *in vitro* through initiating the recruitment of MyD88, IRAK1, IRAK4 and TRAF6 to ST2 (Schmitz et al., 2005). The function of sST2 is not yet defined. However, it has been shown to be up-regulated in inflammatory diseases and to down-regulate mRNA expression of TLR1 and TLR4 in LPS stimulated macrophages *in vitro* (Kumar et al., 1997; Kuroiwa et al., 2001; Oshikawa et al., 2001; Saccani et al., 1998; Sweet et al., 2001; Tajima et al., 2003; Weinberg et al., 2002). Furthermore, administration of sST2 to LPS stimulated macrophages suppresses the production of pro-inflammatory cytokines and attenuates septic shock (Sweet et al., 2001). Interestingly, sST2 also shows significant efficacy in reducing CIA pathology, which has been hypothesised to be due to blocking macrophage activation by binding to a so far unidentified receptor (Leung et al., 2004). However, contrary to that report, administration of sST2 in a murine asthma model reduced the production of IL-4,

IL-5 and IL-13 by IL-33 stimulated splenocytes (Hayakawa et al., 2007), suggesting that soluble ST2 binds IL-33 and/or ST2L, thereby acting as an antagonist.

Similar to ST2, SIGIRR (also known as Toll/IL-1R8 (TIR8)) is a member of the IL-1R family. Unlike other IL-1R family member it consists of a single Ig like extracellular domain and an uncharacteristic long cytoplasmic tail, which includes a TIR domain. In addition, SIGIRR has been shown to have a highly specific expression pattern, being expressed in epithelial cells and immature DCs but not in macrophages (Polentarutti et al., 2003). To date no activation function of SIGIRR has been described and SIGIRR deficient mice display increased inflammation in the DSS-induced colitis model and a higher susceptibility to septic shock, indicating an inhibitory function of SIGIRR on the immune system (Garlanda et al., 2004; Wald et al., 2003; Xiao et al., 2007). Further *in vitro* studies confirmed a role for SIGIRR in regulating TLR/IL-1R signalling. BMDCs from SIGIRR deficient mice display enhanced cytokine production in response to LPS or CpG-DNA but not poly I:C, indicating a MyD88 specific function (Garlanda et al., 2004). Furthermore, over-expression of SIGIRR in Jurkat cells stimulated with IL-1 or IL-18 led to the inhibition of NF-κB (Wald et al., 2003). Its mode of action has been suggested to be as a blocking receptor, as it interacted with IL-1R, TLR4, MyD88, IRAK1 and TRAF6 in HEK293 cells following IL-1 or LPS stimulation, thereby inhibiting IL-1R and TLR4 signalling (Qin et al., 2005). Furthermore, SIGIRR has been shown to exhibit cell type specific functions as it is upregulated by resident intrarenal myeloid cells but down regulated by tubule epithelial cells in response to LPS (Lech et al., 2007). While no general ligand for SIGIRR has been described yet, there is evidence emerging for SIGIRR acting as a signal transducing receptor. Costelloe and colleagues described that IL-1F5 induced IL-4 production in the brain is dependent on SIGIRR, however, so far this dependency of IL-1F5 on SIGIRR has not been observed in other systems and needs to be further investigated (Costelloe et al., 2008). Indications for an involvement of SIGIRR in human diseases come from expression studies, which demonstrated altered expression patterns of SIGIRR mRNA. SIGIRR has been shown to display reduced expression in psoriatic arthritis patients compared to rheumatoid arthritis (RA) (Batliwalla et al., 2005). Furthermore, reduced expression of SIGIRR has been demonstrated in children with asymptomatic bacteriuria while increased expression in monocytes from septic patients has been observed (Adib-Conquy et al., 2006; Ragnarsdottir et al., 2007). However, to date no association of SIGIRR polymorphisms with diseases could be identified (Nakashima et al., 2006).

1.5.5.3 Cytoplasmic TLR inhibitors

Intracellular inhibitors of TLR signalling target all levels of signal transduction. Therefore, the specificity of an inhibitor increases the more receptor proximal it functions. Numerous intracellular TLR inhibitors are described, which include highly specific ones such as MyD88s and SARM as well as more general inhibitors such as A20 (Lang and Mansell, 2007; Liew et al., 2005). In general, these TLR inhibitors function by inducing the degradation of their target, e.g. TRIAD3A, sequestering their target, e.g. IRAK-M, or blocking access for essential signal transducers, e.g. MyD88s.

MyD88s is a short splice version of MyD88, which lacks the intermediate domain between TIR domain and DD (Janssens et al., 2002; Janssens et al., 2003). Over-expression of MyD88s leads to an impairment of NF-κB activation in LPS- but not TNFα-stimulated monocytes (Janssens et al., 2002). Furthermore, MyD88s has been shown to be up-regulated in response to LPS and to form heterodimers with MyD88 (Burns et al., 2003). These heterodimers are preferentially recruited to the TLR instead of MyD88 homodimers, which leads to an ablation of IRAK4 recruitment and subsequently to an inhibition of IRAK1 phosphorylation (Janssens et al., 2003).

SARM is the most recently identified TLR adaptor protein but until recently its function has been elusive. However, a report by Carty and colleagues provide evidence for an inhibitory role of SARM, which is specific for TRIF dependent TLR signalling (Carty et al., 2006). Over-expression studies as well as siRNA knock down of SARM in HEK293 cells and monocytes describe a specific effect on TLR3 and TLR4 induced NF-κB activation and cytokine production. Furthermore, LPS treatment of monocytes induces the expression of SARM, suggesting a negative feedback loop of TRIF dependent TLR signalling by SARM (Carty et al., 2006).

IRAK-M is a member of the IRAK family; however, unlike other family members IRAK-M possesses no phosphorylation capability (Janssens and Beyaert, 2003). The expression of IRAK-M is highly restricted, being specifically expressed in monocytes and macrophages, indicating a specific inhibitory function (Wesche et al., 1999). IRAK-M deficient mice show elevated inflammatory responses to bacterial ligands (Kobayashi et al., 2002). Furthermore, *in vitro* studies have shown that IRAK-M prevents the dissociation of IRAK-4/IRAK-1 complexes from the TLR, thereby preventing further downstream signalling (Kobayashi et al., 2002).

Toll-interacting protein (Tollip) interacts with the IL-1R accessory protein and has been described to be involved in recruiting IRAKs to the TLR complex (Burns et al., 2000). Tollip exists as three isoforms from, which Tollip-1 has been shown to interact with TLR2 and TLR4

(Zhang and Ghosh, 2002). Over-expression of Tollip-1 in HEK293 cells leads to the inhibition of TLR2 and TLR4 induced NF-κB activation (Zhang and Ghosh, 2002). Interaction of Tollip with IRAK-1 results in increased Tollip phosphorylation and the release of Tollip from the complex. Consequently, IRAK-1 becomes ubiquitinated and degraded, thereby terminating signal transduction (Zhang and Ghosh, 2002).

TRIAD3A is a zinc RING finger protein that acts as an E3 ubiquitin protein ligase (Chuang and Ulevitch, 2004). Using a yeast-two-hybrid system, TRIAD3A has been identified to interact with TIR domain containing proteins. Over-expression of TRIAD3A leads to the ubiquitination of TLR4 and TLR9, but not TLR2 or TLR3, and subsequent decreased signal transduction (Chuang and Ulevitch, 2004). Supporting these observations are siRNA knock down studies of TRIAD3A, which leads to an increase in TLR4 and TLR9 but not TLR2 signalling (Chuang and Ulevitch, 2004). In addition, evidence suggests that TRIAD3A targets additional TIR domain containing molecules for degradation, including RIP-1 and Mal, thereby modulating immune responses (Fearns et al., 2006).

The SOCS family of proteins consists of eight members, which are part of a negative feedback regulation in response to cytokine signalling (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Recent data suggests that SOCS-1 is involved in TLR signalling regulation as SOCS-1 has been shown to associate with phosphorylated Mal thereby targeting it for ubiquitination and subsequent degradation (Mansell et al., 2006). Since Mal is utilised by TLR2 and TLR4, it has been demonstrated that the removal of SOCS-1 leads to increased NF-κB activation and IL-6 production induced by these TLRs in macrophages (Mansell et al., 2006).

A20 is a zinc RING finger domain protein whose expression is rapidly increased in response to LPS but also TNFα, indicating a general role in regulating inflammatory responses (Krikos et al., 1992; Opipari et al., 1990). Furthermore, A20 was identified to inhibit the MyD88 dependent as well as the TRIF dependent TLR signalling pathways as A20 deficient mice show increased production of pro-inflammatory cytokines in response to TLR2, TLR3 and TLR9 stimulation (Boone et al., 2004). Regulation of TLR signalling by A20 has been shown to be the result of the de-ubiqutination function of A20, which leads to the cleavage of polyubiquitin chains on TRAF6, rendering it inactive (Boone et al., 2004).

Defects in the regulatory mechanisms described above can lead to excessive inflammation and tissue destruction and ultimately to the initiation of inflammatory diseases.

1.5.5.4 Pathogenic TLR inhibitors

Interestingly, the TLR pathways are also targeted by several viruses and bacteria in order to suppress host immunity. The NS3 protein from hepatitis C virus (HCV) and the N1L proteins from vaccinia virus (VV) block the antiviral response by interacting with TBK-1, thereby inhibiting IRF3 activation (DiPerna et al., 2004; Otsuka et al., 2005). Moreover, the N1L protein and the proteins A46R and A52R from VV down-regulate IL-1/TLR induced activation of NF-κB (Bowie et al., 2000; Stack et al., 2005). TIR domain containing proteins (Tcp) have been identified in virulent strains of *Escherichia coli* and *Brucella melitensis* (Bowie et al., 2000; Harte et al., 2003). These Tcps have been shown to inhibit TLR signaling through binding MyD88, thereby sequestering MyD88 from the TLR (Cirl et al., 2008). The fact that TLR signaling pathways are controlled endogenously, as well as being targeted by pathogens, further highlights their importance in the initiation of the immune response.

1.6 Aims

Extensive research into the involvement of TLRs in the immune response has indicated that they might play a role in the initiation and/or propagation of chronic inflammatory diseases such as RA. Although the TLR signalling pathways have been widely investigated, the majority of these studies have been performed in cells from gene deficient mice or inhibition experiments in transformed cell lines that are genetically abnormal. However, results obtained in our laboratory before this study suggested differences between these systems and primary human cells. Andreakos and colleagues could show that expression of dominant negative MyD88 inhibits TLR4 signalling in HUVECs but not in primary human macrophages (Andreakos et al., 2004). In order to better understand the roles TLRs play in human immune regulation and disease processes, I further investigated the function of MyD88 in primary human DCs. As a consequence of those results, the role and function of the TLR inhibitor SIGIRR was further examined in primary human cells as well as disease models, such as CIA.

The specific objectives of this project were:

- To determine the function of the TLR adaptor molecules in monocyte derived DCs and macrophages by using adenoviral gene transfer of wild-type as well as dominant negative constructs of those molecules.

- As the project evolved, to characterise the role of SIGIRR as an inhibitor of TLR signalling in monocyte derived DCs and macrophages using adenoviral gene transfer as well as siRNA knock down techniques.

- To determine the role and function of SIGIRR in RA disease models including human RA synovial membranes and CIA and to investigate SIGIRR as possible target for RA treatment. In order to achieve this, adenoviral-gene transfer techniques as well as SIGIRR deficient mice have been used.

The characterisation of TLR signalling pathways and specific regulatory components in human cells as well as disease models is crucial in order to establish their potential as targets to treat or prevent inflammatory diseases such as RA.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

All general laboratory reagents and chemicals were purchased from Sigma-Aldrich, Dorset, UK, or BDH Chemicals, Dorset, UK, unless otherwise stated.

2.1.1 Cytokines

Human recombinant cytokines used for cell culture and as concentration standards are listed in Table 2.1.

Table 2.1: Human/murine Recombinant Cytokines

Cytokine	Concentrations	Use	Source
GM-CSF*	50 ng/ml	Tissue culture	Behringerwerke AG, Marburg, Germany
M-CSF*	100 ng/ml	Tissue culture	Genetics Institute Inc., MA, USA
IL-4*	10 ng/ml	Tissue culture	Novartis Pharma AG, Basel, Switzerland
TNFα	10,000-13.71 pg/ml	ELISA/Tissue culture	BD Biosciences, Oxford, UK
IL-6	10,000-13.71pg/ml	ELISA	BD Biosciences, Oxford, UK
IL-8	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
IL-10	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
IP10	10,000-13.71 pg/ml	ELISA	R&D, Minneapolis, USA
IL-1α	20 ng/ml	Tissue culture	R&D, Minneapolis, USA
IL-1β	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
GM-CSF**	20 ng/ml	Tissue culture	PeproTech, London, UK
IL-1α**	20 ng/ml	Tissue culture	PeproTech, London, UK
IL-33**	20 ng/ml	Tissue culture	PeproTech, London, UK
TNFα**	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
IL-10**	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
IL-5**	10,000-13.71 pg/ml	ELISA	R&D, Minneapolis, USA
IL-17**	10,000-13.71 pg/ml	ELISA	BD Biosciences, Oxford, UK
IFNγ**	10,000-13.71 pg/ml	ELISA	PeproTech, London, UK

* gifted reagent, ** murine

2.1.2 Antibodies

Capture and detecting antibodies used for enzyme linked immunosorbent sandwich assay (ELISA) are listed in Table 2.2. Primary antibodies used for western blotting (WB), flow cytometry and immunoprecipitation (IP) are listed in Table 2.3. Secondary antibodies used for WB are listed in Table 2.4. The concentrations/titres for the antibodies used were in accordance with the company's recommendation. Preliminary experiments were performed to determine the optimal titre if data was unavailable.

Table 2.2: ELISA antibodies

Specificity	Concentration	Antibody	Source
TNF	4 μg/ml	Coat	BD Biosciences, Oxford, UK
	0.5 μg/ml	Detect	BD Biosciences, Oxford, UK
IL-6	1 μg/ml	Coat	BD Biosciences, Oxford, UK
	0.5 μg/ml	Detect	BD Biosciences, Oxford, UK
IL-8	2 μg/ml	Coat	BD Biosciences, Oxford, UK
	0.5 μg/ml	Detect	BD Biosciences, Oxford, UK
IL-10	1 μg/ml	Coat	BD Biosciences, Oxford, UK
	1 μg/ml	Detect	BD Biosciences, Oxford, UK
IP10	4 μg/ml	Coat	R&D, Minneapolis, USA
	100 ng/ml	Detect	R&D, Minneapolis, USA
IL-1β	2 μg/ml	Coat	BD Biosciences, Oxford, UK
	0.2 μg/ml	Detect	BD Biosciences, Oxford, UK
IL-10*	4 μg/ml	Coat	BD Bioscience, Oxford, UK
	0.25 μg/ml	Detect	BD Bioscience, Oxford, UK
TNFα*	1 μg/ml	Coat	BD Bioscience, Oxford, UK
	0.25 μg/ml	Detect	BD Bioscience, Oxford, UK
IL-5*	2 μg/ml	Coat	BD Bioscience, Oxford, UK
	1 μg/ml	Detect	BD Bioscience, Oxford, UK
IL-17*	1 μg/ml	Coat	BD Bioscience, Oxford, UK
	1 μg/ml	Detect	BD Bioscience, Oxford, UK
IFNγ*	4 μg/ml	Coat	BD Bioscience, Oxford, UK
	1 μg/ml	Detect	BD Bioscience, Oxford, UK
Streptavidin-HRP	1:400	n.a.	R&D, Minneapolis, USA

* murine

Table 2.3: Western blotting, flow cytometry and immunoprecipitation antibodies

Specificity (human)	Species	Use	Concentration	Source
SIGIRR	Goat	flow cytometry	1:1000	R&D, Minneapolis, USA
TLR4	Mouse	flow cytometry	1:1000	Imgenex, San Diego, USA
TRAM	Rabbit	WB	1:1000	Made in house
MyD88	Rabbit	WB	1:1000	Abcam Ltd, Cambridge, UK
α-Tubulin	Mouse	WB	1:10,000	Sigma, Poole, Dorset, UK
Flag	Mouse	WB/IP	1:1000	Sigma, Poole, Dorset, UK
HA	Mouse	WB/IP	1:2000/5μg/ml	Covance, California, USA
IRF3	Mouse	WB	1:2000	Santa Cruz Biotechnology, Santa Cruz, CA USA
phospho IκBα	Mouse	WB	1:1000	New England Biolabs, Ipswich, MA, USA
phospho JNK	Rabbit	WB	1:1000	New England Biolabs, Ipswich, MA, USA
phospho p38 (Thr180/Tyr182)	Rabbit	WB	1:1000	Cell Signaling Technology, Beverley, MA, USA
total p38 MAPK	Rabbit	WB	1:2000	Prof. J. Saklatvala, Imperial College London, UK
Vimentin-Biotinylated	Mouse	flow cytometry	1:1000	Neo Markers, CA, USA
CD3-FITC	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
CD3-PECy5	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
CD14-PE	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
CD14-FITC	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
CD86-PE	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
IgG1-PE	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
IgG2a-PE	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
IgG1-FITC	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
IgG2a-FITC	Mouse	flow cytometry	1:1000	BD Bioscience, Oxford, UK
IgG-Biotinylated	Goat	flow cytometry	1:1000	Abcam Ltd, Cambridge, UK

* gifted reagent,

Table 2.4: Western blotting secondary antibodies

Specificity	Species	Dilution	Source
Anti mouse IgG F $(ab)_2$ HRP conjugate	Sheep	1:3000	Amersham Biosciences, Little Chalfont, UK
Anti goat IgG HRP conjugate	Rabbit	1:3000	DAKO, Glostrup, Denmark
Anti rabbit IgG F $(ab)_2$ HRP conjugate	Donkey	1:3000	Amersham Biosciences, Little Chalfont, UK

2.1.3 TLR ligands

Toll-like receptor ligands used for cell stimulation are listed in Table 2.5.

Table 2.5: TLR ligands

Receptor(s)	Ligand	Description	Source
TLR 2/6	Malp-2	Macrophage-activating lipopeptide-2	Alexis, Nottingham, UK
TLR3	Poly I:C	Synthetic analogue of double stranded RNA	Amersham Biosciences, Little Chalfont, UK
TLR 4	LPS	Lipopolysaccharide from *Escherichia coli*	Alexis, Nottingham, UK
TLR 5	Flagellin	From *S. typhimurium*	Alexis, Nottingham, UK
TLR7/8	R848	Small antiviral molecules	Invivogen, San Diego, CA, USA

Abbreviations: Lipopolysaccharide (LPS), Macrophage-activating lipopeptide-2 (MALP-2), Pam_3Cys-Ser-$(Lys)_4$. trihydrochloride (Pam_3Cys), polyinosinic- polycytidylic acid (poly I:C)

2.1.4 Inhibitors

Enzyme inhibitors used in cell lysis buffer are listed in Table 2.6. RNA oligonucleotides for RNA interference (RNAi) used in this project are listed in.

Table 2.6: Enzyme inhibitors

Inhibitor	Cellular Target	Source
Protease Inhibitor Cocktail		Sigma, Poole, Dorset, UK
AEBSF (104 mM)	Serine proteases	
Aprotinin (80 μM)	Serine proteases	
Leupeptin (2.1 mM)	Serine and cysteine proteases	
Bestatin (3.6 mM)	Aminopeptidases	
Pepstatin A (1.5 mM)	Acid proteases	
E-64 (1.4 mM)	Cysteine proteases	
Sodium Fluoride	Protein phosphatases	Sigma, Poole, Dorset, UK
Sodium Orthovanadate	Protein phosphatases	Sigma, Poole, Dorset, UK

Table 2.7: RNAi oligonucleotides

Target	Type	Catalogue Number	Source
Non targeting	siControl™	D-001206-13	Dharmacon, Illinois, USA
Human SIGIRR	SMARTpool	M-008082-00	Dharmacon, Illinois, USA

2.1.5 Molecular biology reagents

One-Step RT-PCR Taqman® reagents and fluorescent probes were purchased from Applied Biosystems, California, USA and are listed in Table 2.10. Enzymes and reagents used in virus production are described in the Table 2.8 and Table 2.9. The nuclear acid isolation and purification kits were obtained from Qiagen Ltd., Crawley, West Sussex, UK.

Table 2.8: Restriction enzymes and DNA modifying enzymes

Enzymes	Function	Buffer	Source
EcoR I/ *Not* I	Restriction enzymes	Provided buffer	New England Biolabs, MA, USA
Sma I	Restriction enzymes	NEB4	New England Biolabs, MA, USA
Pac I	Restriction enzyme	Provided buffer	Q-biogene, Cambridge, UK
T4 DNA ligase	DNA Ligase	Provided buffer	New England Biolabs, MA, USA
Polishing kit	PFU polymerase	Provided buffer	Stratagene, TX, USA
Gateway LR clonase enzyme mix	homologous recombination	Provided buffer	Invitrogen, Paisley, UK
shrimp alkaline phosphatase	phosphatase	Provided buffer	Promega, Southampton, UK

Abbreviations: New England Biolabs (NEB)

Table 2.9: Molecular biology reagents and competent bacteria

Reagents	Use	Source
Agarose electrophoresis grade	1% (w/v)	Invitrogen, Paisley, UK
Antibiotics:		
- Ampicillin	100 mg /ml	Sigma, Poole, UK
- Kanamycin	30 mg/ml	Invitrogen, Paisley, UK
- Chloramphenicol	34 mg/ml	Sigma, Poole, UK
Competent bacteria:		
- *E. coli* strain XL-I Blue	n.a.	Stratagene, Cambridge, UK
- *E. coli* strain TOP10	n.a.	Invitrogen, Paisley, UK
SYBR Green II	1:10.000	Molecular probes, Eugene, Oregon, USA
EZ Load 100bp, 500bp or 1 kb molecular ruler	8 μl/ gel	Bio-Rad Laboratories, Hercules, CA, USA
Ethidium bromide	0.1 μg/ml	Sigma, Poole, UK
LB Agar	32 g/ litre	Invitrogen, Paisley, UK
LB Broth base	20 g/ litre	Invitrogen, Paisley, UK

Abbreviations: not applicable (n.a.).

Kits were obtained from Qiagen Ltd., Hilden, Germany.

Table 2.10: Primers

Specificity	Sequence (5'-3')	Use
GAPDH forward	n.a.-proprietary information by company	Taqman PCR Applied Biosystems, CA, USA
GAPDH reverse	n.a.-proprietary information by company	Taqman PCR Applied Biosystems, CA, USA
SIGIRR forward	n.a.-proprietary information by company	Taqman PCR Applied Biosystems, CA, USA
SIGIRR reverse	n.a.-proprietary information by company	Taqman PCR Applied Biosystems, CA, USA
T7 forward	CATTATGCTGAGTGATATCCCG	Sequencing
T3 reverse	TTAATTGGGAGTGATTTCCC	Sequencing

Abbreviations: not applicable (n.a.).

2.1.6 Reagents used for viral purification and titre determination

Seaplaque GTG-agarose and 2x MEM were obtained from Sigma and Gibco, Invitrogen, California, USA, respectively. The polyallomer ultracentrifuge tubes were purchased from Sorvall, Connecticut, USA, and the PD10 columns from Amersham Biosciences, UK. Adeno-X™ Rapid titre kit was obtained from BD Biosciences, New Jersey, USA.

2.1.7 Tissue culture reagents and cell lines

Roswell Park Memorial Institute 1640 medium (RPMI) containing 25 mM L-glutamine, Dulbecco's modified Eagle's Medium (DMEM) with Glucose and L-Glutamine, Hank's Balanced Salt Solution (HBSS), Penicillin/Streptomycin (P/S), and Trypsin/EDTA were obtained as sterile solutions from Cambrex, Nottingham, UK. Heat inactivated (HI) foetal calf serum (FCS) was purchased from Biowest, East Sussex, UK. Cell dissociation solution was purchased from Sigma. MTT (3-[4,5-Dimethylthiazol-2yl]-2,5-diphyltetrazolium bromide3-[4,5-Dimethylthiazol-2yl]-2,5-diphyltetrazolium bromide) (for use in cell viability assays) were obtained from Sigma. Lymphocyte®-H Solution was purchased from Cedarlane Laboratories Limited, Ontario, Canada.

Cell culture was performed in Falcon™ plastics obtained from BD Biosciences, Oxford, UK. Cell lines used in this project are listed in Table 2.11.

Table 2.11: Cell lines

Cell Line	Description	Source
AD-293	Human embryonic kidney (HEK293)-derived AD-293 cells producing the adenovirus E1 gene *in trans*, allowing production of infectious virus particles from ΔE1	Stratagene, California, USA
HSF	Human foreskin fibroblast	Prof J. Saklatvala Imperial College London, UK

2.1.8 Transfection reagents

Monocytes were transfected with RNAi oligonucleotides using the Human Monocyte Nucleofector® Kit (Amaxa Biosystems, Cologne, Germany). All transfections were performed according to the manufacturer's instructions.

2.1.9 Biochemical reagents

All other reagents used for western blotting, immunoprecipitation, flow cytometry, ELISA, and luciferase reporter assays are listed in Table 2.12.

Table 2.12: Biochemical reagents

Reagent	Source
Full range Rainbow molecular weight marker RNP800	Amersham Biosciences, Little Chalfont, UK
Hyperfilm™	Amersham Biosciences, Little Chalfont, UK
PVDF transfer membrane	Perkin Elmer Life Sciences Inc., Massachusetts, USA
Midi-gel	Bio-Rad, Hercules, CA, USA
Streptavidin PE conjugated	BD Bioscience, Oxford, UK
Saponin	Sigma, Poole, UK
ECL™ Western blotting detection reagent	Amersham Biosciences, Little Chalfont, UK
BCA™ Protein assay kit	Pierce, Illinois, USA
TMB (3,3',5,5'-tetramethylbenzinide) peroxidase substrate and peroxidase substrate solution	KPL Inc., Maryland, USA
Re-Blot Western Blot recycling kit (acid strip buffer)	Chemicon International Inc, California, USA
Maxisorb™ ELISA plates	Nunc A/S, Roskilde, Denmark
Bright-Glo™ Luciferase Assay System	Promega, Southampton, UK
γ-^{32}P-ATP	GE Healthcare, BioSciences AB, Upsalla, Sweden
Protein G Sepharose™	GE Healthcare, BioSciences AB, Upsalla, Sweden

2.1.10 Solutions

General Buffers

Phosphate buffered saline (PBS)	4.3 mM Na_2HPO_4 1.4 mM KH_2CO_3 1.4 mM KCL 137 mM NaCl pH 7.2
Tris buffered saline (TBS)	20 mM Tris/Base 137 mM NaCl pH 8.0

Protein Extraction Buffers

Triton lysis buffer	1% (v/v) Triton-X 100 10 mM Tris/Base pH 7.6 150 mM NaCl 1 mM EDTA 100 μM Na_2VO_3* 5 mM NaF* 1:100 protein inhibitor cocktail* (Table 2.7.) *added freshly
Sodium orthovanadate stock solution	100 mM Na_2OV_3 0.0001% (v/v) H_2O_2 pH 10.0 Boiled for 60-90 min Stored at 4°C

SDS-PAGE and Western Blotting Buffers

Running buffer	25 mM Tris/Base 192 mM Glycine 0.1% (w/v) SDS
5x protein gel sample buffer (GSB)	250 mM Tris/HCl pH 6.8 10% (w/v) SDS 50% (v/v) glycerol 12.5% (v/v) β-mercaptoethanol *(Add Tris/HCl, SDS & H_2O and heat to 50°C. Add glycerol, β-mercaptoethanol and bromophenol blue)*

Transfer buffer	25 mM Tris/Base 192 mM glycine 20% (v/v) methanol
Acid stripping buffer	Purchased from Chemicon (Table 1.12)
Washing buffer (TBS/T)	1xTBS 0.1% (v/v) Tween 20
Blocking buffer (milk)	1x TBS/T 5% (w/v) skimmed milk
Blocking buffer (BSA)	1x TBS/T 5% (w/v) BSA

ELISA Buffers

ELISA buffer	1x PBS 0.5% (w/v) BSA
ELISA wash buffer (PBS/T)	1x PBS 0.01% (v/v) Tween 20
ELISA blocking buffer	1x PBS 2% (w/v) BSA
ELISA substrate solution	TMB peroxidase solution (Table 2.13.)
ELISA stop solution	6% (v/v) Sulphuric Acid (H_2SO_4)

Taqman Buffers

Master Mix Reagent	Purchased from Applied Biosystems (part number 4309169)

Adenoviral Purification Buffers

Saturated CsCl solution	~35 ml 0.01M Tris 1 mM EDTA CsCl added until saturated
PBS^{2+}	1x PBS 0.01% (w/v) $CaCl_2$ 0.01% (w/v) $MgCl_2$

1.4g/ml density CsCl	0.534 g CsCl/ml H_2O
1.34g/ml density CsCl	212.5 ml of 1.4 g/ml CsCl in 37.5 ml PBS^{2+}

Luciferase Assay Buffers

Luciferase assay buffer (LAB)	25 mM Tris phosphate pH 7.8 8 mM $MgCl_2$ 1 mM EDTA 1% (v/v) Triton-X 100 1 mM DTT* 0.5 mM ATP* *Added freshly
Luciferase lysis (CAT) buffer	10 mM Tris/Base pH 8.0 1 mM EDTA 150 mM NaCl 0.65% (v/v) NP-40

MTT Buffer

SDS solution for MTT assay	10% (w/v) SDS 10.15 M HCl

Flow cytometry buffers

flow cytometry wash buffer	1 x PBS 1 % (v/v) FCS 0.02% (v/v) NaNO2
flow cytometry fixing buffer	1 x PBS 0.02% (v/v) NaNO2 0.5 % (v/v) paraformaldehyde

Molecular Biology buffers

Tris EDTA buffer (TE)	10 mM Tris-HCl, pH 8.0 1 mM EDTA
6 x DNA gel sample buffer	25 mg Bromophenol blue 25 mg Xylene Cyanole FF 30 % (v/v) Glycerol
5 x Tris borate EDTA buffer (TBE)	0.44 M Tris base 0.44 M Orthoboric acid 10 mM EDTA

2.2 Tissue culture

All cells were cultured at 37°C in 5% CO_2 and 95% humidity.

2.2.1 Endotoxin testing

Prior to their use, all cell culture reagents were tested for the presence of endotoxin by the Limulus Amebocyte assay (LAL) (BioWhittaker, Bershire, UK). A flat-bottomed micro plate was equilibrated to 37°C. A solution of endotoxin (0.1 EU/ml equivalent to 100 pg/ml) was prepared and serially diluted in LPS-free water. 50 µl of either endotoxin solution, test sample, or blank (LPS free water) were added per well. An additional 50 µl of limulus amebocyte lysate were added to each well and the plate was incubated for 10 minutes (min) at 37°C. 100 µl of provided substrate solution (at 37°C) was added to each well and incubated for a further 6 min prior to termination of the reaction by addition of 100 µl stop reagent. Absorbance was read at 410 nm. The sensitivity of the assay was < 10 pg/ml. All assays were kindly performed by Mrs Patricia Green.

2.2.2 Cell lines

All cell lines used in this study are listed in Table 2.11. AD-293 cells were maintained in DMEM supplemented with 10% FCS and 1% P/S. HSF cells, obtained from circumcision specimens, were cultured in DMEM with 5% FCS and 1% P/S. As standard, all cell lines were terminated at a passage number of p11 for HSF cells and p20 for AD-293 cells. All long-term culture cell lines were regularly screened for mycoplasma infections by Ms Parisa Amjadi.

2.2.3 Cryopreservation of cells

Both primary cells and cell lines were stored in liquid N_2 as required. For freezing down of cell lines, cells were grown to 90% confluence, harvested and resuspended in ice-cold freezing medium consisting of 10% (v/v) dimethylsulfoxide (DMSO) and 90% (v/v) FCS at a concentration 5x 10^6 cells/ml in cryovials. Primary cells were resuspended at a concentration of 10x 10^6 cells/ml. Cells were initially frozen at -70°C for up to 1 week and then transferred to liquid N_2 for long-term storage. Cells were thawed quickly and the DMSO removed by washing cells in complete medium prior to culture.

2.2.4 Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation of single donor buffy coats purchased from the North London Blood Transfusion Service, Colindale, UK. Heparinised buffy coats were diluted 1:2 in HBSS and layered onto 20 ml Lymphocyte®-H Solution (Cedarlane Laboratories Limited, Ontario, Canada) and separated at 2200

revolutions per minute (rpm) for 25 min. The layered PBMC were extracted from the interface between the two layers and washed twice in HBSS.

2.2.5 Separation of human peripheral blood monocytes by counter flow centrifugation (elutriation)

Enriched fractions of monocytes were obtained by separation of PBMC by centrifugation at 2500 rpm in a Beckman JE6 elutriator (Beckman Coulter, High Wycombe, UK) in RPMI containing 1% FCS. Elutriation allows separation of cells according to cell size and granularity by exposing the cells to two opposing forces of centrifugal force and fluid flow velocity in a specialised funnel-shaped chamber placed in the centrifuge (Figure 2.1).

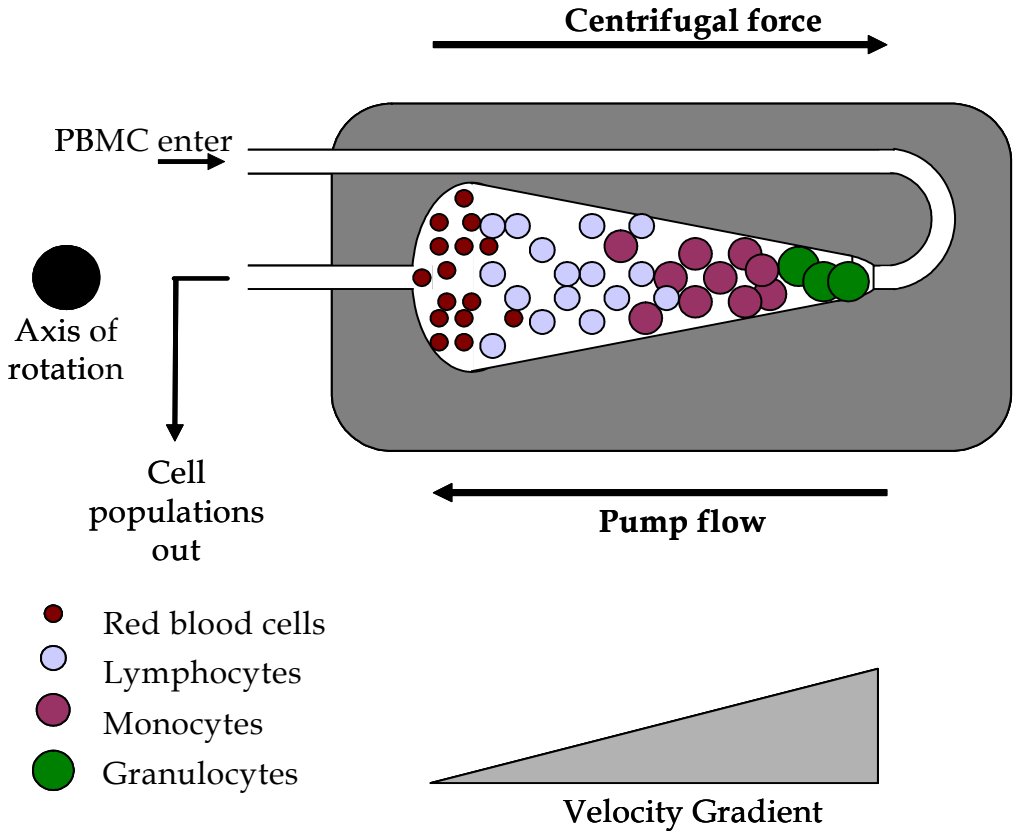


Figure 2.1: Schematic Representation of Centrifugal Elutriation. Illustration shows a cross section of an elutriation chamber, rotating around an axis at 2500 rpm creating a centrifugal force. A peristaltic pump creates an opposite flow. The funnel-shaped chamber causes the velocity of the fluid to decrease, forming a defined gradient. Cells of different sizes and densities migrate to the point where the two opposite forces balance. When the flow rate is increased, cell populations of increasing sizes can be eluted with high purity.

As the cell suspension is pumped into the chamber at a constant flow rate, the fluid velocity decreases as the chamber gets wider, which creates a velocity gradient from the narrow end of the chamber to the widest part. The cells migrate into the chamber under the influence of the

two opposing forces of fluid flow and centrifugation, with the former pushing the cells forward and the latter pushing them backwards.

Cells will halt in a relative position in the chamber, where the effects of the two opposing forces are balanced – this separates the cells according to size as the smaller cells are under a lower centrifugal force than the larger ones, positioning them further within the chamber. Thus, the smallest cells exit the chamber first, with the largest cells exiting last, as the fluid flow rate is gradually increased by 1 ml/min during the elutriation. Monocyte purity was assessed by flow cytometry based on cell size and granularity, with monocyte fractions typically containing >80% $CD14^+$ cells.

2.2.6 Differentiation and culture of human M-CSF and GM-CSF macrophages

Monocytes collected by elutriation (Section 2.2.4) were cultured at 1x 10^6 cells/ml in T175 tissue culture flasks in RPMI containing 10% FCS, 1% (v/v) penicillin/streptomycin and differentiated by adding 100 ng/ml M-CSF or 50 ng/ml GM-CSF for 4 days. After the incubation period, non-adherent cells were removed and adherent cells dissociated from the flask by adding 10 ml cell dissociation solution (Sigma) for 30 min at 37°C. Cell dissociation solution was neutralised by adding 20 ml of complete RPMI, and cells were then collected and washed in complete medium and plated as appropriate. The concentration of TLR ligands and cytokines used to stimulate macrophages are listed in Table 2.13.

Table 2.13: Concentrations of TLR ligands and cytokines used for stimulation

Ligands	Macrophages	Dendritic cells	SF
LPS	10 ng/ml	100 ng/ml	100 ng/ml
Poly I:C	20 μg/ml	20 μg/ml	20 μg/ml
Malp-2	10 ng/ml	100 ng/ml	n.a
R848	100 ng/ml	100 ng/ml	n.a
Flagellin	10 ng/ml	100 ng/ml	n.a.
IL-1α	20 ng/ml	20 ng/ml	20 ng/ml
TNFα	20 ng/ml	20 ng/ml	20 ng/ml

Abbreviations: Lipopolysaccharide (LPS), Macrophage-activating lipopeptide-2 (MALP-2), Polyinosinic-polycytidylic acid (Poly I:C), Resiquimod (R848)

2.2.7 Differentiation and culture of monocyte derived dendritic cells

Freshly elutriated monocytes were differentiated into DCs with a stable immature phenotype. This was achieved by culture of the cells at 1-2 x 10^6 cells/ml in 10 cm culture dishes in RPMI, 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, supplemented with 50 ng/ml GM-CSF and 10 ng/ml IL-4, as previously described (Sallusto and Lanzavecchia, 1994). The cytokines were replenished at day 3, and the non-adherent cells were harvested after 5-7 days. The concentration of TLR ligands and cytokines used to induce dendritic cell maturation are listed in Table 2.13 in Section 2.2.6.

2.2.8 Isolation and culture of rheumatoid arthritis synovial fibroblasts (RASF) from rheumatoid synovial specimens

Synovial tissue specimens were obtained from rheumatoid arthritis patients undergoing joint replacement surgery at Charing Cross (donor ID SM#), Ravenscourt Park (RPH#), Central Middlesex (CM#), Wexham Park/Heatherwood (WP#) and Royal United (Bath) (BU#) hospitals. Ethical approval for the use of this tissue was granted to the Kennedy Institute of Rheumatology (KIR) (RREC 1752) by the Riverside Research Ethics Committee, Charing Cross Hospital, London, UK. Synovial tissue was excised and placed into a tissue culture dish containing 10 ml RPMI supplemented with 10% FCS. The tissue was dissected, homogenised and strained through a cell strainer. Attached extra cellular matrix (ECM) was removed by digestion with collagenase. For the digest, 20 ml RPMI containing 5 mg/ml collagenase and 0.15 mg/ml DNAse type I were added, and cultured at 37°C for 1 hour. Debris was removed by passing the cells through a cell strainer, after which cells were washed and resuspended in RPMI with 10% FCS.

Collection of synovial membranes and isolation of RA synovial cells was kindly performed by Ms Renee Best and Ms Lauren Schewitz at the Kennedy Institute of Rheumatology (KIR). Synovial fibroblasts from rheumatoid tissue (RASF) were obtained by culture of mixed cell populations derived from synovial membranes. Cells were plated at 0.5x 10^6 cells/ml in DMEM containing 10% FCS, 1% P/S and 10 μM Indomethacin (Sigma). Adherent cells were passaged 1:2 with Trypsin/EDTA when confluent and used between passages 3-4. The concentration of TLR ligands and cytokines used to stimulate the fibroblasts are listed in table in Table 2.13 in Section 2.2.6.

2.3 Molecular biology techniques

2.3.1 Restriction enzyme digestion of DNA

Treatment of DNA with restriction enzymes that cleave DNA at specific nucleotide sequences was performed according to the manufacturer's instructions, using the enzymes buffers indicated in Table 2.8 in Section 2.1.5. Where double digestion was carried out, consideration toward buffer compatibility were made in order to maximise the activities of both enzymes whilst minimising non-specific enzyme star activity. All digestions were performed with 1 µl enzyme in total volume of 10- 20 µl for 1 h at 37°C.

2.3.2 DNA fragment purification

Restriction products were resolved on a 1 % (w/v) agarose/TBE gel containing 0.1 µg/ml ethidium bromide or 1/10.000 SYBR Green II, used for DNA visualisation under UV light or a pure light source (Dark reader transilluminator, model DR88M, BRI, Ltd., UK). The fragment of interest was excised from the agarose and purified using a Qiagen gel extraction kit according to the manufacturer's instruction. The purified DNA was eluted with 30 µl double distilled (dd)H2O.

2.3.3 Dephosphorylation of DNA ends

Alkaline phosphatase is an enzyme, which removes 5' phosphate groups from DNA that has been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby improves ligation of other DNA fragments into the vector (e.g. subcloning).

In order to dephosphorylate digested DNA, 1 µl of calf intestinal alkaline phosphatase was added per µg of digested DNA, together with 3 µl of 10 x enzyme buffer (provided with the enzyme). The reaction was carried out in a total volume of 30 µl for 15 min at 37°C, followed by heat inactivation of the enzyme at 65°C for 15 min.

2.3.4 Ethanol precipitation

The most widely used method for concentrating DNA is precipitation with ethanol. The precipitate of DNA that is allowed to form at low temperature is recovered by centrifugation and redissolved in an appropriate buffer. The technique is rapid and is quantitative even with nanogram amounts of DNA.

The DNA solution that is to be treated is mixed with 3 volumes of ice-cold 100 % (v/v) ethanol and 1/14 dilution of 3 M NaAc pH 5.2. After a 30 min incubation on ice, the sample is centrifuges for 10 min at 13,000 rpm (Biofuge Fresco, Kendro Laboratory products Plc,

Bishop's Stortford, UK), and the supernatant is aspirated off. The DNA pellet is then washed with 500 µl 70 % (v/v) ice-cold ethanol and resuspended in either ddH2O or TE buffer.

2.3.5 DNA ligation

Ligations of restriction digested DNA fragments were carried out using approximated vector to insert molar ratios of 1:10 respectively. In general, 1 µl of gel-purified vector was used with 3 µl of purified insert. To this, 1 µl of 10 x T4 DNA ligase buffer and 1 µl of T4 DNA ligase was added and the volume was adjusted to 10 µl with ddH2O. The reaction was carried out at 16°C overnight.

2.3.6 Preparation and transformation of electrocompetent *E.coli*

An XL-I Blue *E. coli* strain was streaked onto an LB-agar plate containing 25 µg/ml tetracycline to give distinct colonies after an overnight incubation at 37°C. Two single colonies were picked and grown overnight in 2 x 20 ml LB media. 10 ml of the overnight culture were each used to inoculate 4 x 500 ml LB cultures that were grown with vigorous shaking until an $OD_{600\ nm}$ of 0.4-0.6 units was reached. The cells were harvested by centrifugation at 3000 rpm at 4°C for 15 min (Sorvall RT-7, Kendro Lab products), and washed in 500 ml ice-cold dH_2O per tube by centrifugation at 3000 rpm at 4°C for 15 min. The pellets were each resuspended in 10 ml ice-cold ddH_2O and combined to two tubes that were each subjected to an additional wash in 500 ml ice-cold dH_2O. Each pellet was further washed with 20 ml of 10 % (v/v) glycerol. The electrocompetent cells were resuspended in an equal weight to volume in g/ml of 10 % (v/v) glycerol and the suspension was divided into 50 µl aliquots, snap frozen using liquid nitrogen and stored at -70°C until use.

For transformation, 1 µl of mini-prep purified plasmid DNA (see Section 2.3.2) or 2 µl of ligation mix was added to 50 µl of electrocompetent cells that was thawed slowly on ice. The mixture was added to an ice-cold 0.2 cm electroporation cuvette, which was pulsed (2.5 kV, 200 Ω, 25 µF) for 5-6 msec using an electro cell manipulator ECM 630 (Qbiogene, Cambridge, UK) according to the manufacturers instructions. After a single pulse, the cuvette was removed and cell recovery was initiated by adding ~600 µl of LB to the cuvette and the cells were transferred to a fresh tube. The cells were incubated at 37°C with gentle shaking for 1 h, followed by spreading onto LB agar plate containing appropriate antibiotics (Table 2.8 in Section 2.1.5) of one fifth of the transformation mix. The plates were left at 37°C overnight and colonies were picked the following day.

2.3.7 DNA isolation by mini – and maxi – preps

Harvesting plasmid DNA transformed into *E. coli* was performed using mini- and maxi- prep kits purchased from QIAGEN, according to the manufacturer's instructions. Briefly, separation of plasmid DNA from bacterial genomic DNA was achieved by alkaline lysis of the bacteria. Pre-cleared lysates were adjusted to high salt concentration and purification was achieved by adsorption of the plasmid DNA onto a silica gel membrane in the provided column. The plasmid DNA was then washed of contaminants, prior to elution into 50 µl ddH_2O. The DNA yield was routinely determined by UV absorption at 260-280 nm on a photometer (GeneQuant, Eppendorf, UK).

2.3.8 RNA isolation

Total RNA was isolated from 10^6 cells using a QiaAmp RNA Blood mini kit (Qiagen GmbH, Germany), following the manufacturer's instruction according to the protocol for isolation of total RNA from cultured cells.

2.3.9 PCR

Polymerase Chain Reaction (PCR) amplification of DNA was performed with *Taq* DNA polymerase, using a MWG-Biotech Primus thermocycler. Each reaction was carried out in a thin walled PCR tube and contained the following:

Reagent	Amount
DNA template	5 µl
10 x PCR buffer	5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTP (1 mM)	1 µl
Forward primer (25 pmol/µl)	1 µl
Reverse primer (25 pmol/µl)	1 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
ddH_2O	To a final volume of 50 µl

Typically, the lid temperature was set for 110 °C and the DNA was denatured at 92°C for 5 min and then for 30 sec per cycle. Primer annealing temperature was usually set at 55 °C, and was carried out over a period of 30 sec. Primer extension was performed at 72 °C and held for 1-4 min, depending on the length of the template (1 min/ 1 kb DNA). The reaction was then subjected to a final 10 min extension at 72°C and held for 4°C with the lid heating off.

Typically 30 cycles were used per reaction. The oligonucleotide primers used for PCR are described in Table 2.10 in section 0.

2.3.10 Real-time PCR - TaqMan®

Real-time PCR is a semi-quantitative variant of polymerase chain reaction (PCR) that measures mRNA levels via fluorescence emitted by specific dyes on specifically designed TaqMan® probes after excitation by laser scanning technology. RT-PCR reactions were set up in 10 µl reactions, with each well containing 5 µl 2x Univerasl PCR Mastermix, 0.25 µl 40x MultiScribe™ and RNase inhibitor Mix, 0.5 µl pre-labelled primer, 2 µl sample mRNA and H_2O. Samples were run in duplicates using the Rotor-Gene 6 (Corbett Research UK Ltd., Cambridge, UK), which was programmed as follows:

Reverse Transcription	20 minutes at 48°C
Denaturation	10 minutes at 95°C
PCR (40 cycles)	
Denaturation	15 seconds at 95°C
Annealing/Extension	40 seconds at 56°C

The number of PCR cycles required for the fluorescence emitted to be greater than the baseline fluorescence is termed the threshold cycle (C_t). Differences in mRNA levels present in the samples and variations in polymerase efficiency are controlled by normalising each sample to the mRNA levels of the housekeeping gene GAPDH.

Figure 2.2 illustrates an exemplary amplification plot obtained by sequence detector software and the method for calculating the relative abundance of template mRNA using the comparative ΔΔCt method.

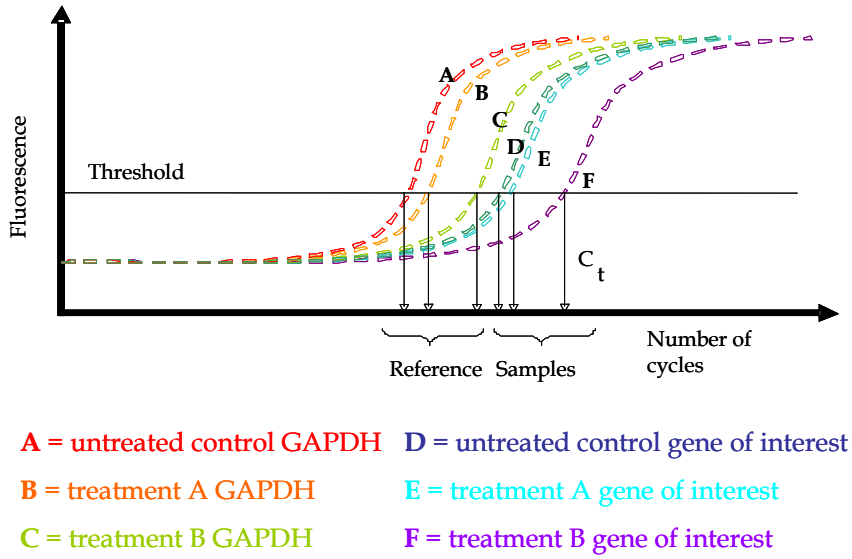


Figure 2.2: Real-time PCR-Comparative Ct Method

The comparative C_t method is also referred to as the $\Delta\Delta C_t$ method, where

$$\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ reference}$$

Here, $\Delta C_{t\ sample}$ is the Ct value for the control sample normalised to the endogenous housekeeping gene GAPDH

$$D - A = \Delta C_{t\ sample}\ (\Delta C_t D)$$

and $\Delta C_{t\ reference}$, which is the C_t value for a reference (treatment) also normalised to GAPDH under the same treatment

$$E - B = \Delta C_{t\ reference}\ (\Delta C_t E)$$

For the treatments illustrated above, the $\Delta\Delta C_t$ values are calculated as follows:

$$\Delta C_t D - \Delta C_t D = \Delta\Delta C_t D \qquad 2^{-\Delta\Delta CtD} = 1$$

$$\Delta C_t E - \Delta C_t D = \Delta\Delta C_t E \qquad 2^{-\Delta\Delta CtE} = x$$

$$\Delta C_t F - \Delta C_t D = \Delta\Delta C_t F \qquad 2^{-\Delta\Delta CtF} = y$$

Values are presented as percent relative to $\Delta\Delta C_t$ sample (=100%).

2.3.11 RNA interference (RNAi)

RNA interference was first discovered in plants, and later in the worm *Caenorhabditis elegans* and its mechanism involves short interfering RNA (siRNA) leading to post-transcriptional gene silencing (PTGS) (Fire et al., 1998). Briefly, RNAi requires several steps to induce degradation of target mRNA and subsequent PTGS. Double stranded RNA (dsRNA) complementary to mRNA of the target gene is synthesised in form of long oligonucleotides (this can vary greatly between species). Long oligonucleotides of dsRNA are then recognised and cut into 21-23 nucleotide siRNAs by an endonuclease called Dicer. Next, the siRNAs are recognised by the

RNA-induced silencing complex (RISC), which links the antisense strand of the siRNA to the specific mRNA. This interaction leads to the subsequent degradation of the target mRNA (Milhavet et al., 2003).

For RNAi in primary human macrophages, freshly elutriated monocytes (typically $5x10^6$ per treatment) were transfected with targeting siRNA or controls (Table 2.7) at concentration ranges from 100-400 nM using the Human Monocyte Nucleofector® Kit (section 2.1.8) according to the manufacturer's instructions. Monocytes were allowed to rest in recovery media (supplied by Amaxa), which was supplemented with 100 ng/ml M-CSF for 24h. After recovery, cells were washed and re-suspended in 10% RPMI with 100 ng/ml M-CSF for 2-3 more days. Differentiated macrophages were then harvested and plated as appropriate.

2.4 Adenoviral production and infection

Adenoviruses are efficient vehicles for delivering genes to cells both *in vitro* and *in vivo*, resulting in high levels of expression of the virally encoded transgene (Horwood et al., 2002). Adenoviruses used in this thesis are listed in Table 2.14. The viruses were constructed using the Gateway® Vector System (Q-BIOgene, CA, USA and Invitrogen, Paisley, UK, respectively) through the homologous recombination of the Ad5 genome with a transfer vector containing the gene of interest. Inserts are driven by a CMV promoter followed by a GFP site. Adenovirus vectors have their E1 and E3 regions of the viral genome deleted, which prevents replication and removes the adenoviral genes known to subvert intracellular signalling (Hayder and Mullbacher, 1996).

2.4.1 Subcloning into transfer vector

Genes of interest were cloned in to the transfer vector pENTR4.3F (made in house, Figure 2.3), by standard molecular techniques (see Section 2.3). Transgene expression was determined in HEK293 cells by lipofectamine mediated transfection (see Section 2.4.3) and confirmed by SDS-PAGE and Western blotting (see Sections 2.5.4 and 2.6.1). The sequence of the cloned cDNA was also confirmed at this stage, by DNA sequencing.

2.4.2 Recombination of transfer vectors with viral genomic DNA

Homologous recombination of the pENTR4.3F vector, containing the gene of interest, with the genomic vector pAd/PL-DEST was performed using the following reaction mix:

Reagent	Amount
300 ng pENTR4.3F	2 µl
300 ng pAd/PL-DEST	2 µl
LR clonase	2 µl
10 x Buffer	2 µl
TE-Buffer	2 µl

The reaction was performed over night at 25°C. The products of the recombination event are expression clone and a by product, known as the donor vector. The desired expression clone is under two forms of selection: antibiotic resistance and negative selection. Selecting for ampicillin resistance eliminates the entry vector (pENTR4.3F) and the by product (both of which contain kanamycin resistance). The presence of the negative selection marker (ccdB gene) eliminates the destination vector (pAd/PL-DEST) and co-integrated molecules. As an

extra control there is another negative selection marker in the form of a chloramphenicol resistance present on the pAd/PL-DEST vector. The resulting vector was transformed into TOP10 *E. coli*. 50 µl of transformed TOP10 *E. coli* were spread on Agarose and resulting colonies were picked after over night incubation at 37°C. The colonies were grown in ampicillin containing LB media and as a control in chloramphenicol media. Vectors from colonies growing in ampicillin containing media only were isolated using maxiprep kit from Qiagen.

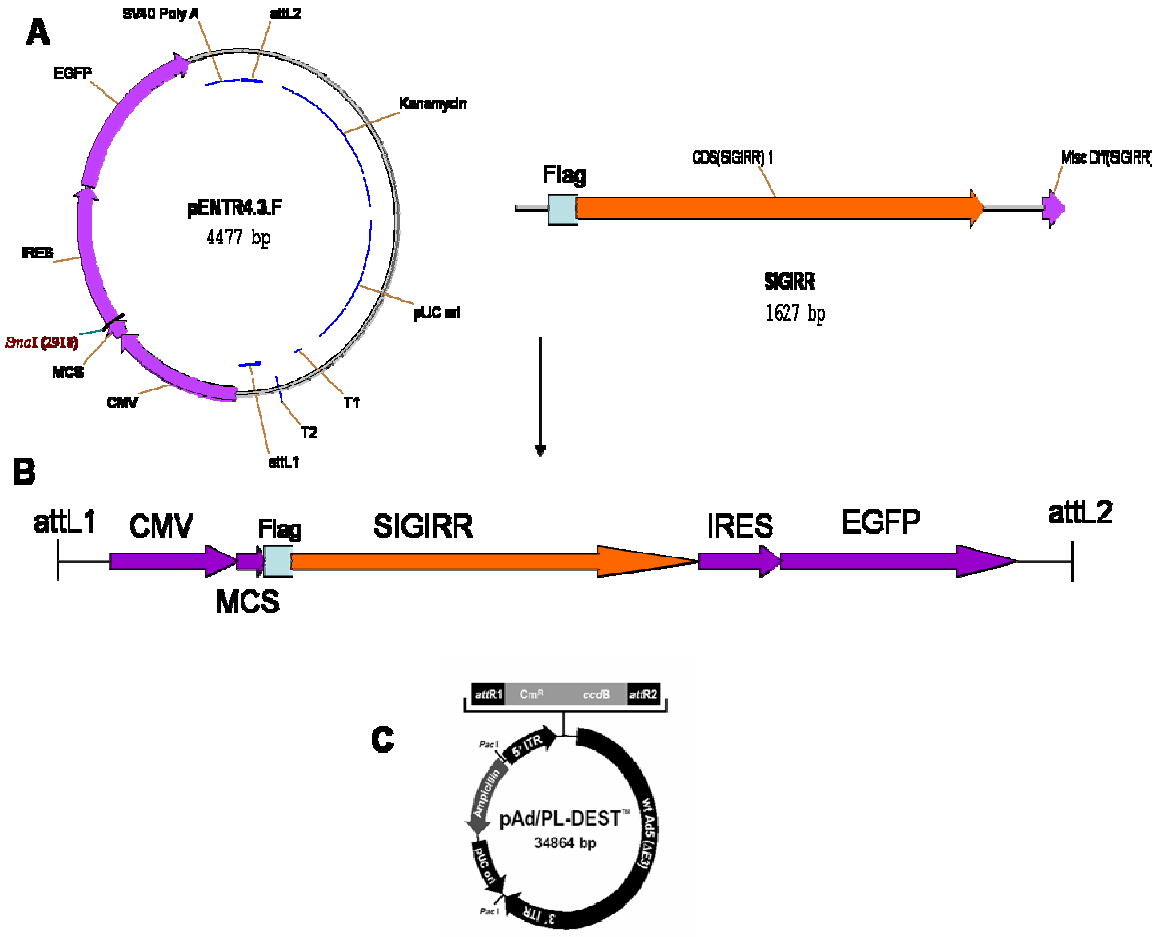


Figure 2.3: Cloning strategy of SIGIRR adenovirus. (A) Flag tagged SIGIRR construct (kindly provided by Prof X. Li, Lerner Research Institute, OH, USA) was ligated into the vector pENTR4.3F, which was cut with the restriction enzyme Sma I. (B) The correct orientation and sequence of the insert was confirmed by sequencing. (C) Following homologous recombination of the entry vector pENTR4.3F and the gateway vector pAd/PL-DEST through the homologous sites attL1/2 and attR1/2 the gateway vector was linearised using Pac I and transfected into HEK293 cells. Flag tagged SIGIRR as well as EGFP are transcribed under the control of the CMV promoter. The IRES permits translation of SIGIRR and EGFP as a single transcript. Abbreviations: cytomegalovirus promoter (CMV), multiple cloning site (MCS), internal ribosome entry site (IRES), enhanced green fluorescent protein (EGFP), Kanamycin resistance gene (Kanamycin), Ampicillin resistance gene (Ampicillin), origin of replication (pUC ori), homologous recombination cites (attL2, attL1, attR1, attR2), adenovirus genome (wt Ad5) .

2.4.3 Transfection of the viral genomic DNA into AD-293 cells

The AD-293 cell line is a transformed HEK cell line that produces the adenoviral E1 genes in *trans*. The E1 gene products originate from early transcribed regions and are involved in mechanisms of viral replication. Transfection of the E1-deleted adenoviral DNA vectors into these cells enables the production of infectious viral particles.

AD-293 cells were seeded per well of a 6 well plate at ~ 90 % confluency (~1 x 10^6) one day prior to the transfection. 3-5 μg of the recombinant viral DNA was digested for 5 h using 4 μl *Pac* I in a total volume of 100 μl. The digest was ethanol precipitated by the addition of 7 μl 3 M NaAc and 300 μl 100 % (v/v) ice-cold ethanol and the mixture was left on ice for 30 min to precipitate, prior to a 5 min spin at 13,000 rpm (Biofuge fresco, Kendro Laboratory products Plc, Bishop's Stortford, UK) at 4°C. The supernatant was removed and 500 μl 70 % (v/v) ice-cold ethanol was added and the pellet was washed by gently tipping the tube. The ethanol was aspirated off and 500 μl prewarmed Optimum transfection media was added, and the pellet gently suspended prior to a 30 min incubation at 37°C. 20 μl Lipofectamine was added to the DNA suspension and a control media, followed by an incubation at room temperature for 20 min. During this time, the cell media was removed and replaced with 1 ml prewarmed Optimum and incubated for an additional 30 min at 37°C. The DNA-lipofectamine complex was added drop-wise to the cells, and the transfection was allowed to occur for 2 h at 37°C, after, which the media was replaced with complete growth medium. Transfected cells were over-laid with DMEM/agarose (see Section 2.4.4) the following day to allow plaque development from the production of infectious viruses.

2.4.4 Agarose/MEM overlay of AD-293 cells

The process of overlaying AD-293 cells with agarose is central to the production and purification of adenoviruses. Overlaying cells with agar provides extra support to the cells, and inhibits contact mediated growth, thus prolonging the longevity of the monolayer. In addition, translocation of the viral particles is restricted to two dimensions (across the monolayer via cell to cell infection). This restriction conferred upon the virus is central to development of plaques, caused by progressive lytic infections of the monolayer. A sterile solution of 1.5 % (w/v) Seaplaque GTG-agarose was prewarmed to approximately 42°C. The agarose solution was mixed 1:1 with 2x MEM media containing 4 % (v/v) FCS and 2 % (v/v) antibiotics (at 37°C), and 3 ml was carefully over-laid onto AD-293 cells in one well of a 6-well plate. Care was taken to avoid damaging the cell monolayer. The agarose/MEM layer on the cells, was allowed to solidify at room temperature for 5 min before continued incubation at 37°C. The cells were then incubated for 5-7 days to allow plaque formation to occur.

2.4.5 Plaque purification and virus recovery

Plaque purification is essential in the *de novo* production of clonal viruses or for the maintenance of good viral stocks. Plaques, developed from plaque assay titration or transfection of recombinant viral genome, were selected microscopically. Approximately 1-5 µl of media from within the plaque was removed using a 10 µl Gilson pipette. The virus containing media was diluted into 500 µl of serum free DMEM and left overnight at 37°C in an incubator to allow viral dissociation from cellular debris or agar constituents. The 500 µl viral media was used to infect one well of a 12-well plate, containing confluent AD-293 cells. Growth media was aspirated and the cells were washed once in serum free DMEM before incubation with the virus containing media for 2 h at 37°C. At the end of the incubation, 500 µl of DMEM containing 4 % (v/v) FCS was added to the cells, to give a final concentration of 2 % (v/v) FCS. Depending upon the number of viral particles isolated from the plaque, the 12-well plate would exhibit a full cytopathic effect (CPE) 3-5 days post infection. CPE is typified by the degeneration of the monolayer, which is caused by cell swelling and detachment due to the adenoviral replication.

2.4.6 Small scale adenoviral amplification

When the AD-293 cells reached CPE, cell lysates were transferred to an AD-293 monolayer with increasing surface area to achieve efficient viral amplification. At full CPE, the media and the cells from the 12- well plate stage were harvested and subjected to three freeze-thaw cycles using liquid nitrogen. This induces cell lysis to ensure the recovery of a maximum amount of viruses in the media. One fifth of this media was diluted to 1 ml with serum free DMEM and was used to infect one well of a 6-well plate for 1 h under serum free conditions. At the end of the incubation, 1 ml of DMEM containing 4 % (v/v) FCS was added to the cells, to give a final concentration of 2 % (v/v) FCS. A reduction in serum concentration was used to reduce the proliferation of the AD-293 cells. Full CPE was normally achieved after 2 days culture, and the freeze-thaw cycle was repeated.

At this stage the adenovirus were screened for expression of the recombinant transgene by SDS-PAGE and Western blotting (see Sections 2.5.4 and 2.6.1). Once over-expression was confirmed, these lysates purified from single-plaques were either used to seed large-scale amplifications of the virus, or were stored at -70°C as archives.

2.4.7 Large scale adenoviral amplification

A confluent T75 flask of Ad-293 cells was infected with the crude lysate made up to 5ml of SF DMEM and allowed to infect for 1-2h.

Table 2.14: Adenoviruses

Adenovirus	Insert cDNA	Source of cDNA
Ad GFP	Green fluorescent protein	Dr. M. Wood, Quantum Biotech, Carlsbad, Canada
Ad MyD88 dn-GFP	MyD88 with truncated death domain and point mutation F56N (Flag-tag)	Dr. K. Burns, Ludwig, Switzerland
Ad MyD88wt-GFP	MyD88 wild type (Flag-tag)	Caroline Andrews, KIRD
Ad Mal wt-GFP	Mal wild type (HA-tag)	Cloned from human MAL image clone purchased from MRC gene service, Babraham Bioincubator, Cambridge, UK
Ad Mal dn-GFP	Mal with point mutation P125H in the TIR domain (HA-tag)	Cloned from MAL image clone purchased from MRC gene service, Babraham Bioincubator, Cambridge, UK
Ad Mal (TIR)	TIR domain of Mal (HA-tag)	T. Bird, J. Sims, Immunex
Ad TRAM dn-GFP	TRAM with point mutation C114H in the TIR domain	Prof K. Fitzgerald, University of Massachusetts, MA, USA
Ad TRAM wt-GFP	TRAM wild type	Corinne Taylor, KIRD
Ad TRIF (TIR)-GFP	TIR domain of TRIF (HA-tag)	Cloned from human TRIF image clone purchased from MRC gene service, Babraham Bioincubator, Cambridge, UK
Ad SIGIRR-GFP	SIGIRR wild type (Flag-tag)	Prof X. Li, Lerner Research Institute, OH, USA
AdLux NFκB*	Firefly luciferase gene driven by 4 tandem repeats of the consensus κ enhancer site	Dr Lynn Williams

Abbreviations: dominant negative (dn); wild-type (wt)

Following infection, 5 ml of 4% DMEM were added to the flask, which was again allowed to reach full CPE before freeze-thawing the lysates 3x as before. For the final step of virus amplification, 10 T175 flasks of AD-293 cells at no more than 80-90% confluence were infected with the crude lysates from the T75 stage made up to 100 ml in SF DMEM (10ml per flask) and again allowed to infect for 1-2 h before adding 10 ml of 4% DMEM to each flask. At this point, the cells must be harvested before the virus causes them to lyse – they must not reach full CPE. Cells were harvested approximately 24-36 h post infection, when they become non-adherent.

2.4.8 Purification of high titre adenoviral stocks

Infected, non-adherent AD-293 cells from the T175 flasks were harvested in 50 ml Falcon™ tubes and washed with SF DMEM. Virus-containing AD-293 cells were spun at 1000 rpm for 5 min, supernatants discarded and cells resuspended in 10 ml 0.1 M Tris (pH 8.0) and freeze thawed 3x as before. Lysates were transferred to a new 50 ml tube and passed through a 19G needle and syringe 3x to shear the chromatin. Following a further spin at 2000 rpm for 5 min, the lysates were collected into a new 50 ml tube and cell debris discarded. Supernatants (containing virus) were made up 11.4 ml with 0.1 M Tris, to which 6.6 ml of saturated CsCl solution were added to achieve a final density of 1.34 g/ml, to enable the adenoviral particles to concentrate into a band under centrifugal force. The solution was mixed thoroughly and transferred into 2 Sorvall ultracentrifuge tubes using a needle and syringe; the tubes were filled to the neck, avoiding bubble formation, and collars screwed tight to seal the tubes for centrifugation. Virus bands were separated by centrifugation at 60,000 rpm overnight (minimum 8 h) at 4°C using the Sorvall RC-M150 GX ultracentrifuge, Kendro Laboratory products, UK. Following centrifugation, a band containing adenovirus was visible in the CsCl gradient, and was collected using a 19G needle and syringe (Figure 2.4).

The viral bands from the two tubes were pooled into a new ultracentrifuge tube and filled by adding 1.34 g/ml density CsCl solution. Virus was separated through a further ultracentrifugation, and virus band recovered as before in <2.5 ml total volume.

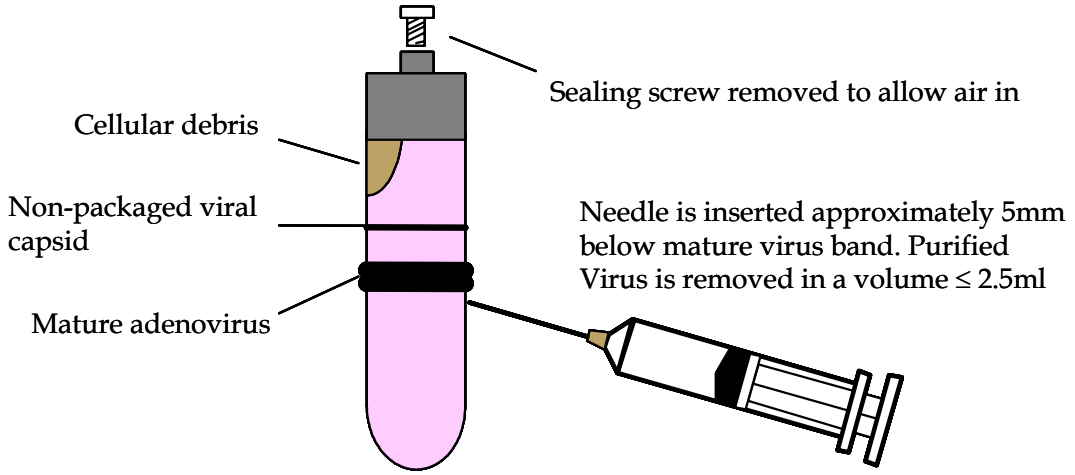


Figure 2.4: Isolation of the mature adenovirus after caesium chloride density-gradient centrifugation. Following ultracentrifugation, mature adenovirus bands at a density of 1.43 g/ml, separating the virus from cellular debris and non-packaged viral particles. The air-tight seal is broken by removing the sealing screw. The viral band was removed form the ultracentrifuge tube using a 5 ml syringe and 19-Gauge needle to puncture the soft tube and removing the viral band in less than 2.5 ml total volume.

CsCl salts were removed from the virus by column chromatography using a PD10 column equilibrated in 25 ml PBS^{2+}. The volume of purified virus was adjusted to 2.5 ml by adding PBS^{2+} and was allowed to enter the column by gravity alone. Elution of purified virus from the column was achieved by addition of 3.5 ml PBS^{2+}. Sterile glycerol was added to the eluted virus to give a final concentration of 10% (v/v), and the virus solution sterilised by passage through 0.45 μm and 0.22 μm filters. Sterilised virus was aliquoted and stored at -70°C. The concentration (titre) of the virus was obtained using the Adeno-X™ Rapid titre kit (Section 2.4.9).

2.4.9 Determination of viral titre with Adeno-X™

The Adeno-X™ Rapid titre kit is an antibody based system used to identify infected cells. The assay takes advantage of the production of viral Hexon proteins for the quantification of viral stocks. Ad-293 cells were seeded in a 12-well plate at 5x 10^5 cells/well in 1 ml of 10% FCS DMEM. A serial dilution was prepared from the viral stock in 2-fold dilutions ranging from 10^{-4} to 10^{-6}. 100 μl of the diluted virus was added drop-wise to the freshly seeded cells, plus one control well without virus, and cells were allowed to infect for 28 h. On the second day, medium was removed and cells allowed to dry before adding 1 ml of ice-cold 100% methanol for 10 min at -20°C to fix the cells. Methanol was removed and cells washed 3x with 1% (w/v) bovine serum albumin (BSA) in PBS. Primary mouse anti-Hexon antibody was added (1ml/well) at 1:1000 in 1% BSA and incubated at 37°C for 1h. Primary antibody was removed, cells washed 3x and 1 ml/well secondary rat anti-mouse antibody added 1:500 in 1% BSA for 1 h at 37°C.

Following the incubation, secondary antibody was removed, cells washed 3x and 0.5 ml/well of diaminobenzidine tetrahydrochloride (DAB) working solution was added and incubated at RT for 10 min. The DAB solution was replaced by 1% BSA and the number of brown (Hexon-positive stained) cells was counted for each viral dilution. A minimum of 3 fields of view were counted using a light microscope with a 20x objective. Each stained cell corresponds to a single infectious unit (ifu). The viral titre was calculated as follows:

$$\text{Virus titre (ifu/ml)} = \frac{\text{(Mean infected cells/field) x (total fields/well*)}}{\text{Volume virus added to the well (ml) x dilution factor } (10^{-N})}$$

* total fields/well based on 20x objective = 573 fields

2.4.10 Assessment of viral replication deficiency

Replication deficiency of the virus was tested by culturing human skin fibroblasts (Table 2.11) in the presence of purified virus for 14 days, after which cell viability was assessed microscopically. The cells are infected, but if the virus is replication deficient, the cells will remain viable over the incubation period.

2.4.11 Adenoviral gene transfer into primary human cells

For adenoviral infection, dendritic cells and macrophages were plated at 1x 10^6 cells/ml, and RASF at 80% confluence. Infections were performed in serum free medium, generally with viruses at a multiplicity of infection (m.o.i.) range between 50:1 and 150:1 for dendritic cells, macrophages and RASF for 2 h. After infection, the medium was replaced with full serum containing RPMI for macrophages and DMEM for RASF. For double infections, cells were infected with adenovirus at an appropriate m.o.i. for 2h, and cells allowed to rest in full medium for 4 h prior to secondary infection as before. Cells were left for produce the viral transgenes for 24 to 48 h prior stimulation. Figure 2.5 shows white and ultra violet (UV) light images of adenoviral infected dendritic cells (Figure 2.5 **A** and **B**) and macrophages (Figure 2.5 **C** and **D**) under a 20x objective. Adenoviral infection efficiency is at ~100%, as assessed by green fluorescent protein (GFP) expression under UV.

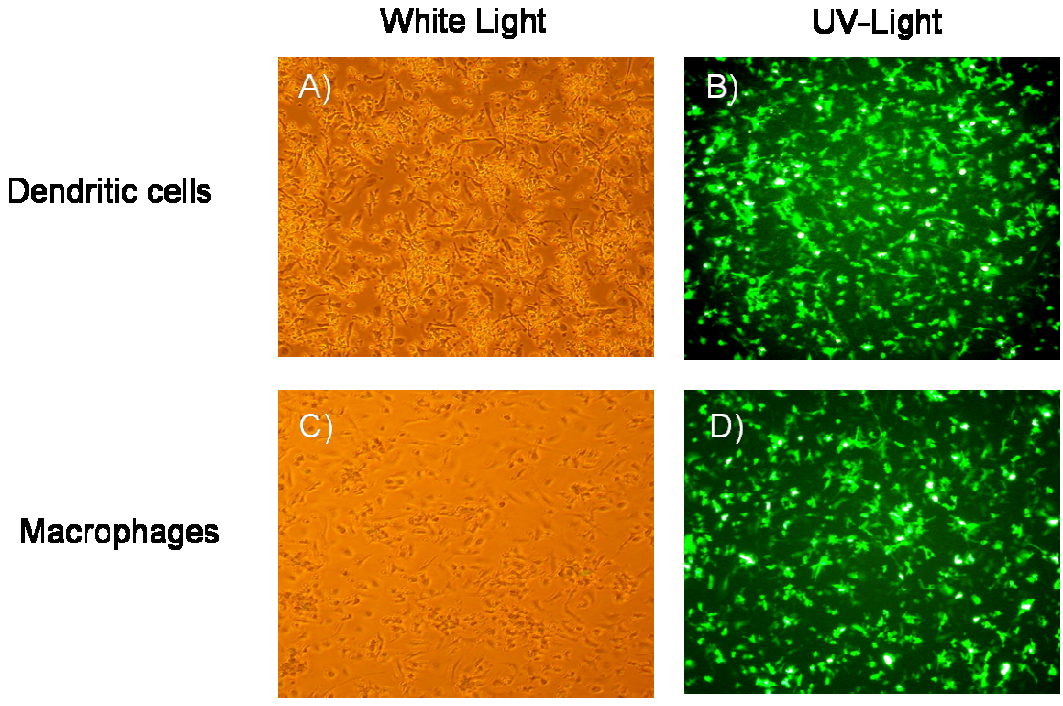


Figure 2.5: Adenoviral infection efficiency as assessed by GFP expression in primary human dendritic cells and macrophages. Primary human dendritic cells (A and B) and macrophages (C and D) were infected with AdSIGIRR at an m.o.i. of 100:1 as described in Section 1.4.12 and allowed to express adenoviral genes for 24 h. Infection efficiency was estimated by GFP expression under the fluorescent light microscope using 20x objective (B and D). Under white light (A and C) dendritic cells and macrophages are healthy and not activated.

2.4.12 Luciferase reporter gene assay

Firefly luciferase has been used as an efficient reporter gene for determining the transcriptional activity of specific gene control elements for several years. The luciferase enzyme catalyses mono-oxygenation of beetle luciferin in the presence of Mg^{2+} and ATP, resulting in the release of photons (fluorescence). This fluorescence can be measured using a luminometer, and the activity of the element regulating the luciferase gene can thereby be quantified. Cells were cultured in a 96-well plate and infected with luciferase constructs (Table 2.12) at an m.o.i. of 50:1, followed by secondary infection with adenovirus (see section 2.4.11), and allowed to express viral transgenes as before. Subsequently, cells were stimulated with LPS for 4 h, at which point supernatants were removed, and cells lysed in 100 µl CAT lysis buffer. As standard, 50 µl of the lysates were mixed with 120 µl LAB containing 1 mM DTT and 0.5 mM ATP. 30 µl luciferin was added immediately prior to analysis in the Wallac MicroBeta Tri Lux luminometer (GMI Inc., Minnesota, USA).

Values were recorded as relative luciferase units (RLU), or as a percentage of the values compared to a control.

2.5 Biochemical techniques

2.5.1 MTT cytotoxicity assay

The MTT cell viability assay is based on the principle that living and metabolically active cells can reduce tetrazolium salts to coloured water-insoluble formazan crystals. The tetrazolium salt is cleaved to formazan by the 'succinate-tetrazolium reductase' system in the respiratory chain of mitochondria and is only active in viable cells. An increasing number of viable cells results in an overall increase in activity of the mitochondrial dehydrogenases. Since a cytotoxic factor will reduce the rate of tetrazolium salt cleavage by a population of cells, this assay can be used to measure the factor-induced cytotoxicity or non-specific cell death. Cells were tested for viability after adenoviral infections (Section 2.4.11). Culture supernatants were harvested and 100 µl of complete medium containing 5 µg/ml MTT (3-[4,5-Dimethylhiazol-2-y]-2,5-diphenyltetrazolium bromide) added to each well and cells cultured for a minimum of 4 h at 37°C. After the incubation, cells were lysed by the addition of 100 µl of 1% SDS and incubated for a further 18 h. Absorbance was read at 620 nm on a Multiskan Biochromatic™ plate reader using Ascent™ 2.4.2 software (Labsystems, Helsinki, Finland).

2.5.2 Preparation of whole cell protein extracts

Preparation of protein extracts was performed in order to investigate events involved in intracellular signalling pathways, using procedures such as SDS-PAGE and western blotting (Sections 2.5.4and 2.6.1). Generally, 1-2x 10^6 cells were washed in ice cold PBS, and lysed in 100 µl lysis buffer containing protease and phosphatase inhibitors (Table 2.6) on ice for 10 min. Following lysis, lysates were collected into 1.5 ml tubes, spun, and lysates without cell debris transferred to a new 1.5 ml tube.

5x GSB was added to give a 1x solution, and lysates further denatured at 100°C for 10 min. At this point, lysates were stored at -20°C or analysed.

2.5.3 Protein concentration estimation

Prior to using the protein extracts from samples with possible high protein variation for any further experimental procedure, the protein concentrations were measured using the BCA™ protein assay kit according to the manufacturer's instructions. For this assay, 2 µl lysate (prior to addition of GSB and denaturation) were diluted in 8 µl H_2O and transferred to a 96-well Maxisorb plate (Nunc, Denmark) in duplicate, along with a series of BSA concentrations as standard. The BCA solutions were mixed at volume ratios of 50:1 (assay reagents A:B) and 200 µl added to each well. Samples were incubated for 1 h at 37°C and absorbance was read at 540

nm on a Multiskan Biochromatic™ plate reader using Ascent™ 2.4.2 software (Labsystems, Helsinki, Finland).

2.5.4 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique commonly used to separate proteins based on their molecular size when an electric field is applied to a gel. SDS is an anionic detergent, which denatures proteins and confers a negative charge to the polypeptide that is proportional to its length. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight of the polypeptides. Samples were denatured prior to electrophoresis by the addition of 5 x gel sample buffer (see Section 2.1.10), and boiling at 95°C for 5 min. A ready to use 10 % Midigel of BioRad was used to separate the proteins. The electrophoresis was performed according to the manufacturers' instructions for 1-2 h using a voltage of 150 V. Molecular weights of the separated proteins could be estimated by comparison to protein standards with known molecular weights, run in parallel to the samples.

2.5.5 Protein transfer

Proteins separated by SDS-PAGE can be transferred onto a membrane, which allows them to be identified using specific antibodies (Table 2.3). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, which was cut to the size of the gel, hydrated in 100% methanol and equilibrated in transfer buffer (Section 2.1.10). The gel was placed onto 2 layers of filter paper pre-soaked in transfer buffer, onto which in turn the equilibrated membrane was placed, avoiding bubble formation between the layers. The membrane was overlaid with further 2 layers of wet filter paper (again, without bubbles), and the assembly placed into a transfer cassette (BioRad, UK). Transfer occurred in a transfer tank (BioRad, UK) filled with pre-cooled transfer buffer (4°C). The transfer cassette was orientated such that the membrane was between the gel and the anode and transfer performed attached to a cooling unit at a constant voltage of 100 V for 2 h.

2.6 Immunological techniques

2.6.1 Western blotting

The membrane containing transferred proteins (Section 2.5.5) was blocked in 5% (w/v) milk powder in TBS/T for 1h and incubated with primary antibody (Table 2.3) in 2% (w/v) BSA or milk for 1-2 h at RT end over end. Following incubations the membrane was washed 3x with TBS/T. After removal of the primary antibody and washing, secondary HRP-conjugated antibody (Table 2.4) was added in 2% (w/v) milk at room temperature (RT) for 1h on an orbital shaker prior to detection using the chemiluminescent substrate solution ECL. The protein bands were visualised using Hyperfilm^{TM}. In most experiments, the membranes were re-probed with different primary antibodies, after the previous ones had been removed by stripping the membrane in mild acid solution (Table 2.12) for 20 min, followed by re-blocking and re-probing with antibodies.

2.6.2 Immunoprecipitation

Immunoprecipitation (IP) allows the extraction of a protein of interest (and any possible associated proteins) from whole cell lysates using specific antibodies, and thus more detailed analyses of protein-protein interactions and activity (e.g. kinase assays). Protein-antibody complexes are removed from solution using beads covered in insoluble antibody binding proteins such as protein A or protein G. Precipitated samples are then denatured and separated by SDS-PAGE as described in Section 2.5.4.

Typically, 5 x 10^6 cells per condition were treated appropriately, washed in ice-cold 1x PBS and lysed for 20 min in 1 ml NP-40 lysis buffer (Section 2.1.10) on ice. Samples were spun at 4°C at 13,000 rpm for 10 min, and 900 µl lysates moved to a fresh pre-cooled 1.5 ml tube. Protein G sepharose beads were washed 3x in lysis buffer, and 50 µl added to each sample for pre-clearing end over end at 4°C for 30 min. Following this, samples were spun at 4°C at 10,000 rpm for 1 min and supernatants removed into new pre-cooled tubes. For further pre-clearing, 5 µg/ml of isotype control antibody was added to each sample and incubated at 4°C end over end for 30 min. Another 50 µl washed beads were added to the samples, which were incubated for a further 30 min at 4°C end over end. Samples were spun at 4°C at 10,000 rpm for 1 min and supernatants removed into fresh pre-cooled tubes. At this point, lysates should be cleared of any particles binding non-specifically to either protein G and/or the antibody isotype. Samples were split and immunoprecipitation with 5 µg/ml specific/isotype control antibody was allowed to proceed for 1.5 h at 4°C end over end. Washed protein G beads were then added to each sample (50 µl each) and samples incubated a further hour at 4°C end over end before spinning at 10,000 rpm for 1 min at 4 °C. Supernatants were removed, and beads (with bound

antibody-protein complexes) were washed 3x with lysis buffer prior to addition of 500 mM NP-40 lysis buffer for denaturing and separation from the beads. Samples were separated by SDS-PAGE as described in Section 2.5.4.

2.6.3 ELISA

The concentration of secreted cytokines in the media surrounding stimulated cells was measured by enzyme linked immuno-sorbent sandwich assay (ELISA). This technique uses specific antibodies to capture and detect cytokines with the use of a colorimetric assay. The antibodies used for ELISA are listed in Table 2.2. Between each incubation step, plates were washed 3x with PBS/T. 100 µl capture antibodies were coated onto 96-well Maxisorb plates (Nunc, Denmark) in PBS and left at 4°C overnight. The wells were then blocked with 2% (w/v) BSA in PBS (200 µl) for 1h at RT, and 100 µl of samples loaded in 0.5% (w/v) BSA in appropriate dilutions alongside a serial dilution of recombinant cytokine (Table 2.1) and incubated at RT for 2 h on an orbital shaker. Minimal levels of detection (LD) for each cytokine are ≤ 13,71 pg/ml based on minimum concentration of standards (Table 2.1). Samples were removed and plates washed prior to addition of 100µl biotin-conjugated detection antibody in 0.5% (w/v) BSA for 1 h at RT. Plates were washed again and incubated with 100 µl/well Streptavidin-HRP 1:400 in 0.5% BSA for a further hour at RT. Following the washing steps, all wells were developed by adding TMB peroxidase substrate. The reaction was stopped with H_2SO_4, and the absorbance read at 450 nm on Multiskan Biochromatic™ plate reader using Ascent™ 2.4.2 software (Labsystems, Helsinki, Finland).

2.6.4 Immunofluorescence-staining and flow cytometry

Flow cytometry is a technique that is commonly used for determining the presence of cell surface or intracellular proteins on a single cell basis. Cells labelled with antibodies are passed through the light of one or more lasers, both scattering light and emitting fluorescence. The light scatter provides some information regarding the morphology of the particles, which can be analysed using forward and sideward scatter. The fluorochrome coupled to the secondary antibody emits light when excited by a laser, and the light intensity correlates to the amount of antigen present on the cell. Antibodies used in this study are summarised in Table 2.3**.** In these studies an IgG isotype control was used, see Table 2.1.

In this project DCs were harvested using pre-warmed cell dissociation solution, for 20 min at 37°C. $1x10^6$ cells/condition were then washed in ice-cold flow cytometry wash buffer (see Section 2.1.10). To determine the location of the protein of interest, the cells were surface- as well as intracellular stained.

2.6.5 Surface staining

Non-specific staining was blocked by incubating the cells in flow cytometry wash buffer containing species-specific serum for 20 min on ice. Then cells were incubated with 10 μg/ml of the primary antibody (see Table 2.3) for 1 h on ice. In the case of directly conjugated antibodies, the cells were washed 3 times with flow cytometry wash buffer and fixed using 0.5% formaldehyde before analysis. For secondary conjugated antibodies the cells were washed 3 times with flow cytometry wash buffer and incubated with 2 μg/ml of the secondary antibody (Table 2.12) for 30 min on ice. After incubation cells were washed 3 times with flow cytometry wash buffer and fixed using 0.5% formaldehyde.

2.6.6 Intracellular staining

The cells are fixed by incubation in 0.5 % formaldehyde for 5-10 min on ice followed by triple washing with flow cytometry wash buffer. The cells were permeabilized by incubation in flow cytometry wash buffer containing 0.1 % saponin. The cell were incubated for 1 h with 10 μg/ml of the primary antibody, washed 3 times with flow cytometry wash buffer containing 0.1 % saponin and incubated with 2 μg/ml of the secondary antibody. After 3 washing steps in flow cytometry wash buffer containing 0.1 % saponin the cells were re-suspended in flow cytometry wash buffer and analysed as described in Section 2.6.4.

2.7 Mouse techniques

2.7.1 Induction and assessment of arthritis

Collagen-induced arthritis is the most widely used animal model for the evaluation of novel therapeutic strategies for rheumatoid arthritis. The disease is induced by immunisation of genetically susceptible strains of mice or rats with type II collagen in adjuvant. Susceptibility to CIA is associated with MHC class II genes, although non-MHC genes also play a role. Both B and T lymphocytes are important in the pathogenesis of collagen-induced arthritis, with the peak of the T cell response occurring around the time of disease onset. Histopathological assessment of the joints of animals with collagen-induced arthritis reveal a proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, the formation of an erosive pannus, cartilage degradation, and fibrosis. As in human rheumatoid arthritis, a number of both pro- and anti-inflammatory cytokines are expressed in the joints of mice with collagen-induced arthritis, including TNFα and IL-1β, IL-6, IL-1Ra, IL-10 and TGFβ.

The type II collagen from cartilage was purified and prepared for injection by Dr. Julia Inglis using the method based on the studies of Miller (Miller, 1972) and Herbage *et al* (Herbage et al., 1977). Type II collagen was dissolved at 4 mg/ml in 0.1M acetic acid overnight at 4◦C, with vigorous stirring. The complete Freund's adjuvant (CFA) used was produced by grinding *M. tuberculosis* (H37 RA, Difco) with a pestle and mortar to produce a fine powder, followed by suspending it in incomplete Freund's adjuvant (IFA, Difco) (approx. 3 mg *M. tuberculosis*/ml of IFA). The dissolved type II collagen was emulsified with an equal volume of CFA on ice, using a syringe. For immunisation, mice were sedated by intraperitoneal injection of 100 µl of 10% (v/v) Hypnorm®, diluted in distilled water followed by the shaving of the rumps of the mice. The emulsion was injected by Dr. Julia Inglis intradermally at 2 sites at the base of the tail using a glass syringe or a latex-free syringe and a 27 gauge needle. Each mouse received 0.1 ml of emulsion in total. The mice were sacrificed on day 14 and lymph node, spleen, bone marrow and blood removed or monitored for arthritis every day from day 14 after immunisation. The peak time of arthritis onset is around day 30. To compare the clinical severity of arthritis a scoring system was used where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, 3 = ankylosis. Each limb is graded in this way, giving a maximum score of 12 per mouse. In addition, paw swelling was monitored using calipers (Poco 2T, Kroeplin). To compare histological severity, paws were removed at post mortem, fixed in buffered formalin (10% v/v), then decalcified in EDTA in buffered formalin (5.5% w/v). The tissues were then embedded in paraffin, sectioned and stained with haematoxylin and eosin. The severity of arthritis was graded as mild, moderate or severe based on the following criteria: mild = minimal synovitis, cartilage loss and bone erosions limited to discrete foci; moderate =

synovitis and erosions present but normal joint architecture intact; severe = synovitis, extensive erosions, joint architecture disrupted.

2.7.2 Measurement of anti-collagen IgG

Serum levels of anti-collagen IgG provide a marker of the magnitude of the humoral anti-collagen response whereas levels of IgG1 and IgG2a serve as *in vivo* markers of Th2 and Th1 responses, respectively. To measure IgG levels, a stock solution of type II collagen in 0.05M Tris-HCl, 0.2M NaCl (pH7.4) at 1 mg/ml was prepared. An ELISA plate was coated with type II collagen at 2-5 μg/ml in 0.05M Tris-HCl, 0.2M NaCl (pH7.4) and incubated overnight at 4$_o$C. Following the coating the plate was blocked for 1h at room temperature with 2% BSA and incubated with test sera (diluted in PBS/Tween 20) for 2h at room temperature. The levels of anti-collagen IgG can vary enormously between mice and it is important to serially dilute samples to ensure that comparisons are made based on the linear portion of the titration curve. The starting dilution used was 1/100, with seven three-fold dilution steps. A standard serum sample, obtained from pooled serum from collagen-immunised, wild type C57/B6 mice, was used on each plate. The plate was washed 6 times with PBS/Tween 20 and the IgG levels detected using HRP-conjugated anti-mouse IgG1 or IgG2a (BD Biosciences). Plates were developed with TMB substrate, the reaction stopped with 4.5N H_2SO_4 and read at 450 nm.

2.7.3 Analysis of T cell responses

The proliferative responses of T cells were measured by incorporation of [^{3}H]thymidine in response to stimulation of lymph node cells and splenocytes with type II collagen and anti-CD3. Moreover, cytokines in culture supernatants were measured by ELISA. The inguinal lymph node and the spleen were removed from collagen-immunised mice at day 14 after immunisation. The lymph nodes and the spleen were pushed through cell strainer using a syringe plunger followed by three washes in HBSS. The red blood cells in the spleen were lysed, using red cell lysis buffer (Sigma Aldrich), for 5 min at room temperature. Cells were resuspended at 5 x 10^6 cells/ml in complete medium (RPM1 1640 containing 10% (v/v) heat-inactivated foetal calf serum or 1% (v/v) mouse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2x10^{-5} M 2-mercaptoethanol and 20 mM L-glutamine) and cultured for 72h in the presence or absence of type II collagen (50 μg/ml) or anti-CD3 (100 ng/ml, eBioscience). Supernatants were removed for measurement of IL-5, IL-10, IFNγ, TNFα and IL-17 by ELISA as described in Section 1.6.3. To determine the rate of T cell proliferation, cells were pulsed with [^{3}H]thymidine (Amersham) and cultured for a further 16 h before being harvested and incorporated radioactivity assessed.

2.7.4 B and T cell purification and activation

A single cell suspension was prepared from lymph nodes and spleens as described above. B cells were positively enriched using anti-IgM MACS micro beads, and T cells using anti-CD4 MACS micro beads, according to the manufacturer's guidelines (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was assessed by flow cytometric analysis (B cells >90% $CD19^+$, T cells >90% $CD4^+$). Cells were cultured at 1 x 10^5 cells/ml in 200µl complete RPMI, as described above, in a flat bottom 96-well plate and cultured for 72 h. B cells were stimulated with IgM (10 µg/ml; BD) or LPS (10 µg/ml, Alexis), and T cells were stimulated with 10 µg/ml plate-bound anti-CD3 (eBiosciences) plus 1 µg/ml soluble anti-CD28 (eBiosciences). In addition T cells were cultured in the absence or presence of IL-1α (20 ng/ml, PeproTech) or IL-33 (20 ng/ml, PeproTech). For proliferation assays, 48 hours after stimulation cells were pulsed with 1 µCi 3H thymidine per well for 18 h. Cells were then harvested and plates assessed for thymidine incorporation. Each assay was performed on a minimum of 2 occasions. IFN-γ, IL-17 and IL-5 levels were assessed in the 48hr culture medium by ELISA after cells were restimulated on Day 5 after culturing with anti-CD3/CD28, with or without IL-1α or IL-33.

2.7.5 *In vitro* culture of Bone marrow cells

In order to obtain bone marrow derived dendritic cells the femurs were removed and the bone marrow washed out using a syringe. The cells were cultured for 7 days in complete media containing 20 ng/ml GM-CSF (PeproTech). Cells were re-pulsed with 20 ng/ml GM-CSF on day 3 of *in vitro* culture. On day 7 of *in vitro* culture the floating cells were collected and re-plated at 1x10^5 Cells/ml in a 96-well plate. Cells were stimulated with 100 ng/ml LPS or 100 ng/ml Malp2 for 18 h before supernatants were collected and cytokines measured by ELISA (see Section 2.6.3).

2.8 Statistical techniques

Mean, standard deviation (SD) and standard error of the mean (SEM) were calculated using GraphPad Prism version 4.03 (GraphPad Software, Inc.). Statistical significance was assessed using the Student's t-test due to small sample sizes, linear regression or two-way ANOVA (Grafen 2002).

Due to donor variation in primary human cells, data cannot generally be pooled unless converted into percentage of controls for each set of experiments. For representative data, SD is shown for each triplicate. SEM is shown for pooled data of $n \geq 3$. Statistical analyses using Student's t-test were performed on pooled percentage values for $n \geq 3$, due to donor variation.

CHAPTER 3

EVIDENCE FOR A MECHANISM OF MYD88 TO SUPPRESS DENDRITIC CELL ACTIVATION

3.1 Introduction

In recent years, the TLR signalling pathway has been described to be one of the major inducers of inflammatory mediators in response to PAMPs, which are conserved molecular structures present in microbes but not the host (Akira and Takeda, 2004). TLR signalling leads to the activation of macrophages and DCs. As a consequence macrophages and DCs produce cytokines and chemokines and increase their capacity to phagocytose (Leon et al., 2005) (Lee and Kim, 2007). In addition DCs become able to present antigens on MHC II complexes on their surface. Consequently these antigens are presented to T cells in the lymph node, thereby initiating the adaptive immune response (Leon et al., 2005).

Our current knowledge of TLR signalling mainly stems from two sources; knock out mice and cell lines. In these models TLR signalling has been described to be dependent on four adaptor molecules. MyD88, the first one to be described, has been shown to be ubiquitously used by all TLRs with the exception of TLR3. Mal is used by TLR2 and TLR4, TRIF by TLR3 and TLR4 and TRAM only by TLR4. A fifth adaptor molecule, SARM, has been implicated as a negative regulator of TRIF dependent signalling (Akira and Takeda, 2004; O'Neill, 2006; O'Neill and Bowie, 2007). The recruitment of those adaptor molecules is one of the earliest events following TLR stimulation. It leads to the induction of a signalling cascade, ultimately resulting in the activation of NF-κB, MAPK and IRFs (O'Neill, 2006). The signalling cascades induced by TLRs are further divided into MyD88 dependent signalling, leading to an early activation of NF-κB, and TRIF dependent signalling, leading to the activation of IRFs and a late induction of NF-κB (O'Neill, 2006).

Until recently, it has been thought these principles of TLR signalling and its regulation apply to human and mouse. Differences in the signalling mechanisms between different cell types have until recently not been considered. However, our group has reported differences in TLR4 signalling between cells of human myeloid and non-myeloid origin (Andreakos et al., 2004). Investigating the role of MyD88 in primary human myeloid and non-myeloid cells, our group found that a dominant negative construct of MyD88 is able to inhibit TLR4 signalling in HUVECs but not in macrophages (Andreakos et al., 2004). Given TLR4 is the most complex TLR, as it utilises all 4 adaptor molecules, this result led me to investigate the role of MyD88 in "simpler" TLR signalling pathways in macrophages. Furthermore, this study investigated the effect of dominant negative MyD88 in another myeloid cell, the dendritic cell.

The findings in this chapter challenge the current belief of a common TLR signalling pathway utilised by most cell types and highlight the possibility of cell type specific regulation in accordance with the cell type's function. Moreover, the results presented in this chapter suggest a MyD88 dependent mechanism, through which DCs are kept in an immature phenotype.

3.2 Results

3.2.1 Adenoviral constructs encoding TLR adaptor proteins for functional investigations were generated at the Kennedy Institute

To begin this study I collected the adenoviral constructs already available at the KIR (Figure 3.1). In order to investigate the function of the TLR adaptor molecules in primary human cells, adenoviral constructs, expressing both wild type (wt) and dominant negative (dn) forms of MyD88, Mal and TRAM, have been generated at the KIR (Mrs Brenda Mutch, Dr Anna Lundberg) (Figure 3.1). These constructs allow reliable over-expression of the encoded gene in primary human cells. This has been a widely used method to study protein function in those cells before the advent of siRNA. In addition to wild-type constructs of the adaptors MyD88, Mal and TRAM, mutant constructs have been developed. MyD88 dn contains a point mutation (Phe56Asn) and a deletion in the DD, which renders it incapable of signal transduction as shown before (Andreakos et al., 2004; Burns et al., 1998). Mal dn and TRAM dn contain a point mutation of the conserved proline 125 to histidine in the TIR domain and both constructs have been shown to inhibit TLR4 signalling in RASF and HUVECs but not human macrophages (Andreakos et al., 2004; Sacre et al., 2007c). In addition adenoviral deletion constructs, encoding the TIR domain of Mal and TRIF only, have been generated (Figure 3.1).

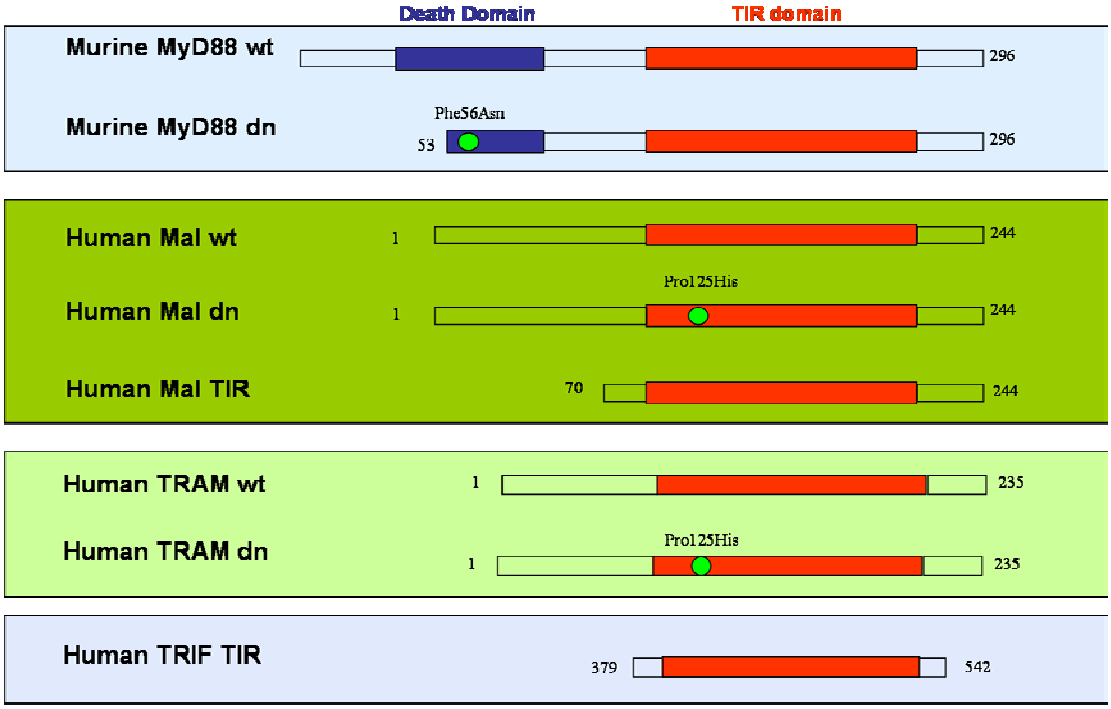


Figure 3.1: Wild type- (wt), dominant negative- (dn) and deletion-constructs (TIR) of TLR adaptor proteins. Murine MyD88 wt is the only TLR adaptor protein containing a death domain (DD). The murine dominant negative construct of MyD88 contains an N-terminal deletion of 52 amino acids and the point mutation Phe56Asn in the DD. Human Mal dn and human TRAM dn contain a point mutation of the conserved proline in the TIR domain (Pro125His). Moreover, constructs consisting of the TIR domain only of human Mal and human TRIF have been made (Mal TIR and TRIF TIR).

3.2.2 MyD88 dominant negative over-expression inhibits TLR4 and IL-1R signalling in RASF

To further investigate the difference between cells of myeloid and non-myeloid origin this study began by confirming previous experiments performed in our laboratory by over-expressing murine MyD88 dn in RASF stimulated with LPS, IL-1, poly I:C and TNFα. The RASF used in this study stain positive for the fibroblast marker Vimentin while no contamination with monocytes/macrophages (CD14) or T cells (CD3) could be detected (Figure 3.2).

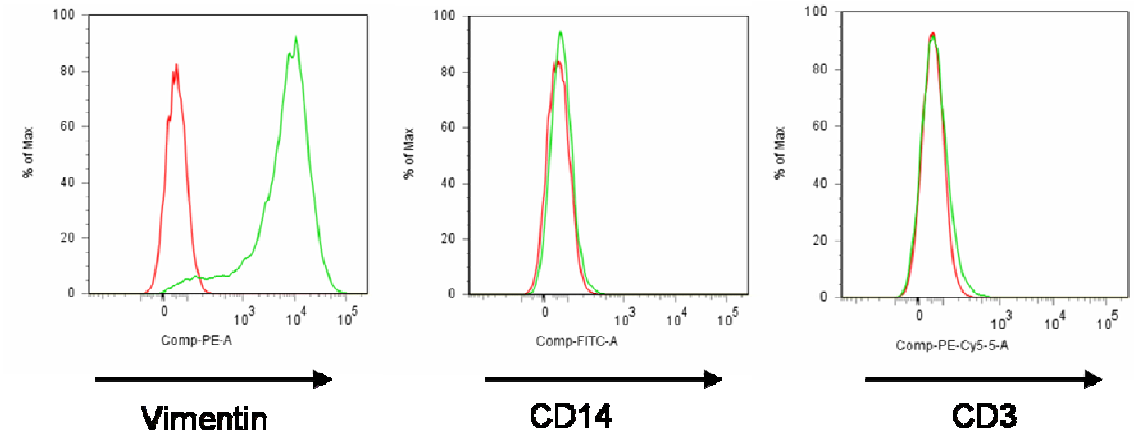


Figure 3.2: Rheumatoid arthritis synovial fibroblasts (RASF) are Vimentin positive while no contaminations with monocytes/macrophages or T cells can be detected. RASF were used from passage 3 after expansion in DMEM 10% FCS. Cells were collected using cell dissociation buffer before being permeabilized using 0.1% Saponin and stained for Vimentin (Neo Markers, CA, USA), CD14 (BD Bioscience) and CD3 (BD Bioscience). Red line, Isotype control; green line, antigen specific staining.

Over-expression of murine MyD88 dn in RASF resulted in a significant inhibition of IL-6 production in response to LPS or IL-1 but not poly I:C or TNFα (A, Figure 3.3). The vitality of the cells, assessed by MTT assay, was unaffected by the over-expression of murine MyD88 dn (B, Figure 3.3).

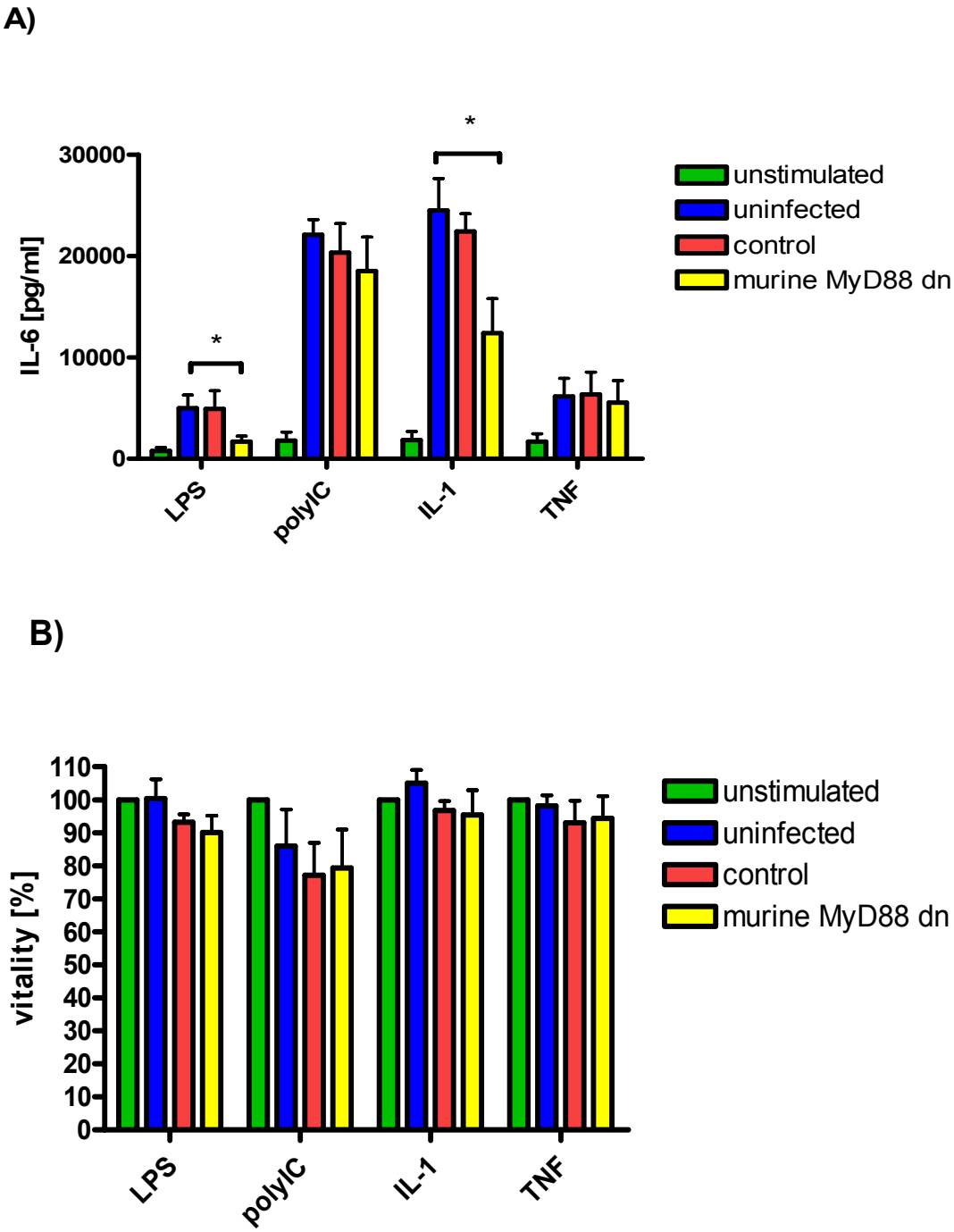


Figure 3.3: Murine MyD88 dn inhibits LPS and IL-1 induced cytokine expression in Synovial Fibroblasts (LPS p = 0.02, IL-1 p = 0.0298). RASF were used from passage 3 after expansion in DMEM 10% FCS (A). Cells were left uninfected, or infected (300:1 m.o.i.) for 1 h in serum-free medium with adenoviruses expressing green fluorescent protein (control) or murine MyD88 dn. 24 h post infection, cells were stimulated with 10 ng/ml LPS, 20 µg/ml poly I:C, 20 ng/ml IL-1α or 20 ng/ml TNFα for 18 h, where after supernatants were collected and IL-6 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 3, data represents 3 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***. B) After supernatants were collected, cells were incubated with MTT over night, lysis performed and OD574 measured. Data is normalised to un-stimulated cells and expressed as mean $\pm$ SEM. n = 3, data represents 3 independent experiments pooled.

The impairment of IL-6 production in response to LPS and IL-1 (A, Figure 3.3) is due to an inhibition of NF-κB as over-expression of murine MyD88 dn led to a significant inhibition of NF-κB in response to LPS or IL-1 as was shown by using an NF-κB luciferase reporter assay (Figure 3.4). Although, the efficency of the second infection with MyD88 dn can not be quantitated, its effect seems to be specific to LPS and IL-1 as poly I:C as well as TNFα induced NF- κB activation is unaffected.

While this result once more shows that the murine MyD88 dn is functional in non-myeloid human cells it is unclear what effect its over-expression has on TLR signalling, other than TLR4, in human monocyte derived macrophages.

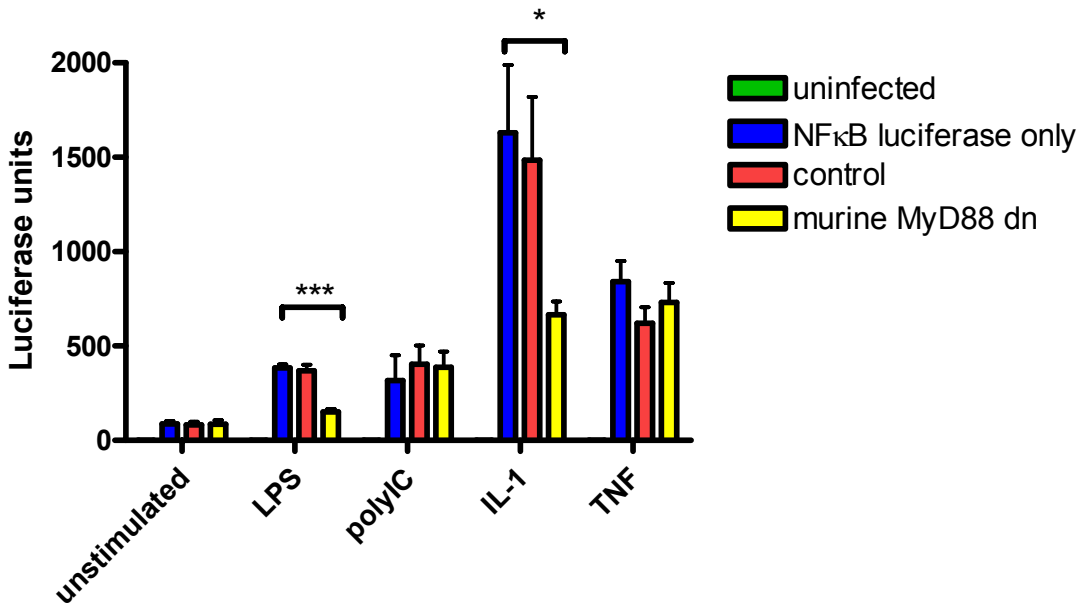


Figure 3.4: Murine MyD88 dn inhibits LPS and IL-1 induced NF-κB activation in Synovial Fibroblasts, (LPS p = 0.0001, IL-1 p = 0.03). Cells were left uninfected, or infected (300:1 m.o.i.) for 1 h in serum-free medium with adenoviruses expressing NF-κB luciferase reporter. After over night incubation in DMEM 10% FCS cells were left uninfected, or infected (300:1 m.o.i.) for 1 h in serum-free medium with adenoviruses expressing green fluorescent protein (control) or MyD88 dn. 24 h post infection, cells were stimulated with 10 ng/ml LPS, 20 µg/ml poly I:C, 20 ng/ml IL-1α or 20 ng/ml TNFα for 18 h, where after cells were lysed and assayed for luciferase activity. Data are expressed as mean $\pm$ SEM. n = 4, data represents 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

3.2.3 MyD88 dominant negative over-expression does not inhibit TLR4 signalling in human monocyte derived macrophages but does inhibit TLR2, TLR5 and TLR7/8

In contrast to RASF, macrophages are professional immune cells whose main function is to recognise and eliminate pathogens. Macrophages belong to the myeloid cell lineage and differentiate from $CD34^+$ haematopoietic progenitor cells. The monocyte derived macrophages used in this study express the monocyte/macrophage marker CD14 and show no contamination with T cells (CD3) following isolation from peripheral blood mononuclear cells and *in vitro* culture (Figure 3.5).

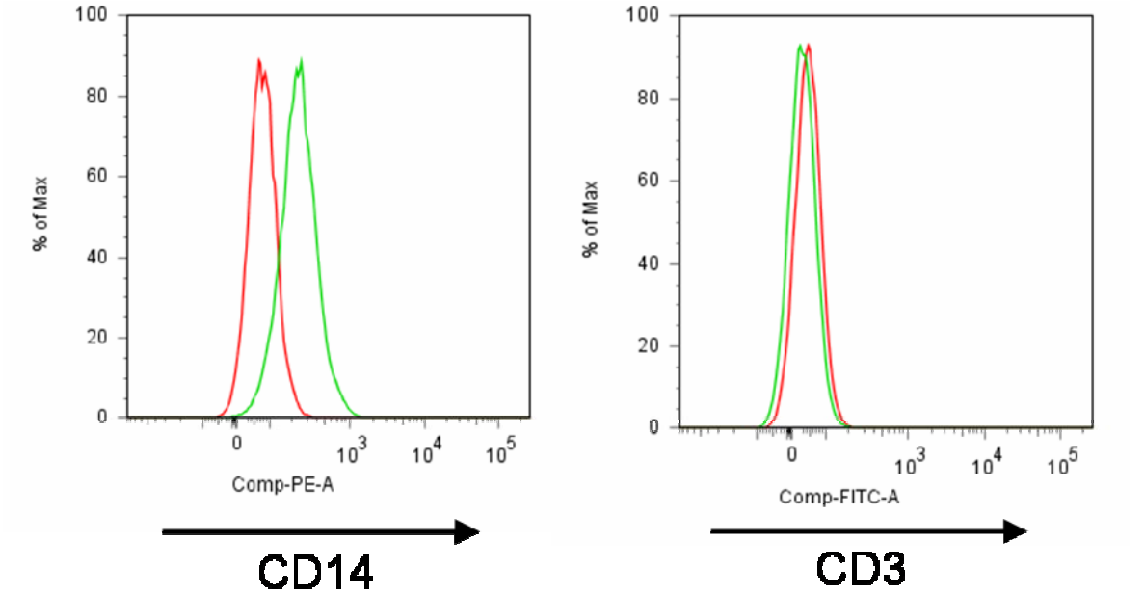


Figure 3.5: M-CSF differentiated macrophages stain positive for CD14 while being negative for CD3. Macrophages were used at day 4 of *in vitro* differentiation with 100 ng/ml M-CSF in RPMI 5% FCS. Macrophages were collected using cell dissociation buffer before being stained for CD14 (BD Bioscience) and CD3 (BD Bioscience). Red line, Isotype control; green line, antigen specific staining.

Over-expression of murine MyD88 dn in macrophages did not result in an inhibition of TLR4 signalling (A and B, Figure 3.6), which is in accordance with results published before (Andreakos et al., 2004). Interestingly, while it has been demonstrated before that TLR4 signalling is unaffected by murine MyD88 dn over-expression IL-1R signalling has been shown to be impaired in human macrophages (Andreakos et al., 2004). Since the IL-1R, unlike TLR4, only utilises MyD88 for signal transduction, I further investigated TLRs, which show similar signalling characteristics.

TLR2 (Malp-2) signalling, using MyD88 and Mal, as well as TLR5 (Flagellin) and TLR7/8 (R848) signalling, utilising only MyD88, were impaired in macrophages over-expressing murine MyD88 dn (Figure 3.6). Over-expression of MyD88 dn significantly inhibited the

production of TNFα and IL-6 by R848 stimulated macrophages. Furthermore, expression of MyD88 dn resulted in an inhibition of TNFα and significant inhibition of IL-6 following stimulation with Flagellin (B, Figure 3.6) and Malp2 (B, Figure 3.6). The cell vitality of macrophages was not affected by the over-expression of MyD88 dn (C, Figure 3.6).

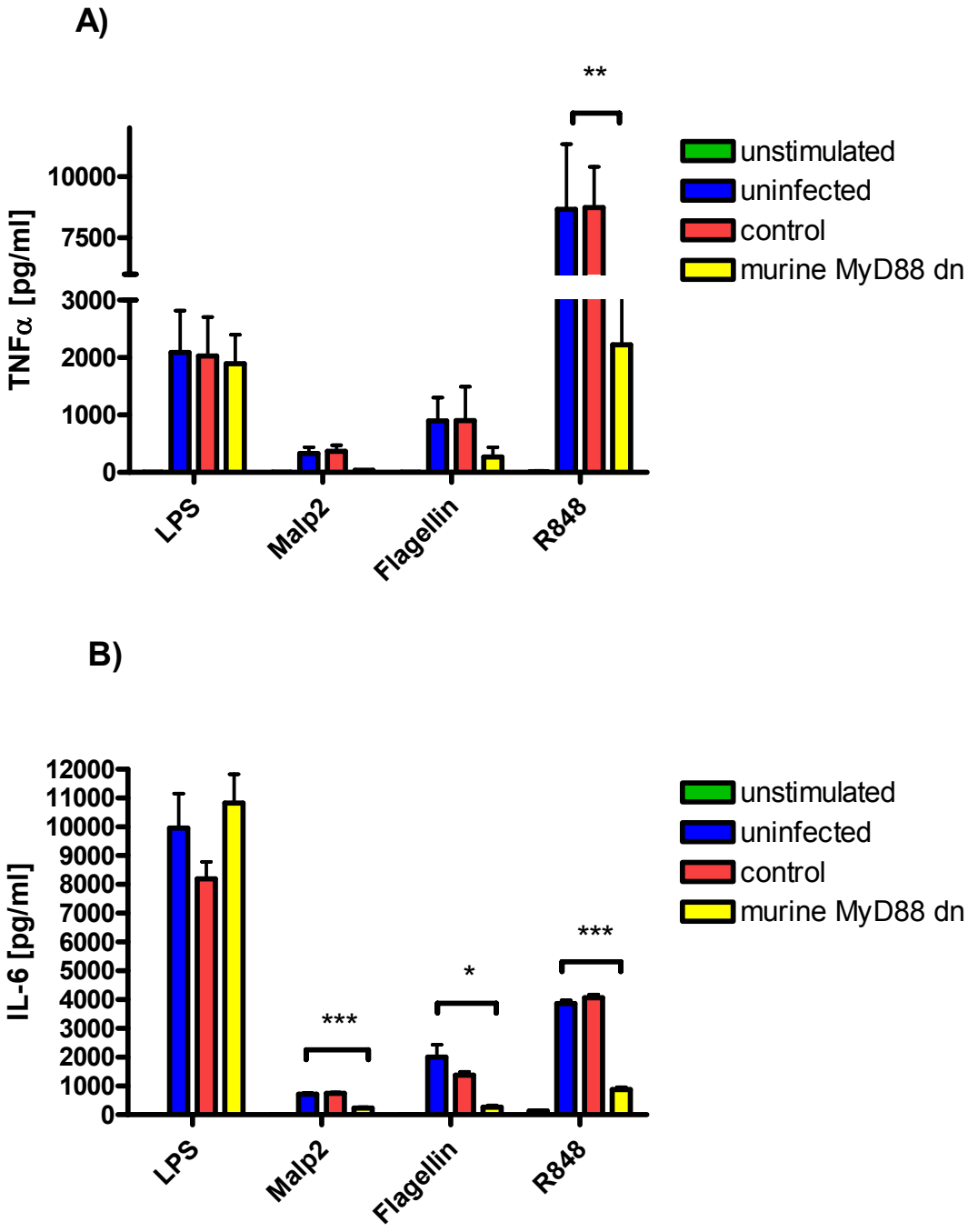


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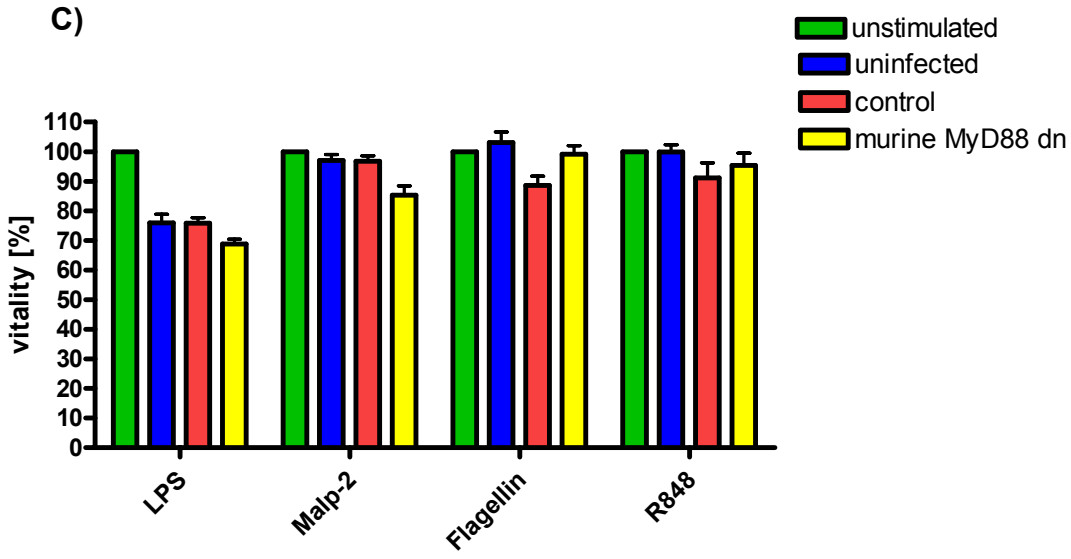


Figure 3.6: Murine MyD88 dn inhibits TLR-2 (IL-6 p = 0.0003), TLR-5 (IL-6 p = 0.0277) and TLR-7/8 (TNFα p = 0.0083 and IL-6 p = 0.0003) but not TLR4 signalling in primary human monocyte derived macrophages. (A and B) Cytokine induction assayed in culture supernatant by ELISA. Macrophages were used at day 4 of *in vitro* differentiation with 100 ng/ml M-CSF in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 1 h in serum-free medium with adenoviruses expressing β-galactosidase (Ad β-gal), murine MyD88 dn. 24 h post infection, cells were stimulated with 20 µg/ml Malp2, 10 ng/ml Flagellin or 100 ng/ml R848 for 18 h, where after supernatants were collected and TNFα (A) and IL-6 (B) induction measured by ELISA. Data are expressed as mean $\pm$ SEM. n = 3, this data is 3 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ *** C) After supernatants were collected for ELISA assay cells were incubated with MTT over night, lysed and OD574 measured and data normalised to un-stimulated cells. Data is normalised to un-stimulated cells and expressed as mean $\pm$ SEM. n = 3, data represents 3 independent experiments pooled.

Although this shows that the murine dominant negative construct of MyD88 is functional in human myeloid as well as non-myeloid cells it is intriguing that TLR4 signalling is not suppressed in human macrophages. Current knowledge suggests that TLR4 is the TLR with the most complex signalling transduction (O'Neill, 2006). It is the only TLR to utilise four of the five described adaptor molecules and is part of a multi-receptor complex consisting of CD14, MD-2 and LBP. Although the described complexity in TLR4 signalling could be due to the simple fact that TLR4 has been the first mammalian TLR to be described, it could also be an indication why TLR4 is the only TLR tested in this study, which is not inhibited by the over-expression of a MyD88 dn. The usage of all four adaptors raises the possibility of redundancy between the adaptors especially MyD88/Mal and TRIF/TRAM. However, the combined expression of MyD88 dn, Mal dn and TRAM dn performed in our group also resulted in no inhibition of TLR4 signalling (Dr. Anna Lundberg, unpublished data). Another explanation could be the involvement of co-receptors and the induction of so far elusive signalling pathways independent of MyD88. TLR4 is part of a receptor complex. These co-receptors are necessary for a complete induction of TLR4 signalling as was shown in case of smooth LPS and its dependency on CD14 to signal (Jiang et al., 2005b). It is intriguing, that while RASF are

$CD14^-$ the human macrophages used in this study are $CD14^+$. Given these differences I further investigated the role of MyD88 in TLR4 signalling in a myeloid cell, which does not express CD14, the dendritic cell.

3.2.4 Over-expression of MyD88 dn induces and super-induces IL-6 and TNFα production in un-stimulated and LPS stimulated human dendritic cells

Characteristic of immature DCs is their ability to up-regulate co-stimulatory molecules in response to TLR stimulation, which results in a mature phenotype (Adams et al., 2005). The monocyte derived DCs display this ability by up-regulating CD86 in response to LPS (Figure 3.7). Furthermore, DCs used in this study do not express CD14 (Figure 3.7) and no contaminations with residual T cells from the monocyte isolation process could be detected in the DC cultures used (Figure 3.7).

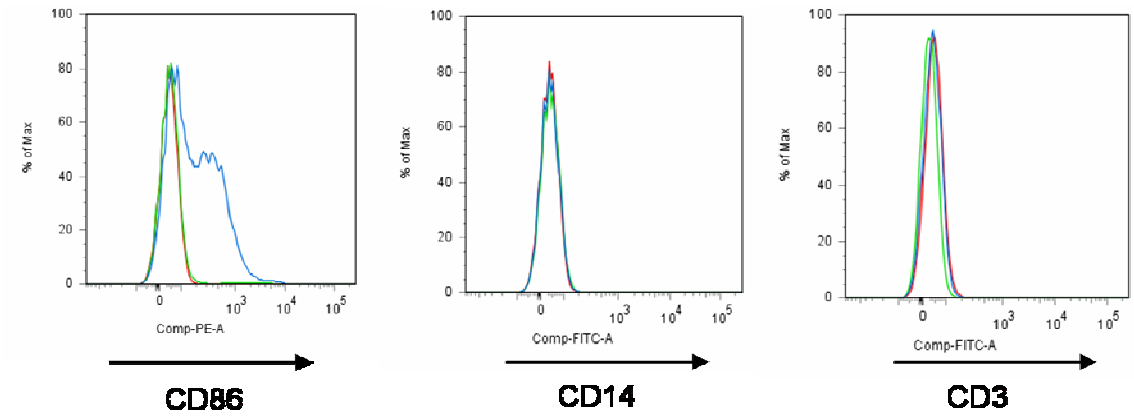


Figure 3.7: Monocyte derived DCs up-regulate co-stimulatory molecule CD86 while staining negative for CD14 and CD3. DCs were used at day 5 of *in vitro* differentiation using 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. DCs were collected and left un-stimulated or stimulated using 10 ng/ml LPS for 18 h before being stained for CD86 (BD Bioscience), CD14 (BD Bioscience) and CD3 (BD Bioscience). Red line, Isotype control; green line, staining of un-stimulated cells; blue line, staining of stimulated cells.

To confirm that the adenoviruses used in this study over-express the gene of interest in DCs, cells were infected and the over-expression examined by western blot. The infection of DCs with adenoviruses encoding wild type-, dominant negative- and deletion (TIR)-constructs of TLR adaptor proteins (Figure 3.1) resulted in the over-expression of these proteins (Figure 3.8).

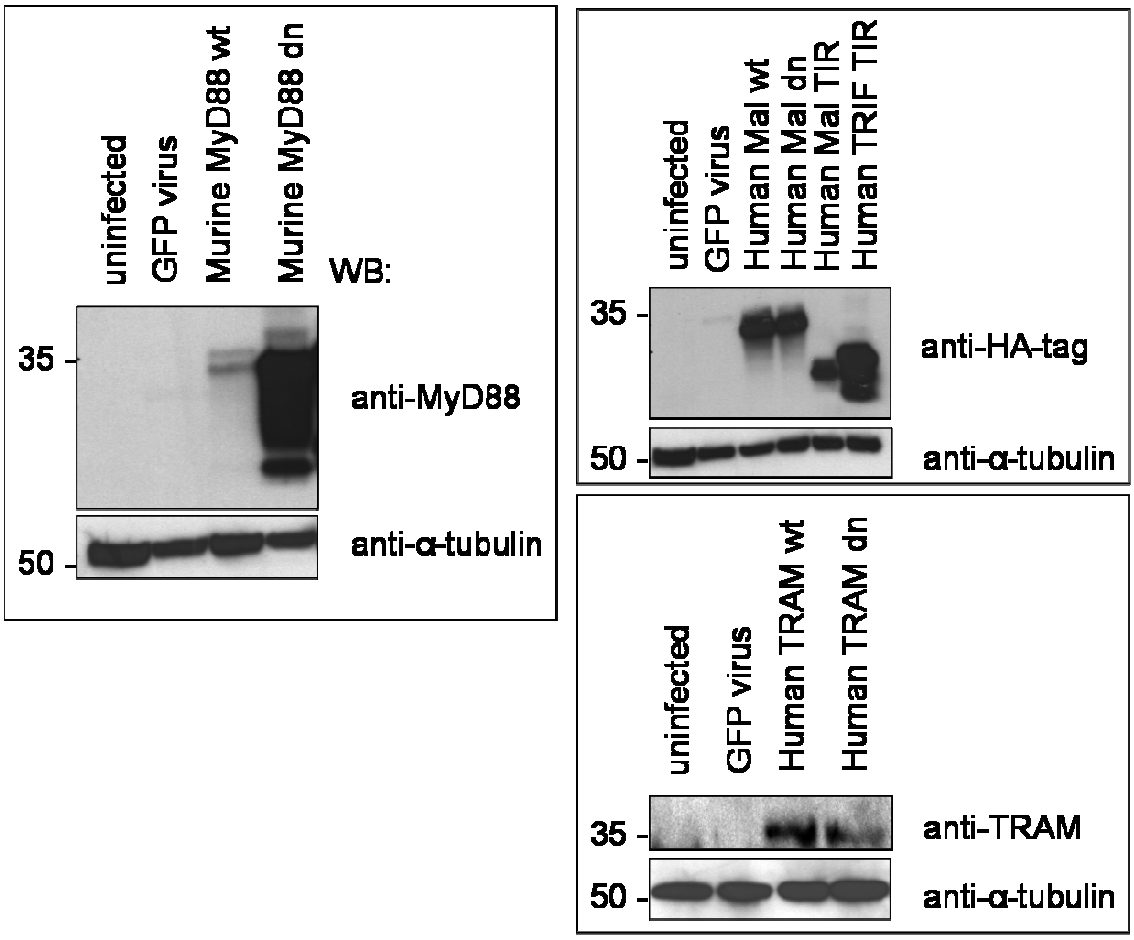


Figure 3.8: Adenovirus constructs are expressed in DCs at an m.o.i of 100:1. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing green-fluorescent protein (GFP), murine MyD88 wt, murine MyD88 dn, Mal wt, Mal dn, Mal TIR, TRIF TIR, TRAM wt and TRAM dn. 24 h post infection cells were lysed with 1 % Triton X 100 lysis buffer and cell lysate was separated by SDS-PAGE. Western blot for MyD88 was performed using an rabbit anti-MyD88 antibody (abcam, Cambridge, UK). The HA-tagged Mal and TRIF constructs were detected using mouse anti-HA (Covance, Berkeley, CA, USA) and TRAM wt and TRAM dn were detected using rabbit ant-TRAM antibody (made in house). As a loading control the membranes were striped and re-probed for α-tubulin using a mouse anti-α-tubulin antibody (Sigma, Poole, Dorset, UK). As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa).

Having shown the expression of the TLR adaptor constructs in DCs, the cells were infected with murine MyD88 dn and subsequently stimulated with LPS (Figure 3.9). However, murine MyD88 dn did not inhibit LPS induced cytokine production. On the contrary, the over-expression of MyD88 dn significantly induced IL-6 (A, Figure 3.9) and TNFα (B, Figure 3.9) production, in the absence of any stimuli. Furthermore, stimulation of MyD88 dn over-expressing DCs with LPS resulted in significant super-induction of IL-6 (A, Figure 3.10) and TNFα (B, Figure 3.10).

Given this unexpected result, the role of Mal and TRAM was further investigated in TLR4 signalling by DCs. Therefore, DCs were infected with human Mal dn or human TRAM dn,

which did not result in an induction or super-induction of IL-6 (A, Figure 3.9) or TNFα (B, Figure 3.9).

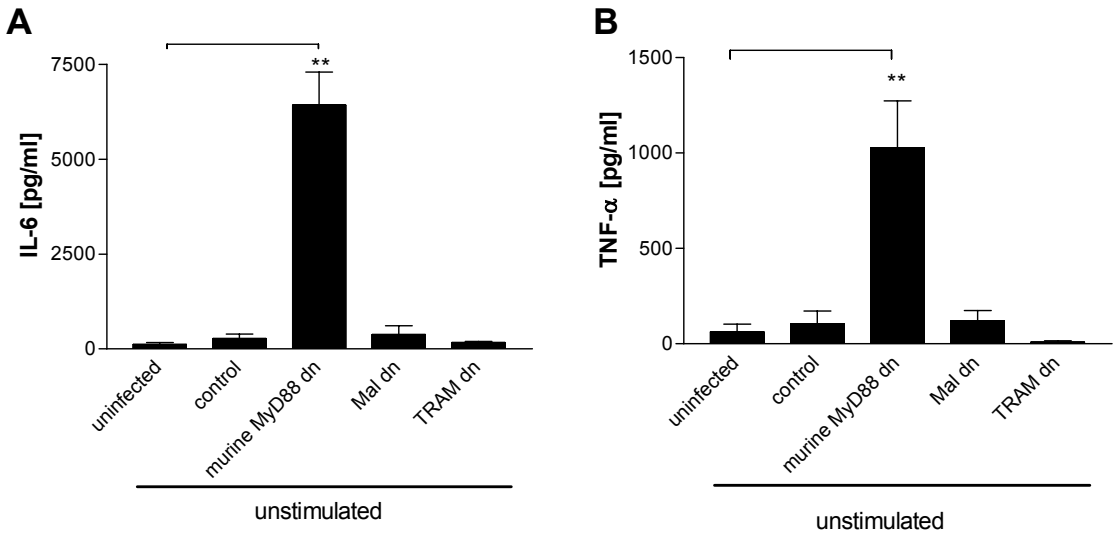


Figure 3.9: Expressing murine MyD88 dn significantly induces IL-6 (A, p = 0.0029) and TNFα (B, p = 0.0079) production in un-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 dn, Mal dn and TRAM dn. 32 h post infection supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean ± SEM. n = 4, data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

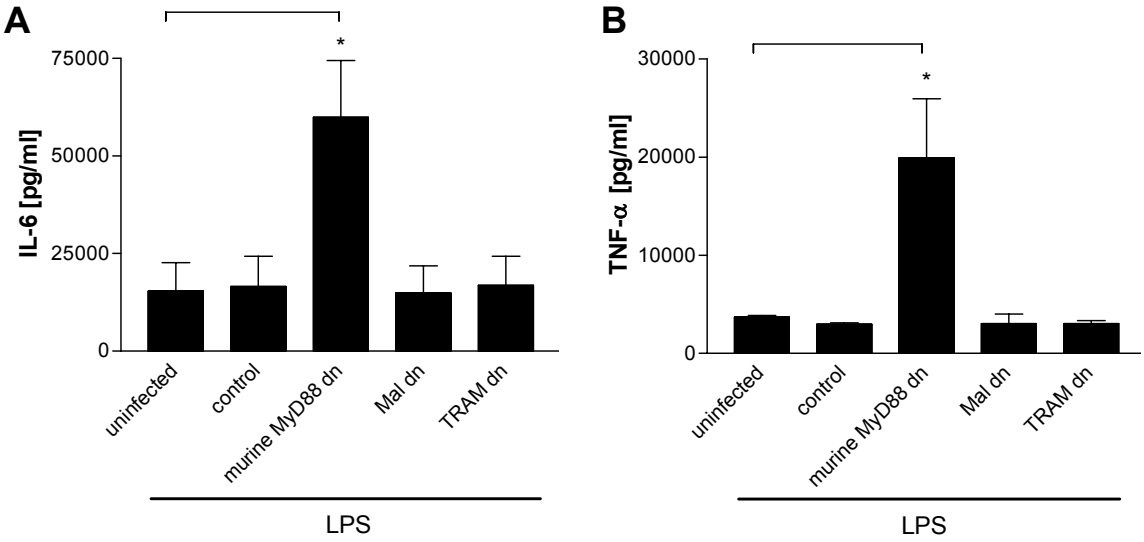


Figure 3.10: Expressing murine MyD88 dn significantly super-induces IL-6 (A, p = 0.0448) and TNFα (B, p = 0.0392) production in LPS-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 dn, Mal dn and TRAM dn. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean ± SEM. n = 4, data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

To further study the observation of cytokine induction in DCs expressing murine MyD88 dn, the cells were infected with adenovirus over-expressing murine MyD88 wt, human Mal wt and human TRAM wt. Unlike MyD88 dn, MyD88 wt did not induce IL-6 (A, Figure 3.11) and TNFα (B, Figure 3.11) in un-stimulated DCs. However, over-expression of Mal wt and TRAM wt led to a significant induction of IL-6 (A, Figure 3.11) and TNFα (B, Figure 3.11). Moreover, stimulation with LPS resulted in significant super-induction of IL-6 (A, Figure 3.12) and TNFα (B, Figure 3.12) in TRAM wt over-expressing DCs. Although a trend of IL-6 and TNFα super-induction was observed in Mal wt over-expressing cells it did not reach statistical significance due to donor variability and limited number of repeats.

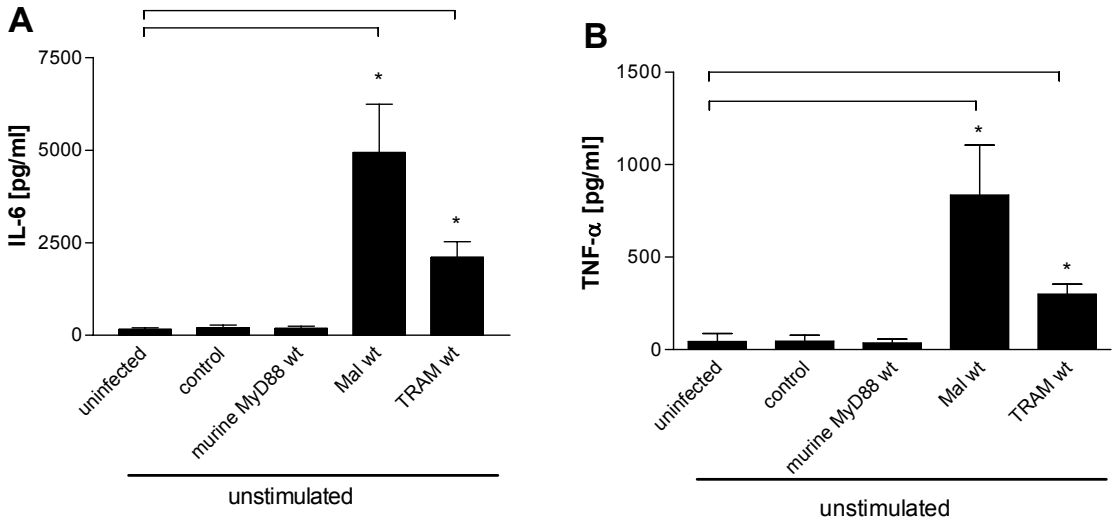


Figure 3.11: Over-expressing Mal wt and TRAM wt significantly induces IL-6 (A, p = 0.0164 and p = 0.0113 respectively) and TNFα (A, p = 0.0251 and p = 0.0385 respectively) production in un-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 wt, Mal wt and TRAM wt. 32 h post infection supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4; data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

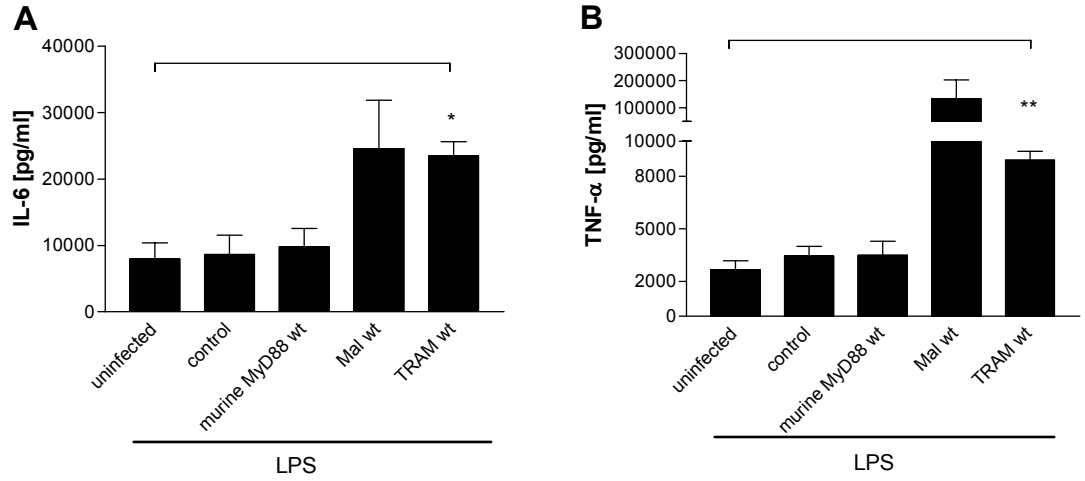


Figure 3.12: Over-expressing Mal wt and TRAM wt super-induces IL-6 (A, p = 0.0755 and p = 0.0351 respectively) and TNFα (B, p = 0.0767 and 0.0043 respectively) production in LPS-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 wt, Mal wt and TRAM wt. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4; data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

Although, the over-expression of MyD88 wt in DCs is low compared to MyD88 dn, which could be one reason for the results obtained, MyD88 wt expression is still significantly higher compared to background levels (Figure 3.8).

Another possible explanation for the observations made could lie in the structural differences of the constructs used. While human Mal wt and human TRAM wt, containing a wild type TIR domain in the absence of a DD, induced and super-induced IL-6 and TNFα in un-stimulated and LPS stimulated DCs, Mal dn and TRAM dn, containing a point mutation in the TIR domain, did not. These results may suggest an important role of the TIR domain in DC activation. Therefore, the effect of over-expressing Mal TIR and TRIF TIR constructs, consisting of the TIR domain only, was further investigated (Figure 3.13 and Figure 3.14).

3.2.5 Expression of a wild type TIR domain induces and super-induces IL-6 and TNFα in human dendritic cells

The expression of the TIR domain of Mal and TRIF resulted in the significant induction of IL-6 (A, Figure 3.13) and in the case of TRIF TIR TNFα (B, Figure 3.13) in un-stimulated DCs.

As well as inducing IL-6 (A, Figure 3.13) and TNFα (B, Figure 3.13) in un-stimulated DCs, stimulation with LPS resulted in the significant super-induction of IL-6 (A, Figure 3.14) and TNFα (B, Figure 3.14) in case of Mal TIR. Although a trend of IL-6 and TNFα super-induction

was observed in TRIF TIR over-expressing cells it did not reach statistical significance due to donor variability and limited number of repeats.

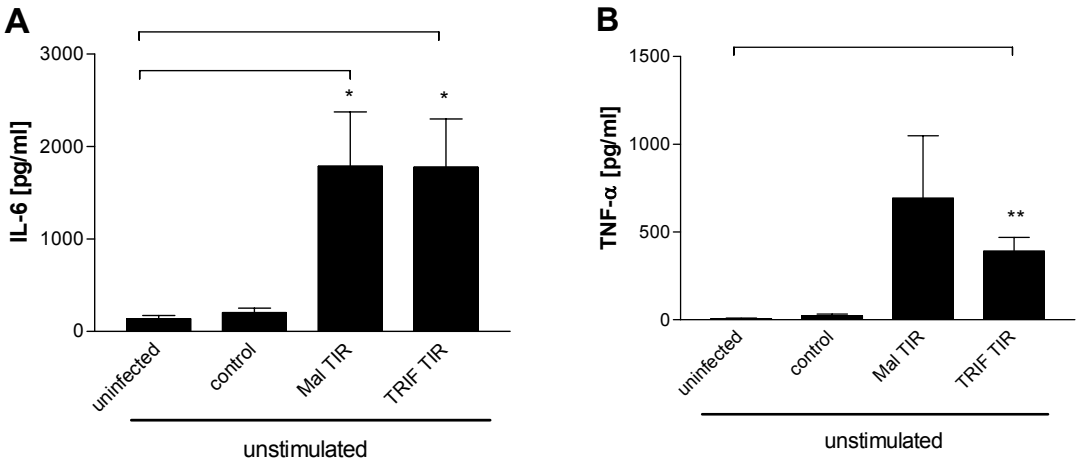


Figure 3.13: Expressing Mal TIR and TRIF TIR induces IL-6 (A, p = 0.0298 and p = 0.0217 respectively) and TNFα (B, p = 0.0758 and p = 0.0087 respectively) production in un-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), Mal TIR and TRIF TIR. 32 h post infection supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean ± SEM. n = 4; data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

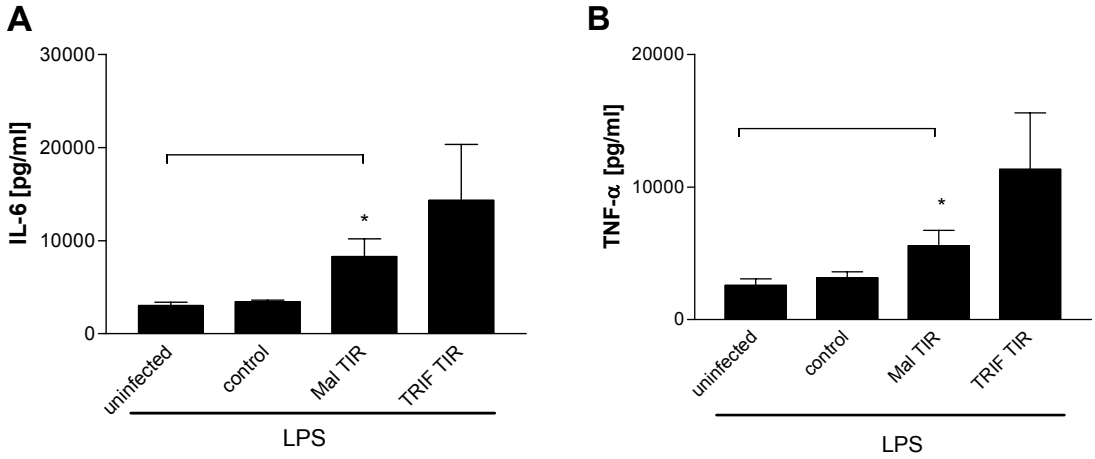


Figure 3.14: Expressing Mal TIR (p = 0.0498 and p = 0.044 respectively) and TRIF TIR (p = 0.0873 and p = 0.061 respectively) super induces IL-6 (A) and TNFα (B) production in LPS-stimulated dendritic cells. Cytokine induction assayed in culture supernatant by ELISA (IL-6: A, TNFα: B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), Mal TIR and TRIF TIR. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean ± SEM. n = 4; data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

These results further indicate the importance of a TIR domain in the induction of cytokines in un-stimulated DCs. However, since murine MyD88 wt over-expression does not result in cytokine induction, although it has an intact TIR domain, it might also suggest a role for the death domain. Furthermore, since the MyD88 constructs used, are of murine origin, the observations made could be a species related artefact, although they do function in human macrophages and RASF as predicted (Figure 3.3, Figure 3.4 and Figure 3.6).

3.2.6 Human MyD88 death domain leads to the super-induction of TNFα in response to LPS

In order to be able to exclude any species related effects and to further examine the role of the death domain, human MyD88 constructs were generated at the KIR (Mrs Brenda Mutch) (Figure 3.15). The human MyD88 mutation construct contains a point mutation of the conserved proline (Pro200His) in the TIR domain. The MyD88 DD construct is TIR domain deleted but still contains the DD as well as the intermediary domain between DD and TIR domain (Figure 3.15).

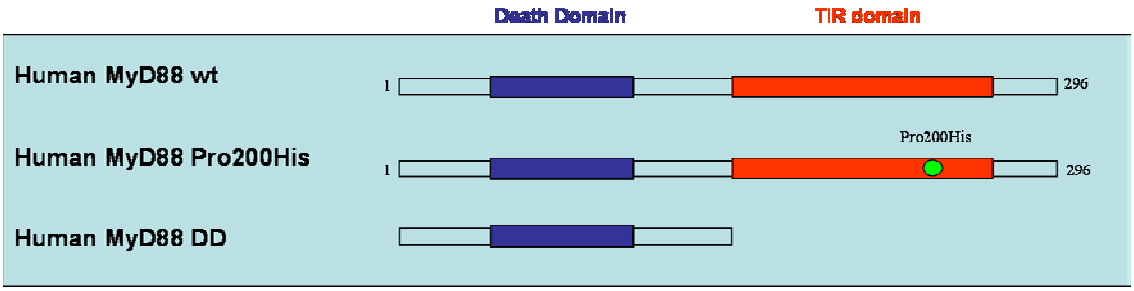


Figure 3.15: Wild type- (wt), point mutated- (Pro200His) and deletion-constructs (DD) of human MyD88. Human MyD88 wt is the only construct containing a Toll/Interleukin-1 receptor (TIR) domain. The human dominant negative construct of MyD88 contains a point mutation in the conserved praline (Pro200His) in the TIR domain. The MyD88 death domain (DD) construct is TIR domain deleted.

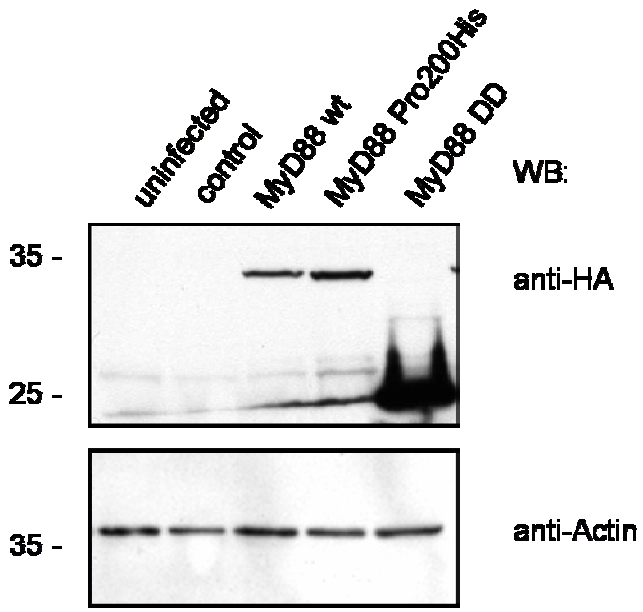


Figure 3.16: Adenoviral constructs encoding human MyD88 wt, MyD88 Pro200His and MyD88 DD are over-expressed in DCs. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing green-fluorescent protein (GFP), human MyD88 wt, human MyD88 Pro200His and MyD88 DD. 24 h post infection cells were lysed with 1 % Triton X 100 lysis buffer and cell lysate was separated by SDS-PAGE. Western blot for MyD88 was performed using mouse anti-HA (Covance, Berkeley, CA, USA). As a loading control the membranes were striped and re-probed for β-actin using a mouse anti-β-actin antibody (Sigma, Poole, Dorset, UK). As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa).

Having confirmed the over-expression of human MyD88 wt, human MyD88 Pro200His and human MyD88 DD (Figure 3.16), DCs were infected with MyD88 wt and subsequently stimulated with LPS. The over-expression of human MyD88 wt in DCs did not lead to an induction or super-induction of TNFα in response to LPS (Figure 3.17). This result is in accordance with murine MyD88 wt over-expression and would suggest that a TIR domain together with a DD is not able to activate human DCs.

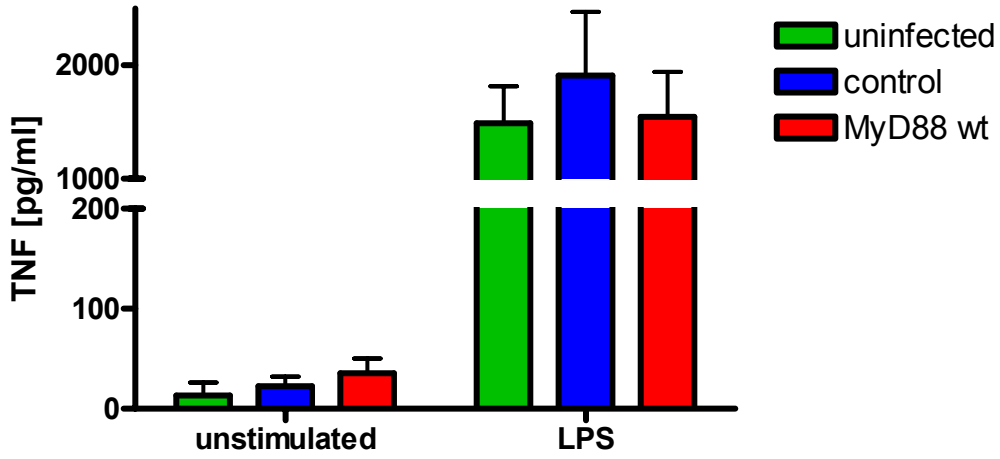


Figure 3.17: Over-expressing human MyD88 wild type (wt) does not induce or super-induce cytokine production in DCs. TNFα induction assayed in culture supernatant by ELISA. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 wt. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα induction assayed by ELISA. Data are expressed as mean ± SEM. n = 5 data is 5 independent experiments pooled.

Consistent with the results using Mal dn and TRAM dn, shown in Figure 3.9 and Figure 3.10, the disruption of a TIR domain also does not induce or super-induce TNFα production in DCs as was observed using the human MyD88 Pro200His construct (Figure 3.18).

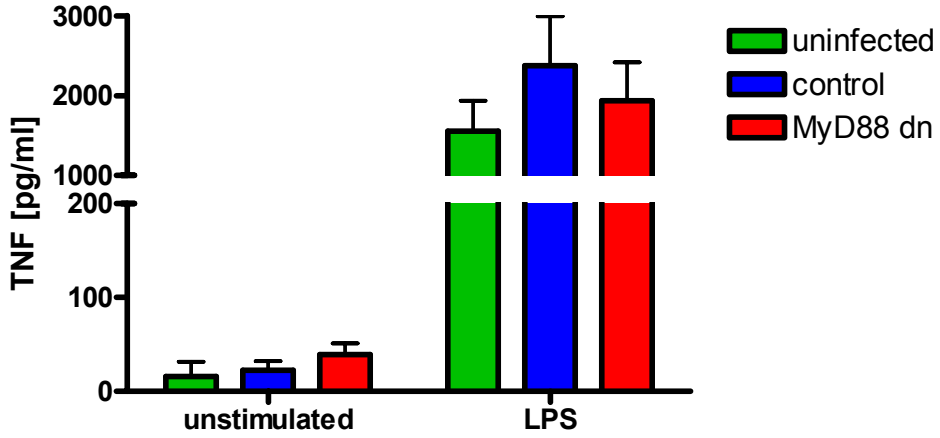


Figure 3.18: Over-expressing human MyD88 Pro200His does not induce or super-induce cytokine production in DCs. TNFα induction assayed in culture supernatant by ELISA. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 Pro200His. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean ± SEM. n = 5; data is 5 independent experiments pooled.

Although the over-expression of a DD alone is not sufficient to significantly induce TNFα production without stimuli, it is sufficient to significantly super-induce TNFα production in LPS stimulated human DCs (Figure 3.19).

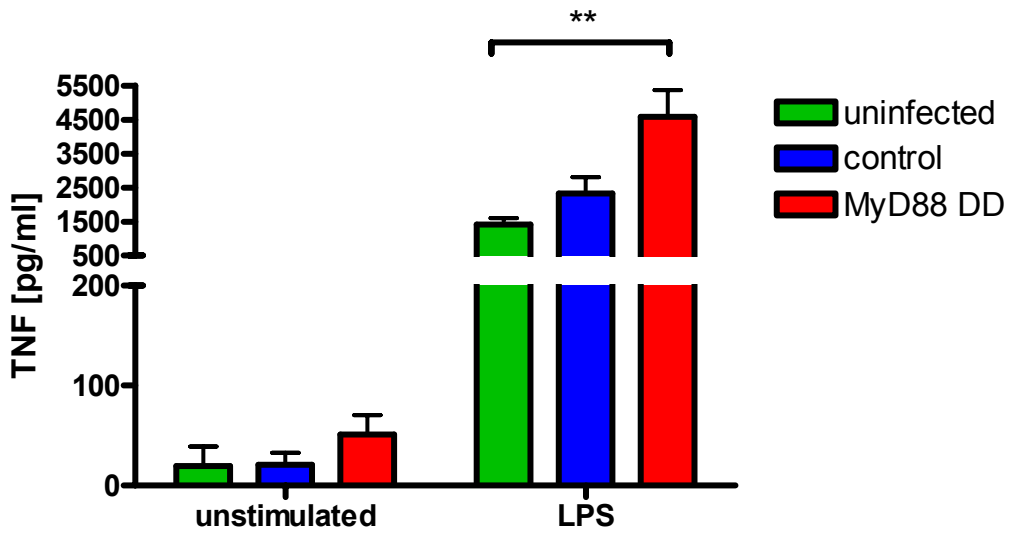


Figure 3.19: Over-expressing human MyD88 death domain (DD) does significantly super-induce cytokine production in DCs (p= 0.048). TNFα induction assayed in culture supernatant by ELISA. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 DD. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4; data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

These results confirm the observations made using the murine MyD88 constructs. Furthermore, they show that similar to a TIR domain the DD is able to increase activation of human DCs following TLR4 stimulation.

3.2.7 MyD88 dn induces the production of TNFα by BMDCs

Given the unexpected result of human DC specific activation by over-expression of a TIR domain this study further investigated if this mechanism exists in mice as well. Therefore, murine BMDCs and BMDMs were infected with murine MyD88 wt or MyD88 dn and cytokine production measured (Figure 3.20).

While the over-expression of MyD88 dn significantly induced the production of TNFα in BMDCs without stimulation, it had no effect on BMDMs (Figure 3.20). This result is consistent with observations made in human DCs, where over-expression of MyD88 dn did induce cytokine production without stimulation but had no effect on human macrophages (Figure 3.6 and Figure 3.9). Furthermore, the over-expression of MyD88 wt had no effect on TNFα

induction by BMDCs or BMDMs (Figure 3.20) again reflecting the observations made in human DCs (Figure 3.11). Taken together, these results suggest, that the DC specific activation by MyD88 dn over-expression is not restricted to humans but is also present in murine models.

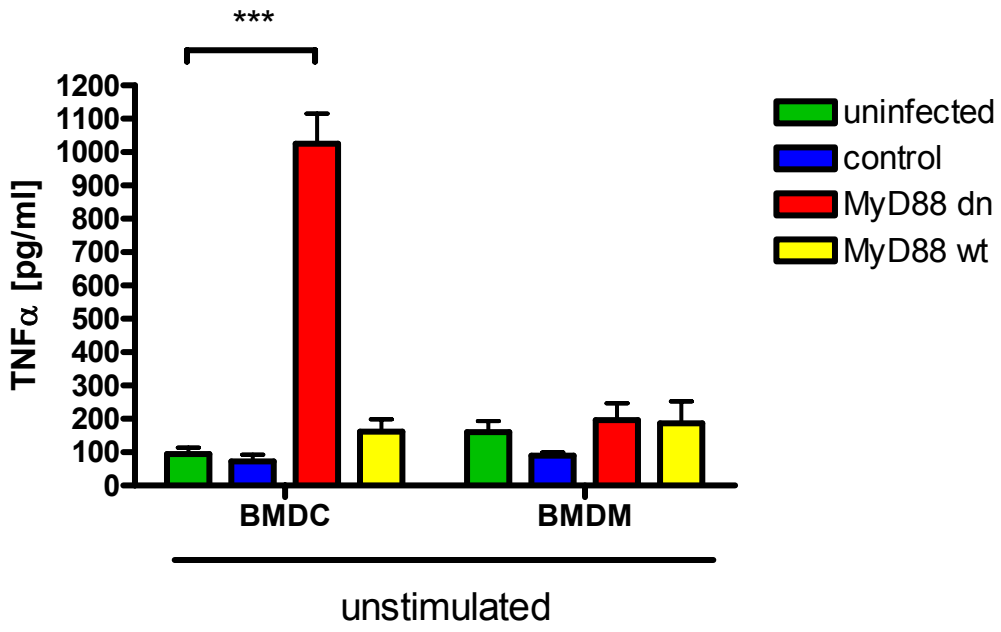


Figure 3.20: MyD88 dn over-expression significantly induces TNFα production in un-stimulated BMDCs ($p \leq 0.0001$) but not BMDMs. TNFα induction assayed in culture supernatant by ELISA. BMDCs and BMDMs were derived from the femurs of wild-type C57Bl/6 mice. BMDCs were cultured for 7 days *in vitro* in the presence of 20 ng/ml GM-CSF in RPMI 5% FCS. After 7 days non-adherent cells were collected and infected with adenoviral constructs. BMDMs were cultured for 7 days in the presence of 10 ng/ml GM-CSF in RPMI 5% FCS. Following 7 day culture adherent cells were collected. BMDCs and BMDMs were left uninfected, or infected (300:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), MyD88 dn or MyD88 wt. 32 h post infection supernatants were collected and TNFα induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4, data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

3.2.8 Co-expression of MyD88 wt reverses the inducing effect of MyD88 dn

It is curious, that while MyD88 wt is not able to induce DC activation, murine MyD88 dn is, as both of these constructs contain a wild type TIR domain. Therefore, it is necessary to investigate if the activation of human and murine DCs by MyD88 dn is a consequence of an inhibition of MyD88 wt activity. In order to examine whether the activation of DCs, by expressing murine MyD88 dn, can be reversed by its wild-type form, murine MyD88 dn and murine MyD88 wt were co-expressed in human DCs.

The induction of IL-6 and TNFα as a result of expressing MyD88 dn was significantly abrogated by the co-expression of MyD88 wt in un-stimulated cells (A, B, Figure 3.21). In LPS

stimulated DCs the levels of IL-6 as well as TNFα super-induced by MyD88 dn expression were reduced to the levels produced by uninfected cells. This suggests that at an m.o.i ratio of MyD88 dn to MyD88 wt of 1:2, MyD88 wt is indeed reversing the activating effect of MyD88 dn (A, B, Figure 3.21). These results may suggest that the over-expression of a TIR domain disrupts an inhibitory signal, which is dependent on MyD88.

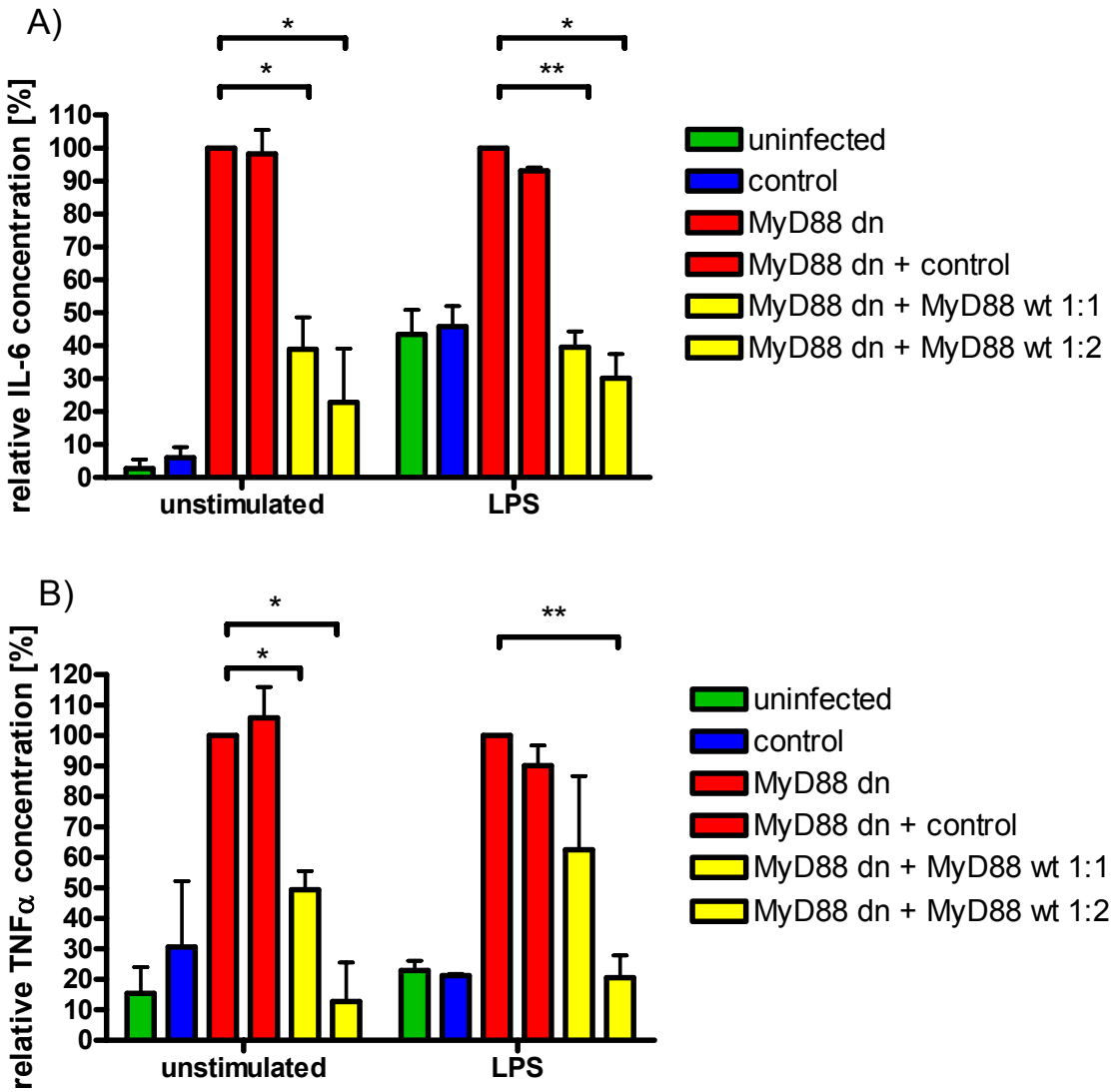


Figure 3.21: Expression of murine MyD88 wt significantly abrogates murine MyD88 dn induced IL-6 (A)(un-stimulated p = 0.0243 and p = 0.0418; stimulated p = 0.0061 and p = 0.0109) and TNFα (B) (un-stimulated p = 0.0143 and p = 0.0208; stimulated p = 0.0084) production in human DCs. IL-6 (A) and TNFα (B) induction assayed in culture supernatant by ELISA. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), murine MyD88 wt, murine MyD88 dn or combinations of them. A m.o.i. of 100 was used for each virus when used at a ratio of 1. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα and IL-6 induction assayed by ELISA. Data are normalised to MyD88 dn only infected cells and are expressed as mean $\pm$ SEM. n = 3; data is 3 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

3.3 Conclusions

The results presented in this chapter suggest a so far unknown unique role of TLR4 signalling in human macrophages as over-expression of MyD88 impairs TLR2, TLR5 and TLR7/8 signalling in those cells but not TLR4. Furthermore, the data presented here indicate a DC specific inhibitory mechanism that depends on MyD88 and keeps immature DCs in an inactive state.

When investigating the role of the TLR adaptor molecules in RASFs, HUVECs and human macrophages, it became apparent that there are differences between cells of myeloid and non-myeloid origin (Andreakos et al., 2004). While, murine MyD88 dn, which was reported to inhibit TLR signalling, diminished TLR4 and IL-1 signalling in RASFs as expected, TLR4 signalling was unaffected in human macrophages. This supports data published by our group, which compared HUVECs and macrophages and found similar results (Andreakos et al., 2004). Further investigation of TLR signalling in macrophages showed that TLR4 signalling in macrophages is exceptional, since TLR2, TLR5 and TLR7/8 driven cytokine induction could be inhibited by over-expressing murine MyD88 dn. This observation is still part of ongoing investigations in our laboratory. A possible explanation could be redundancy between the adaptor molecules, as TLR4 has been shown to be the only TLR to use all four adaptor molecules. However, unpublished work from our group shows that neither Mal dn, TRAM dn nor TRIF dn expression nor the expression of a combination of all four dn adaptor constructs can inhibit TLR4 signalling in human macrophages (unpublished results, Dr. Anna Lundberg). Therefore, TLR adaptor molecules are utilised differently in murine systems, cell lines and primary human cells. This is in accordance with a recent study describing TRAM as an adaptor of TLR4 as well as TLR2 in RASF, while no involvement of TRAM in any TLR signalling pathway could be observed in human macrophages (Sacre et al., 2007c). While the possibility of redundancy between TLR adaptor molecules in human macrophages has been excluded by co-expression of the dominant negative adaptor constructs, the possibility of alternative signalling transducers being utilised remains. CD14 is a well established co-receptor, necessary for TLR4 signalling. However, recent studies described that functions of CD14 are specific to the chemotype of LPS (Jiang et al., 2005b). This might indicate that CD14 has a more predominant function in TLR4 regulation than previously thought. Since RASFs used in this study do not express CD14 and TLR4 signalling is inhibited by MyD88 dn and in contrast human macrophages express CD14 but TLR4 signalling is unaffected by MyD88 dn, this study further investigated TLR4 signalling in a myeloid cell type, which is CD14 negative, the DC.

Unexpectedly the over-expression of murine MyD88 dn in DCs led to an induction of TNFα and IL-6 without stimulation. Furthermore, LPS stimulation led to a super-induction of these

cytokines. In contrast, Mal dn and TRAM dn had no effect on cytokine production in un-stimulated and LPS stimulated DCs. This unexpected result led to the investigation of TLR4 signalling using the wild type constructs of MyD88, Mal and TRAM. However, while murine MyD88 dn does induce TNFα and IL-6, murine MyD88 wt does not. Moreover, Mal wt and TRAM wt both induce and super-induce these cytokines in DCs.

The common structural feature of the constructs inducing TNFα and IL-6 without further stimuli is a wild type TIR domain (Figure 3.1), which might be responsible for this observation. This could be confirmed by using the deletion constructs of Mal and TRIF, which contain the TIR domain only. The only exception to this pattern is wild type MyD88, which is the only adaptor protein with a wild type death domain. In order to investigate the role of a DD further a human wild type, a human point mutation and a human deletion construct of MyD88 were generated at the KIR (Ms Brenda Mutch). The results obtained by over-expressing human MyD88 wt confirmed previous results using murine MyD88 wt as it does not induce or super-induce cytokine production in DCs. This excludes the possibility of an effect related to species differences. Moreover, the over-expression of human MyD88 Pro200His, with a point mutation in its TIR domain, had also no effect on cytokine induction. While over-expression of the DD domain on its own super-induces TNFα, it does not lead to a significant induction of TNFα without any stimuli. Thus showing that while the DD is able to increase cytokine production in LPS stimulated DCs it is not as potent as a TIR domain to induce cytokine production in un-stimulated DCs.

3.3.1 Potential models to explain the activation of DCs by TIR domains

TIR domain over-expression leads to induction and super-induction of TNFα and IL-6 in human DCs. This unexpected result might be explained by a DC specific negative regulation of TLR signalling. Based on this premise I proposed three models to explain this observation (Figure 3.22).

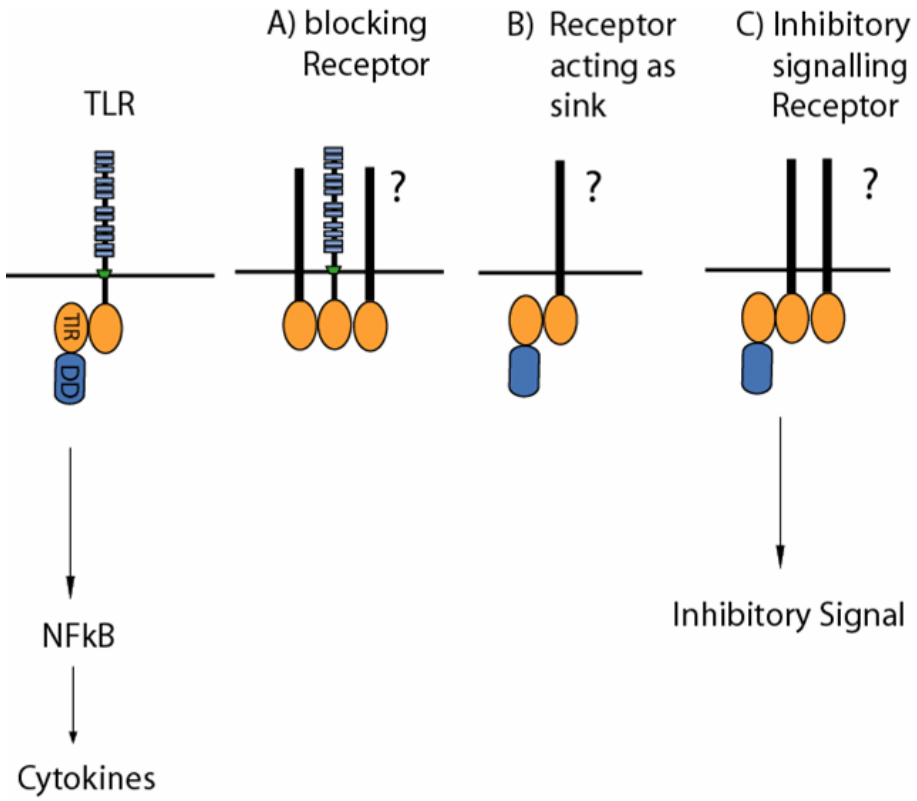


Figure 3.22: Role of a hypothetical, dendritic cell specific, inhibitory receptor for TLR signalling. LPS stimulation of human DCs induces the expression of TNFα and IL-6. Over-expression of a TIR domain results in the induction of TNFα and IL-6 without further stimuli implicating a DC specific negative regulation of TLR signalling, which is disrupted by the over-expression of an intact TIR domain. A) This inhibition could be through a receptor blocking access to the TLR. Over-expression of a TIR domain would release the TLR and could induce signalling. B) Another model would propose a TIR domain containing receptor acting as a sink and thereby keeping MyD88 away from the TLR. C) A third model proposes a TIR domain containing receptor, which constitutively induces an inhibitory signal in immature DCs. This signal is dependent on MyD88. Abbreviation: TIR (Toll/IL-1R), DD (death domain), TLR (toll-like receptor), NF-κB (nuclear factor κB)

A TLR blocking receptor could restrict access to the TLR, thereby keeping the adaptor proteins from interacting with the TLR and impairing the signal. By over-expressing a TIR domain the blocking receptor would be replaced and the TLR would be accessible for the adaptor molecules. Consequently an activating signal would be induced. This model, however, does not explain why over-expression of murine or human MyD88 wt, containing an intact TIR domain, do not induce cytokine production (A, Figure 3.22).

A TIR domain containing molecule could act as a sink thereby sequestering TLR adaptor molecules away from the TLR and inhibiting TLR signalling. By over-expressing a TIR domain the sequestered adaptor molecules are released and can bind the TLR, consequently inducing a signal. However, this model, again, does not explain why over-expression of murine and human MyD88 wt does not induce cytokine production (B, Figure 3.22).

Therefore, a third model proposes a TIR domain containing receptor, which constitutively induces an inhibitory signal, which is dependent on MyD88. Over-expression of a TIR domain

would displace MyD88 from the inhibitory receptor, thereby impairing the inhibitory signal (C, Figure 3.22). Consequently, the immature DC becomes activated and induces cytokine production. Over-expression of MyD88 wt would have no effect, since the inhibitory signal is dependent on MyD88 (B, Figure 3.23). Over-expression of a DD domain, while being able to super-induce cytokine production through blocking the DD domain of MyD88, is not able to replace MyD88 from the inhibitory receptor and thus is not potent enough to induce cytokine production without further stimuli (C, Figure 3.23).

A prediction from this third model is that MyD88 wt should compete with other TIR domain containing molecules for access to the inhibitory receptor when being over-expressed. Therefore, the cytokine inducing effect of MyD88 dn should be reversible by co-expression with MyD88 wt. That this is the case could be demonstrated in Figure 3.21, where an increase in m.o.i. of MyD88 wt leads to a titratable reduction of IL-6 and TNFα induced by the expression of murine MyD88 dn. This observation further strengthens the hypothesis of a DC specific constitutive inhibitory signal, which is disrupted by the over-expression of a TIR domain (Figure 3.23).

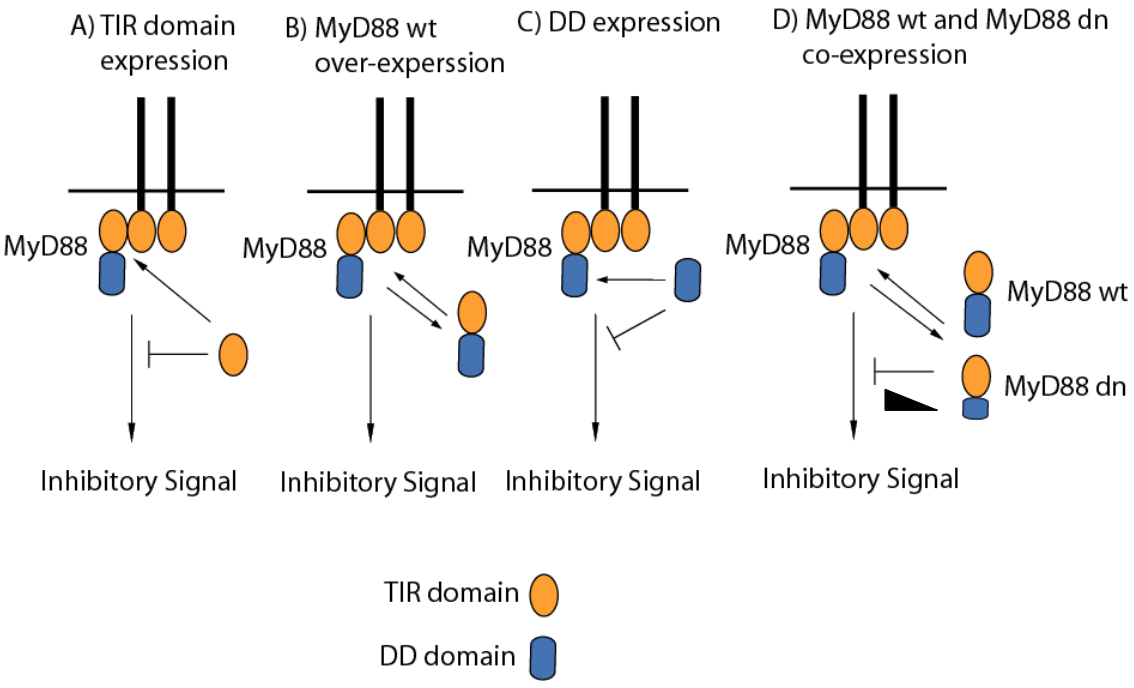


Figure 3.23: Characteristics of a constitutive inhibitory signal induced by MyD88 in dendritic cells. A) Expression of a wild type TIR domain in dendritic cells leads to the displacement of MyD88 from the inhibitory receptor. Consequently the inhibitory signal is impaired and leads to the activation of DCs. B) However, over-expression of MyD88 wt in DCs has no effect since it only replaces MyD88 wt with MyD88 wt. C) While the expression of the DD domain is able to block the DD domain of MyD88 it is not able to displace MyD88 from the inhibitory receptor and therefore, has not the same potency as a TIR domain to activate DCs D) The co-expression of MyD88 wt with MyD88 dn is able to gradually reverse the induction of cytokines in DCs through competing for the TIR domain of the inhibitory receptor.

Several other predictions can be made from this model. The proposed inhibitory receptor must be expressed in immature DCs but not in macrophages as no cytokine induction is observed in macrophages following the expression of MyD88 dn. Since this constitutive inhibitory signal would need to depend on MyD88 it follows that, MyD88 must be bound to the inhibitory receptor in a constitutive manner without stimuli present. Besides having an activating function in the case of TLR signalling, MyD88 would also need to be able to transduce an inhibitory signal. This would require an unexpected functional versatility of MyD88. A recent report describing the involvement of MyD88 in IFN-γR signalling would support a multifaceted function of MyD88 (Sun and Ding, 2006). Although IFN-γR possesses no TIR domain, IFNγ induced stabilization of IP10 mRNA depends on MyD88 to activate p38 MAPK (Sun and Ding, 2006). Moreover, it has been shown that TIR domain containing adaptor molecules can have inhibitory roles instead of signalling inducing functions. A study by Carty *et al.* describes inhibitory functions of SARM, which are specific for TRIF dependent signal induction (Carty et al., 2006).

TLRs recruit adaptor molecules through dimerisation following ligand binding (Akira and Takeda, 2004). The model proposed above would suggest that MyD88 binds with a higher affinity to the TIR domain of the inhibitory receptor in the un-stimulated state, most likely because the inhibitory receptor is constitutively dimerised. Following TLR stimulation, MyD88 dissociates from the inhibitory receptor and is recruited to the TLR, thereby allowing the activation of the DC.

The model of a constitutive inhibitory signal specific for DCs would propose a mechanism, which keeps immature DCs in an inactivated state. This would increase the threshold necessary to activate a DC and thereby to activate the adaptive immune response. Such a mechanism would control the risk of unwanted and exaggerated inflammatory responses.

Since TLR signalling leads to a strong inflammatory response it is necessary to be controlled at all levels of the signalling pathway (Lang and Mansell, 2007; Liew et al., 2005). Most TLR inhibitors described, however, do not show a dendritic cell specific expression pattern. SIGIRR, an IL-1R family member, is the exception. SIGIRR was shown to be highly expressed in murine and human epithelial cells and immature DCs but not in macrophages (Polentarutti et al., 2003). Furthermore, SIGIRR also has been shown to be an inhibitor of TLR signalling in mice (Garlanda et al., 2004; Qin et al., 2005; Wald et al., 2003). This makes SIGIRR a possible candidate for the model proposed in this chapter and therefore, became the focus of my investigation.

CHAPTER 4

SIGIRR ACTS AS A TLR INHIBITOR IN PRIMARY HUMAN MYELOID CELLS

4.1 Introduction

Based on the results obtained in Chapter 3, an inhibitory mechanism specific for DCs has been proposed, which is disrupted by the over-expression of a TIR domain. This model would provide a mechanism that keeps DCs from becoming activated in situations where excessive inflammation would be damaging to the host.

In order to keep a potent inflammatory response in check the signalling pathways inducing it must be tightly regulated. Inhibitory mechanisms of TLR signalling have been described at all levels of signal transduction. In the case of TLR2 and TLR4, extracellular decoy receptors have been shown to function as antagonists, inhibiting the binding of the ligand to the receptor. Intracellular inhibitors of TLR signalling include TRIAD3A, MyD88s, IRAK-M, TOLLIP and A20 (reviewed in (Lang and Mansell, 2007; Liew et al., 2005)). These inhibitors regulate TLR signalling by different mechanisms including the ubiquination and consequent degradation of TLRs, e.g. TRIAD3A, blocking access to the TLR, e.g. IRAK-M and TOLLIP, or sequestering signalling transducers, e.g. MyD88s.

Transmembrane inhibitors include the IL-1R family members SIGIRR and ST2 as well as the TLR4 homologue RP105. Unlike SIGIRR and ST2, RP105 does not posses an intracellular TIR domain. The IL-1R family member ST2 exists in two isoforms, the membrane bound ST2L and the secreted sST2 (Lang and Mansell, 2007). ST2L inhibits IL-1R and TLR signalling by sequestering MyD88 and Mal through its TIR domain in murine macrophages (Brint et al., 2004). On the other hand, sST2 has been shown to act as an antagonist to ST2L in T cells by sequestering its ligand IL-33 (Hayakawa et al., 2007). Similar to ST2, SIGIRR is also a member of the IL-1R family. Its mode of action has been described to be a blocking receptor, as it interacted with IL-1R, TLR4, MyD88, IRAK1 and TRAF6 in HEK293 cells, thereby inhibiting IL-1R and TLR4 signalling (Qin et al., 2005). In addition to containing a TIR domain, SIGIRR, unlike all the above described inhibitors, has been shown to have a very specific expression pattern, being expressed in murine and human monocytes and immature DCs but not in macrophages (Polentarutti et al., 2003).This makes SIGIRR a candidate for the inhibitory mechanism proposed in chapter 3.

So far, investigations on SIGIRR have concentrated on its expression and function in mice models. In order to investigate SIGIRR in primary human cells this chapter examines the expression pattern in primary human myeloid and non-myeloid cells. Furthermore it investigates the function of SIGIRR in human monocyte derived DCs as well as macrophages and describes the signalling pathways regulated by SIGIRR. The results in this chapter describe SIGIRR as an inhibitor of the MyD88-dependent as well as MyD88-independent TLR signalling pathway in primary human cells.

4.2 Results

4.2.1 SIGIRR mRNA is expressed in primary human monocytes and dendritic cells but is differentially expressed in macrophage subtypes

The first prediction of the inhibitory mechanism proposed in Chapter 3 is a dendritic cell specific expression of a TLR inhibitory molecule. In case of SIGIRR this expression pattern has been described in murine as well as human cell systems in a study by Polentarutti *et al.* (Polentarutti et al., 2003). To confirm these results, real-time PCR was performed on mRNA extracted from primary human monocytes, M-CSF differentiated macrophages, GM-CSF differentiated macrophages, DCs, RASF and human skin fibroblasts (Figure 4.1).

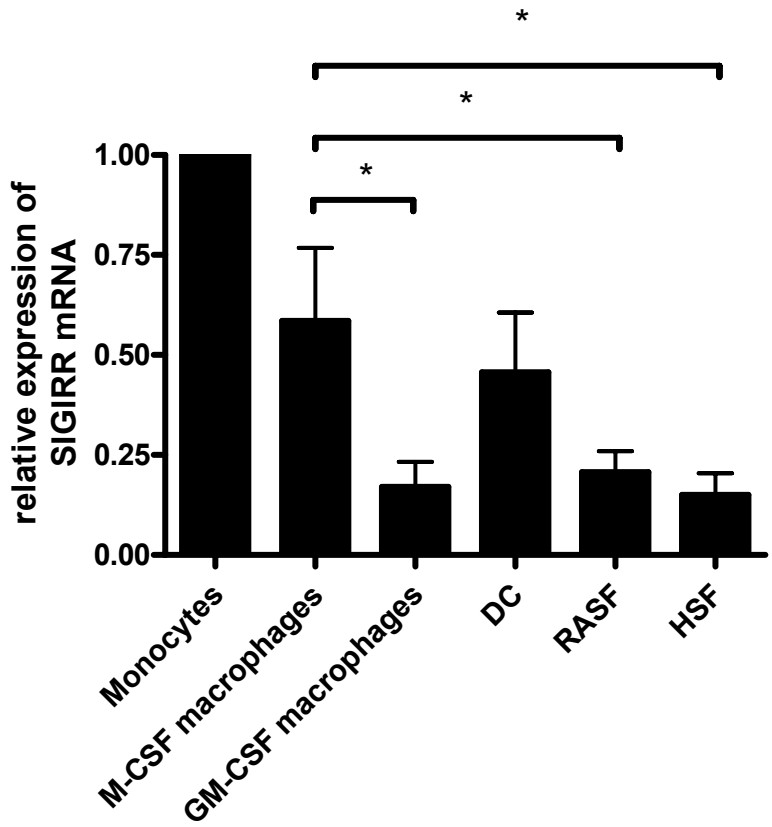


Figure 4.1: SIGIRR is expressed in primary human monocytes, M-CSF differentiated macrophages and DCs and expression is significantly reduced in GM-CSF differentiated macrophages (p = 0.0378), RASF (p = 0.0473) and HSF (p = 0.0315). Monocytes were isolated from peripheral blood mononuclear cells by elutriation. mRNA from $5x10^6$ purified monocytes was extracted. The remaining monocytes were differentiated into macrophages using 100 ng/ml M-CSF or 50 ng/ml GM-CSF for 4 days or dendritic cells using 50 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 days in 10% FCS RPMI. RASF and HSF were used from passage 3 after expansion in DMEM 10% FCS. mRNA from $5x10^6$ cells was extracted and Taqman PCR performed using GAPDH as a housekeeping gene. Data is normalised to GAPDH and expressed relative to monocytes. Data are expressed as mean $\pm$ SEM. n = 4, this data is 4 independent experiments pooled $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***. Abbreviations: DC, dendritic cell; RASF rheumatoid arthritis synovial fibroblast; HSF, human skin fibroblast.

The results obtained by Taqman PCR show the already described highly specific expression pattern of SIGIRR in myeloid and non-myeloid cells (Figure 4.1). SIGIRR mRNA is expressed at high levels in monocytes, to which mRNA levels of the other cells investigated has been normalised. The mRNA expression is substantially reduced during differentiation to macrophages using M-CSF and to dendritic cells. Therefore, contrary to previous reports, M-CSF differentiated macrophages used in this study express similar amounts of SIGIRR as DCs. Furthermore, macrophages differentiated using GM-CSF show a further significant reduction in SIGIRR mRNA expression. Similarly to GM-CSF differentiated macrophages, RASF as well as HSF express lower levels of SIGIRR mRNA compared to M-CSF differentiated macrophages or DCs (Figure 4.1).

4.2.2 Western blot does not allow the specific detection of SIGIRR protein

To date the expression pattern of SIGIRR has not been investigated at the protein level. In order to confirm the differential expression of SIGIRR mRNA in human cells, western blots were performed.

However, the anti-human SIGIRR antibodies commercially available do not detect SIGIRR specifically by western blot (Figure 4.2).

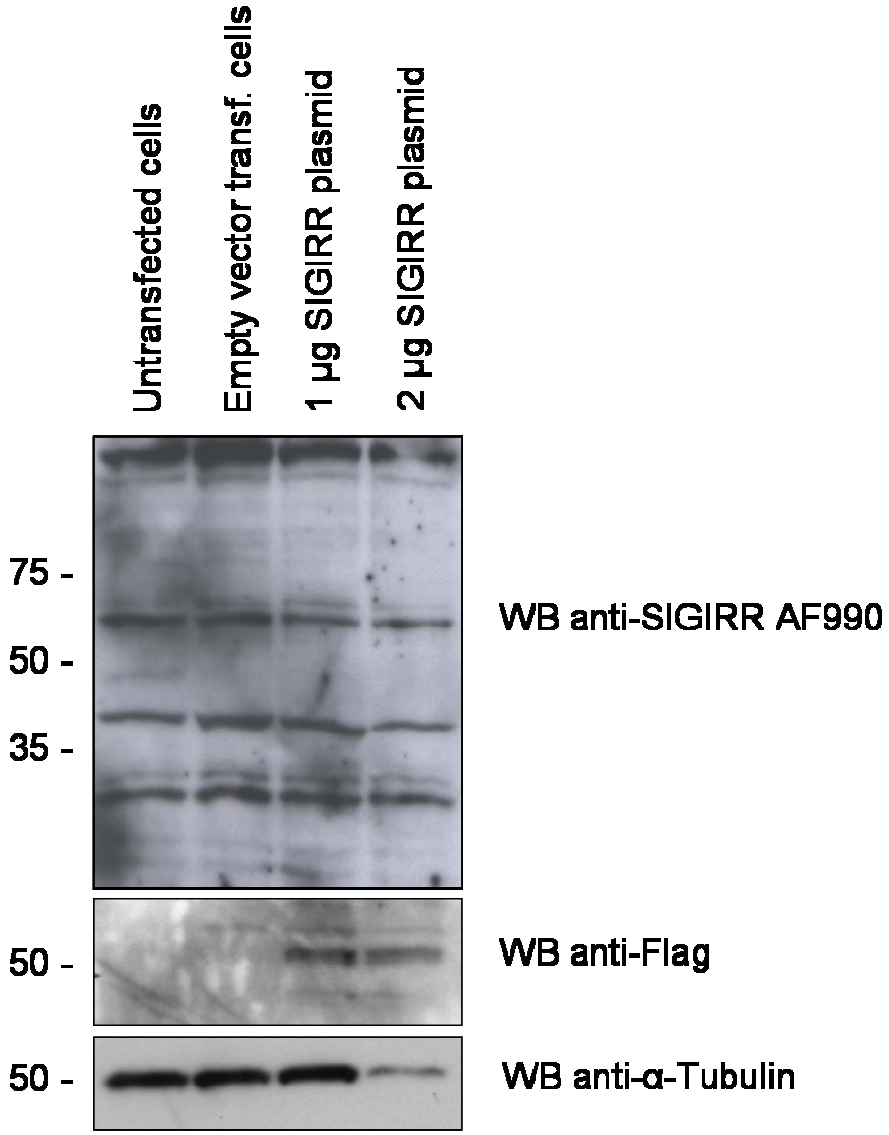


Figure 4.2: SIGIRR is not detected by western blot in whole cell lysate of HEK293. HEK293 cells were transfected with 1 µg and 2 µg of pENTR4.3F plasmid encoding Flag tagged SIGIRR. Cells were left for 24 h, where after they were lysed in 1% Triton X 100, the protein concentration measured by BCA assay and lysate separated by SDS-PAGE. A total amount of 50 µg of protein was loaded per condition. As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa). Membrane was stained using polyclonal anti-human SIGIRR antibody AF990 (R&D, Minneapolis, USA). Membrane was stripped and counter-blotted for Flag (Sigma, Poole, Dorset, UK) and as a loading control α-tubulin (Sigma Poole, Dorset, UK).

HEK293 cells were transfected with 1 µg or 2 µg of pENTR4.3F vector encoding Flag tagged SIGIRR (kindly provided by Prof X. Li, Lerner Research Institute, OH, USA). After 24 h of over-expression the cells were lysed and proteins separated by SDS-PAGE. The membrane was stained using a polyclonal anti-human SIGIRR antibody. However, no specific band was detected using this antibody (Figure 4.2). By counter-blotting the membrane, using an anti-Flag antibody, the over-expression of SIGIRR in these cells could be confirmed (Figure 4.2). Therefore, this antibody, as well as others tested, does not detect SIGIRR by western blot.

Triton X 100 as well as RIPA lysis buffer was used to lyse the cells. Using PVDF or nitrocellulose membranes to transfer the protein also showed no improvement in the quality of the western blot. Beside the polyclonal anti-SIGIRR antibody AF990, the following antibodies could also not specifically detect SIGIRR by Western Blot (data not shown):

- anti-SIGIRR, ProSci, Poway, California, USA, Cat. No. 3367
- anti-TIR8, Abcam, Cambridge, UK, Cat. No. ab22053
- anti-SIGIRR, Abcam, Cambridge, UK, Cat. No. ab13717
- anti-SIGIRR, R and D, Minneapolis, Minnesota, USA, Cat. No. MAB990

Although, one publication does show the detection of SIGIRR protein in HEK293 cells by using the polyclonal anti-SIGIRR antibody AF990, I was not able to reproduce these results (Qin et al., 2005).

4.2.3 SIGIRR protein expression can be specifically detected by Flow cytometry and confirms mRNA expression pattern

Flow cytometry is another method to examine protein expression. Unlike western blots, flow cytometry requires the recognition of the natural epitope by the antibody. Therefore, it is possible that while some antibodies do not detect the denatured epitope, they are able to detect a protein specifically by Flow cytometry.

Monocytes, M-CSF as well as GM-CSF differentiated macrophages and DCs were stained for SIGIRR on the cell surface. This did not result in a positive staining (Figure 4.3). Therefore, cells were permeabilized and stained for SIGIRR intra-cellular. Interestingly, permeabilization of monocytes, M-CSF differentiated macrophages and DCs resulted in a positive staining for SIGIRR while there was no SIGIRR expression detectable in permeabilized GM-CSF differentiated macrophages (Figure 4.3). These results are in accordance with the expression of SIGIRR mRNA detected by TaqMan PCR (Figure 4.1).

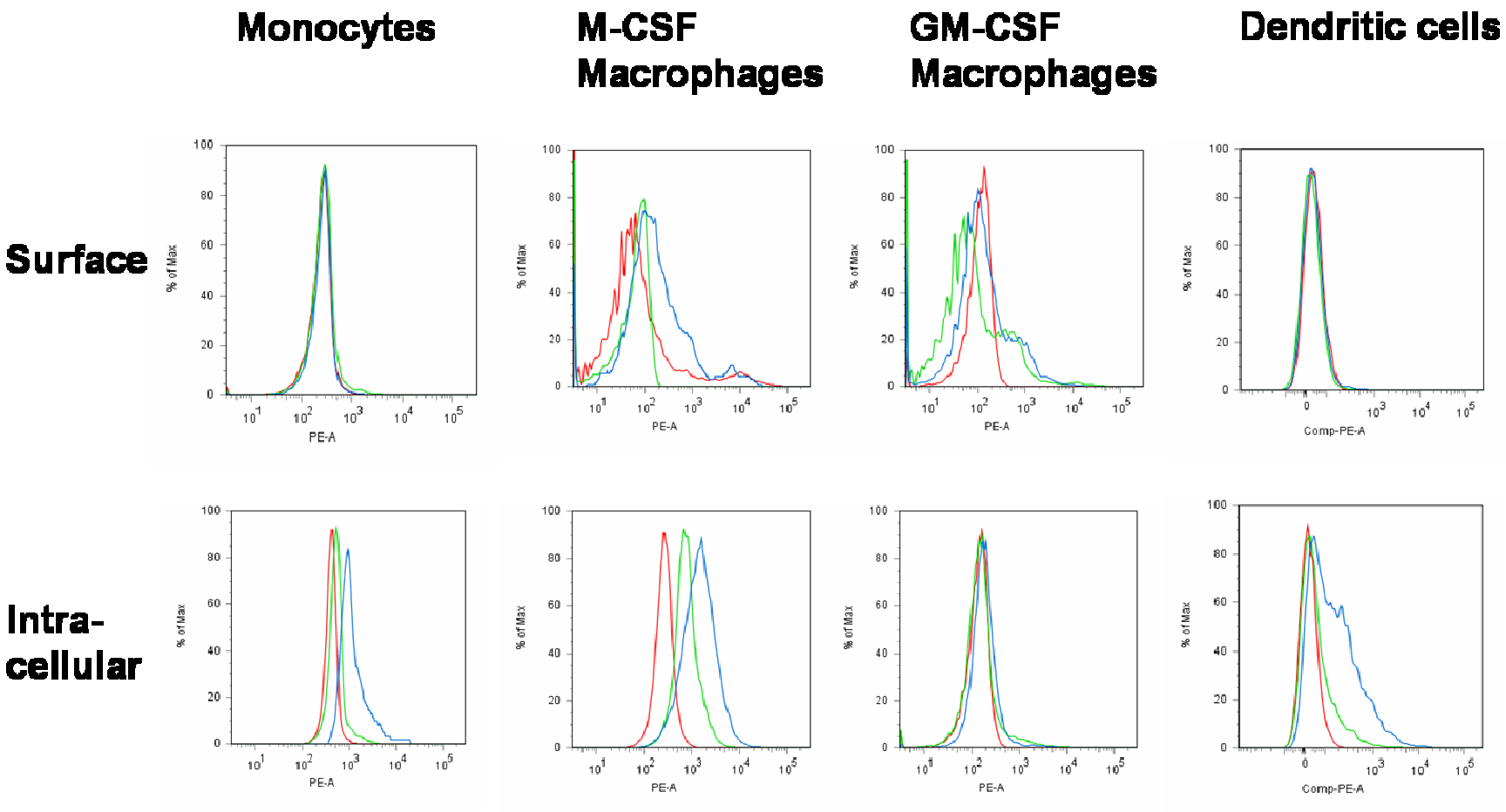


Figure 4.3: Monocytes and M-CSF differentiated macrophages as well as DCs express SIGIRR intra-cellular. Monocytes were isolated from peripheral blood mononuclear cells by elutriation. Cells were stained using BAF990 anti-SIGIRR antibody (R&D, Minneapolis, USA). The remaining monocytes were differentiated into macrophages using 100 ng/ml M-CSF or 50 ng/ml GM-CSF for 4 days or dendritic cells using 50 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 days in 10% FCS RPMI. Cells were stained for SIGIRR using, polyclonal antibody BAF990. Red line, unstained cells; Green line, isotype control stained cells; blue line, SIGIRR stained cells. Experiment shown is representative of 3 independent experiments.

Although, the Isotype control used in this experiment did not indicate unspecific staining and the GM-CSF macrophages stained negative for SIGIRR, there is still the possibility of unspecific binding of the SIGIRR antibody used, which would lead to false positive results. This can be especially the case in permeabilized cells.

To exclude the possibility of unspecific staining an adenovirus expressing Flag-tagged, wild type, human SIGIRR was generated to over-express SIGIRR in DCs and macrophages (Figure 4.5).

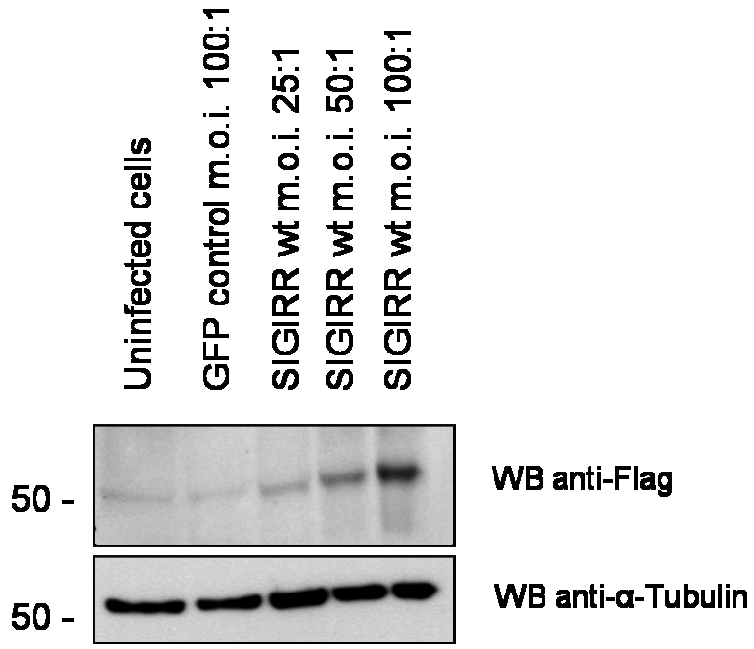


Figure 4.4: DCs over-express SIGIRR wt after adenoviral infection at an m.o.i of 100:1. Dendritic cells were used at day 6 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected (cells), or infected for 2 h in serum-free medium with adenoviruses expressing GFP and SIGIRR wt (25:1, 50:1, or 100:1 m.o.i.). 24 h post infection cells were lysed with 1 % Triton X 100 lysis buffer and cell lysate was separated by SDS-PAGE. Western blot was performed using a mouse anti-Flag antibody (SIGMA, Poole, Dorset, UK). As a loading control the membranes were striped and re-probed for α-tubulin using a mouse anti-α-tubulin antibody (Sigma, Poole, Dorset, UK). As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa).

Infection of DCs with an adenovirus expressing SIGIRR wt led to a titratable over-expression of SIGIRR in these cells as observed by Western Blot for Flag (Figure 4.4). In order to assess anti-SIGIRR antibody specificity, flow cytometry of SIGIRR over-expressing DCs was performed (Figure 4.5).

DCs over-expressing SIGIRR were surface stained for SIGIRR using BAF990 antibody, which resulted in a small fraction of SIGIRR over-expressing DCs being stained positive for SIGIRR

(Figure 4.5). However, permeabilization of the cells followed by SIGIRR staining, showed, that the majority of DCs over-express SIGIRR intra-cellular (Figure 4.5).

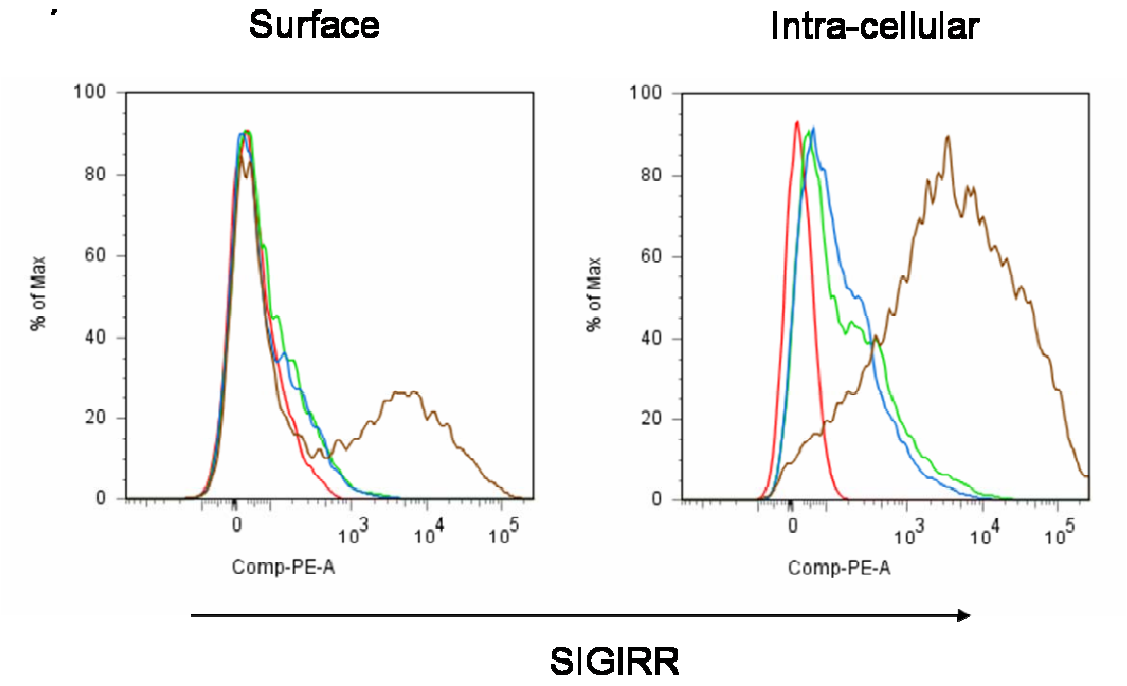


Figure 4.5: Anti-human SIGIRR antibody BAF990 recognises SIGIRR specifically. SIGIRR is expressed in DCs intracellularly. DCs were used at day 5 of *in vitro* differentiation using 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. Cells were left for 24 h before being stained for SIGIRR using biotynulated, polyclonal antibody BAF990 (R&D, Minneapolis, USA). Red line, SIGIRR over-expressing, unstained cells; Green line, SIGIRR over-expressing, isotype control stained cells; blue line, control virus infected, SIGIRR stained cells; brown line, SIGIRR over-expressing, SIGIRR stained cells. Experiment shown is representative of 2 independent experiments.

The expression pattern of SIGIRR protein and mRNA described in this chapter might exclude SIGIRR as a DC-specific TLR inhibitor since it is expressed in M-CSF differentiated macrophages (Figure 4.1 and Figure 4.3). However, several interesting questions arise, which have so far not been answered in the literature. Does SIGIRR function as a TLR inhibitor in primary human cells? Does the cellular localisation have any influence on the function of SIGIRR as a TLR inhibitor in these cell types? Since an adenoviral construct expressing SIGIRR wt has been generated, it was possible to address these questions for the first time in primary human cells.

4.2.4 SIGIRR wt over-expression inhibits TLR and IL-1R signalling in DCs

SIGIRR has already been shown to inhibit TLR and IL-1R signalling *in vivo* in mice as well as *in vitro* in cell lines (Garlanda et al., 2004; Wald et al., 2003). In order to examine if SIGIRR functions as an inhibitor of TLR signalling in primary human cells, an adenoviral vector, over-expressing SIGIRR wt, was infected into primary human DCs (Figure 4.7 and Figure 4.8). The over-expression of SIGIRR wt at m.o.i. of 25, 50 and 100 has been assessed (Figure 4.3) and subsequently DCs were infected at an m.o.i. of 50 and 100. At these m.o.i. adenoviral infection of DCs had no effect on cell viability as assessed by MTT assay (Figure 4.6).

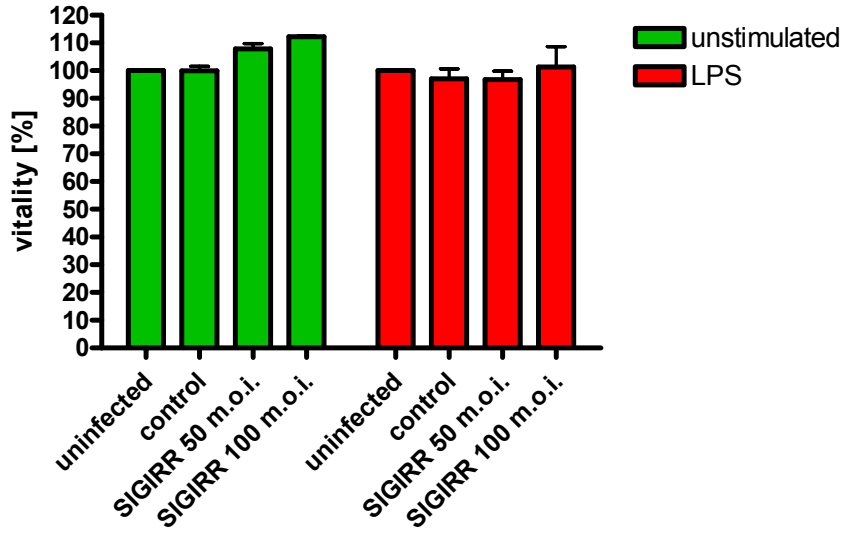


Figure 4.6: SIGIRR over-expressing DCs show no reduced vitality. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (50:1 or 100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 24 h post infection cells were stimulated with 100 ng/ml LPS. After further 18 h cells were incubated with MTT over night, lysis performed and OD574 measured. Data are normalised to uninfected cells and expressed as mean $\pm$ SEM. n = 3, data is 3 independent experiments pooled.

SIGIRR wt over-expression in human DCs significantly inhibits the production of TNFα (A, Figure 4.7) as well as IL-10 (B, Figure 4.7) at an m.o.i. of 100 in response to LPS, MALP-2, Flagellin and R848. Although a trend of inhibition of TNFα induced by Flagellin or IL-1α was observed it did not reach statistical significance due to donor variability and limited number of repeats. No significant levels of TNFα or IL-10 were being produced by human DCs in response to poly I:C (A and B, Figure 4.7). The inhibition of TNFα production in response to TLR stimuli as well as IL-1 is titratable. Furthermore, while TLR and IL-1R signalling is inhibited by SIGIRR over-expression, TNFα induced IL-10 production is unaffected, suggesting functional specificity of SIGIRR inhibition (B, Figure 4.7).

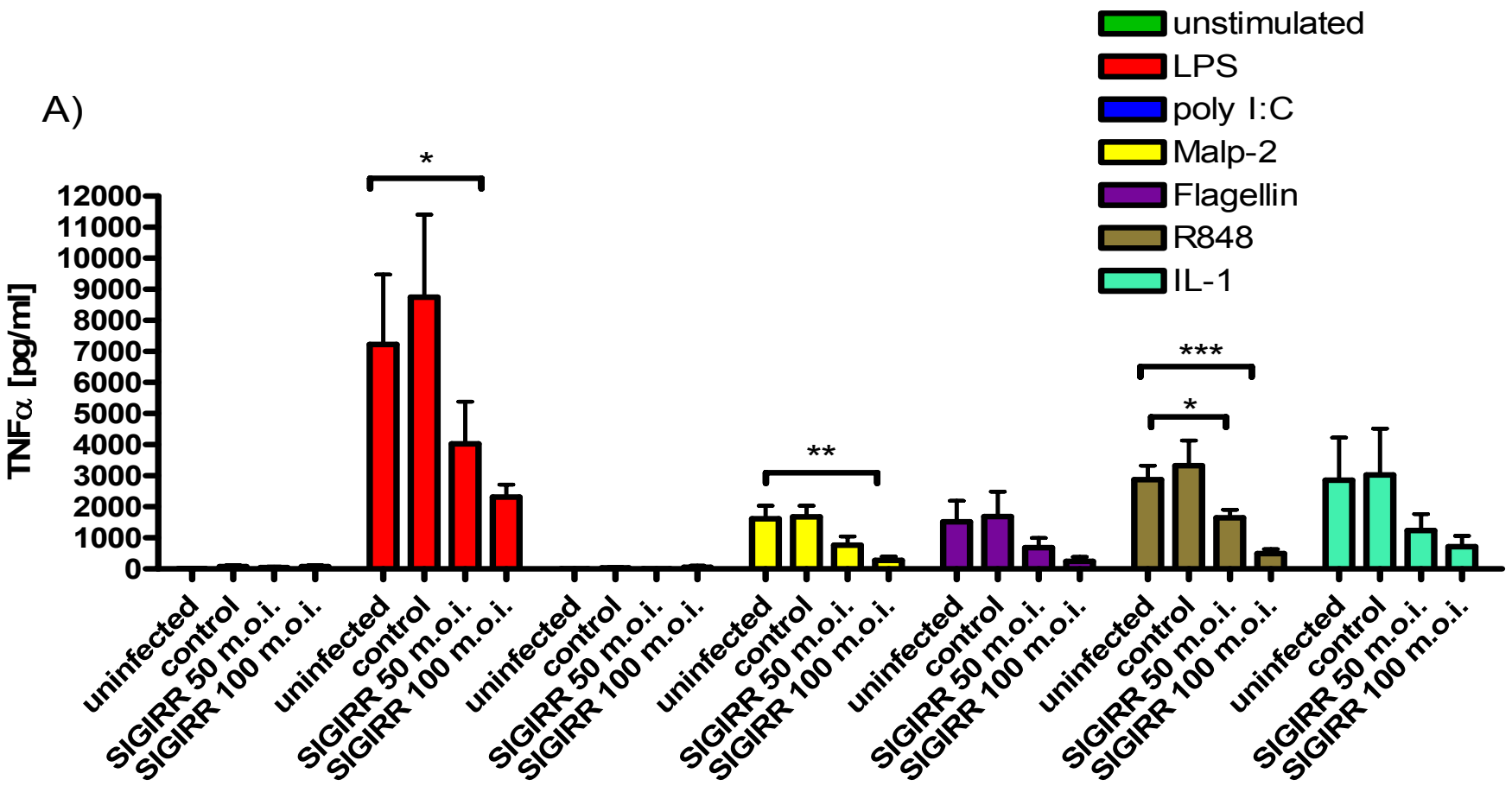


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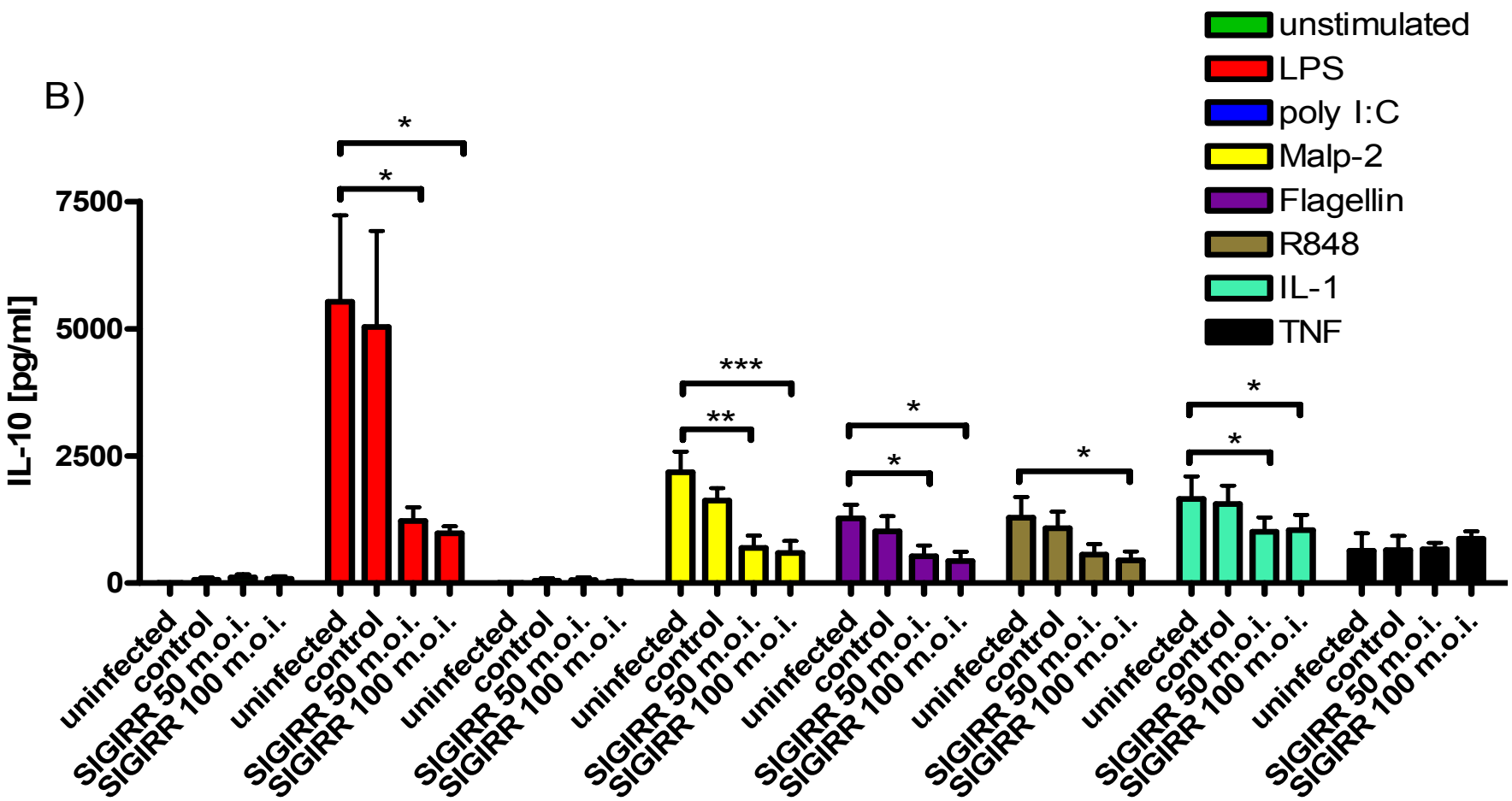


Figure 4.7: SIGIRR wt inhibits TLR4, TLR2, TLR5, TLR-7/8 and IL-1R signalling in primary human monocyte derived DCs. Cytokine induction assayed in culture supernatant by ELISA (TNFα, A; IL-10, B). Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (50:1 or 100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 24 h post infection cells were stimulated with 100 ng/ml LPS, 20 µg/ml poly I:C, 100 ng/ml MALP-2, 100 ng/ml Flagellin, 100 ng/ml R848, 20 ng/ml IL-1α or 20 ng/ml TNFα. After further 18 h supernatants were collected and TNFα and IL-10 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 5, data is 5 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

Induction of TNFα and IL-10 is dependent on the MyD88 induced pathway, whereas IP-10 induction depends on TRIF induced signalling, which is utilised by TLR3 and TLR4. In order to investigate if SIGIRR over-expression also inhibits the TRIF pathway, IP10 levels were measured in cell supernatants (Figure 4.8).

As well as inhibiting the MyD88 dependent pathway, SIGIRR over-expression in DCs also inhibited the TRIF dependent pathway (Figure 4.8). LPS and poly I:C induced production of IP10 was significantly inhibited by the over-expression of SIGIRR in a titratable manner (Figure 4.8). As expected, no significant levels of IP10 were produced in response to MALP-2, Flagellin, R848, IL-1 and TNFα.

These results, for the first time, show, that SIGIRR is an inhibitor of the MyD88 dependent TLR signalling pathway in primary human cells, thereby, confirming published data in mice. Moreover, they also show an inhibitory function of SIGIRR in the TRIF dependent pathway.

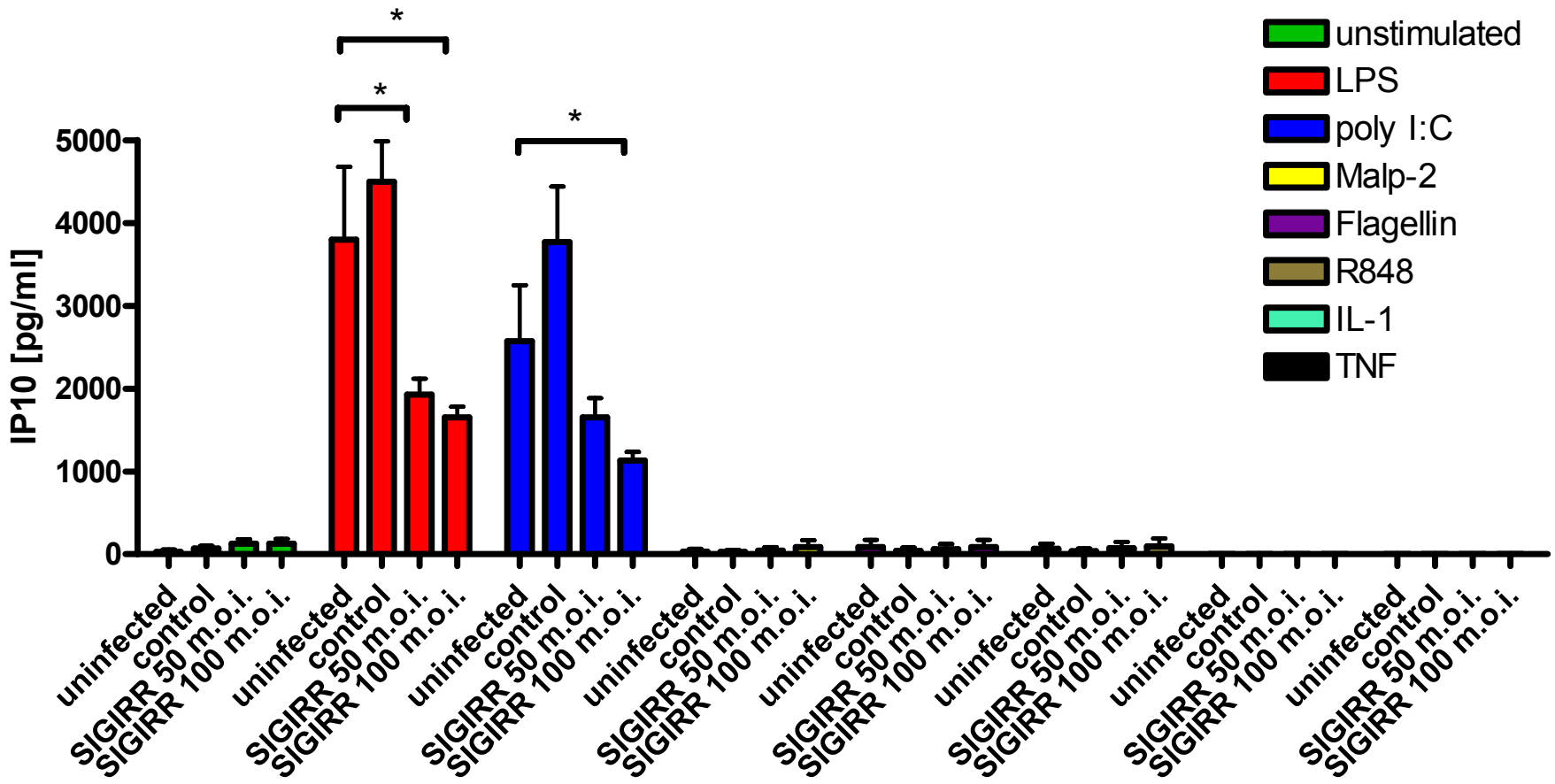


Figure 4.8: SIGIRR wt inhibits TLR3 and TLR4 induced IP10 production in primary human monocyte derived DCs. IP10 induction assayed in culture supernatant by ELISA. Dendritic cells were used at day 5 of *in vitro* differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (50:1 or 100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 24 h post infection cells were stimulated with 100 ng/ml LPS, 20 µg/ml poly I:C, 100 ng/ml MALP-2, 100 ng/ml Flagellin, 100 ng/ml R848, 20 ng/ml IL-1α or 20 ng/ml TNFα. After further 18 h supernatants were collected and IP10 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 5, data is 5 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

4.2.5 SIGIRR wt over-expression inhibits TLR and IL-1R signalling in M-CSF macrophages

To further establish the role of SIGIRR as an inhibitor in primary human cells, SIGIRR wt was over-expressed in M-CSF differentiated macrophages, where it was shown to be expressed previously (Figure 4.1 and Figure 4.5). Subsequently, M-CSF differentiated macrophages were stimulated with LPS, MALP-2, Flagellin, R848, IL-1α or TNFα and the levels of TNFα, IL-10 and IP10 measured in response to these stimuli (Figure 4.9).

Similar to the observations made in DCs, SIGIRR inhibits TNFα and IL-10 production in response to TLR4 (LPS), TLR2 (Malp-2), TLR5 (Flagellin), TLR7/8 (R848) and IL-1R (IL-1α) stimuli in macrophages (Figure 4.9). The inhibition of TNFα production is titratable and is significantly inhibited at an m.o.i. of 100 in response to LPS, Flagellin and R848 (A, Figure 4.9). Moreover, IL-10 production is significantly inhibited at an m.o.i. of 100 in response to LPS, Malp-2, Flagellin and R848 (B, Figure 4.9). Although a trend of inhibition of TNFα induced by Malp-2 or IL-1α was observed it did not reach statistical significance due to donor variability and limited number of repeats. While SIGIRR over-expression inhibits all TLRs tested, it does not inhibit TNFα induced IL-10 production (B, Figure 4.9). No significant levels of TNFα and IL-10 were produced in response to poly I:C in human macrophages. In order to investigate the effect of SIGIRR over-expression on TLR3 signalling, IP10 levels in cell supernatants were examined (C, Figure 4.9). Similar to DCs, TLR4 as well as TLR3 induced IP10 production was significantly inhibited at an m.o.i. of 100 in SIGIRR over-expressing macrophages (C, Figure 4.9). No significant levels of IP10 were produced in response to other TLR stimuli demonstrating its dependence on the TRIF dependent pathway (C, Figure 4.9).

These results suggest that SIGIRR, analogous to its function in human DCs, regulates TLR signalling in human M-CSF differentiated macrophages. In order to investigate this observation further, I established siRNA knock down of SIGIRR in M-CSF differentiated macrophages.

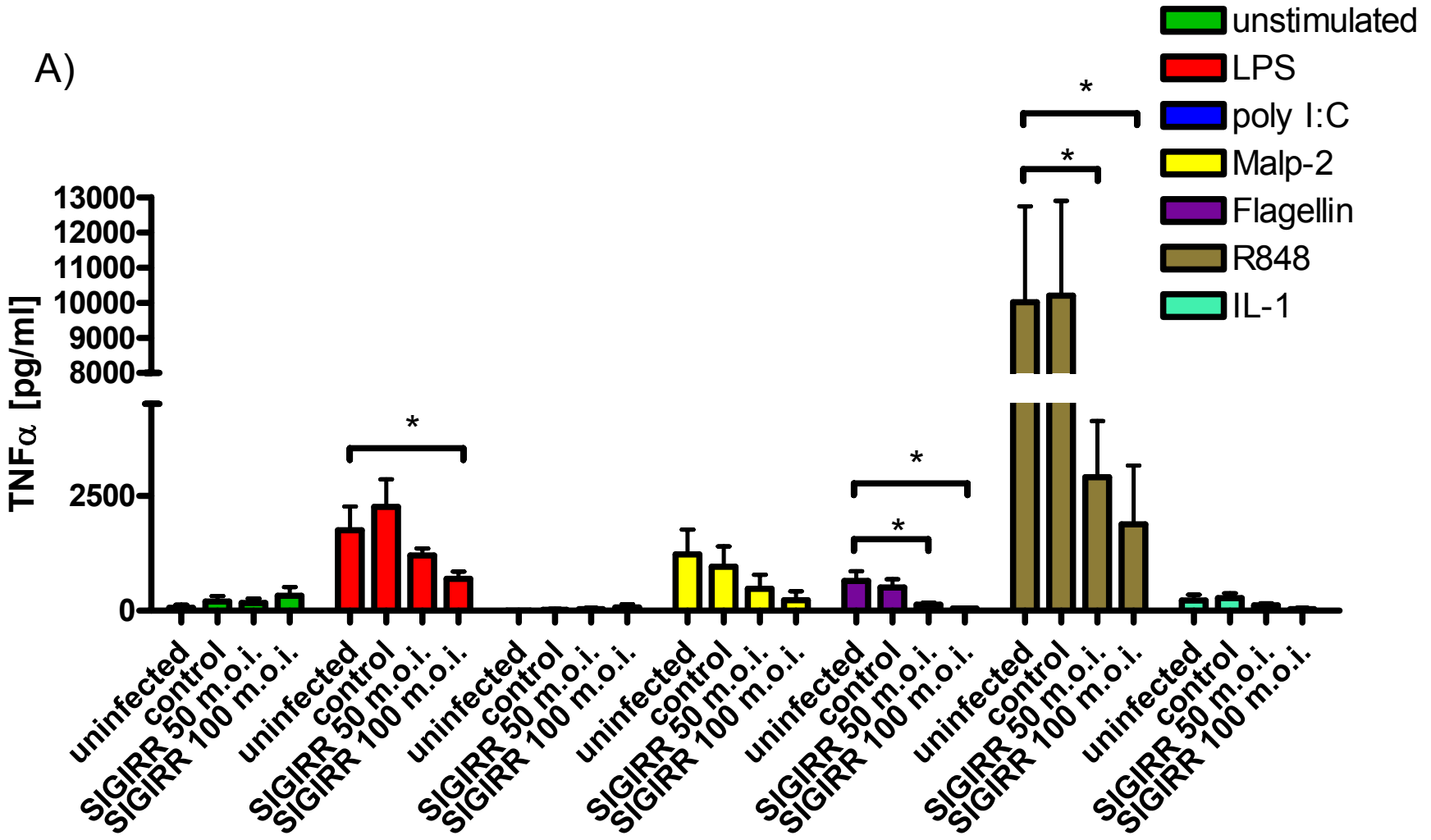


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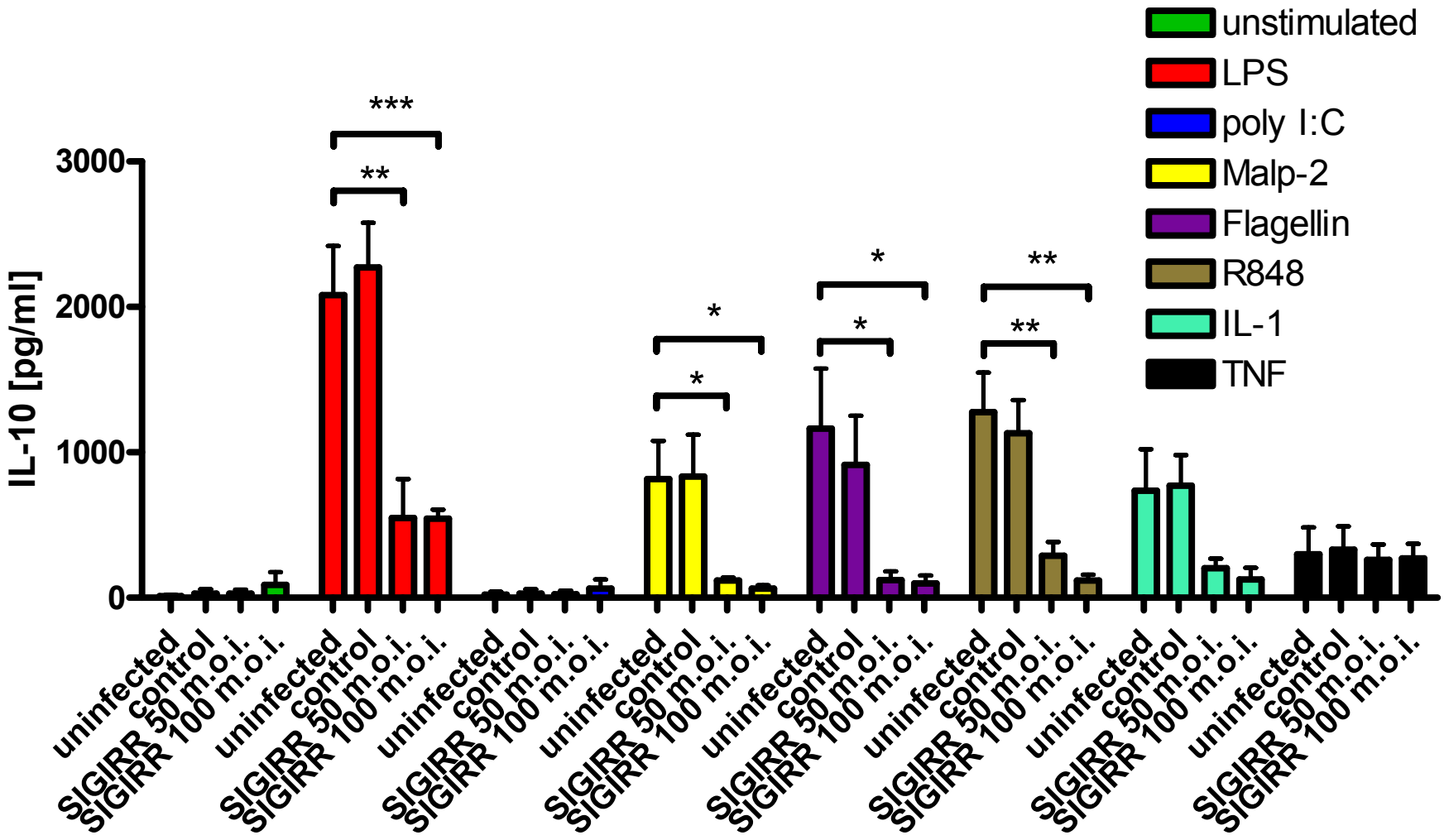


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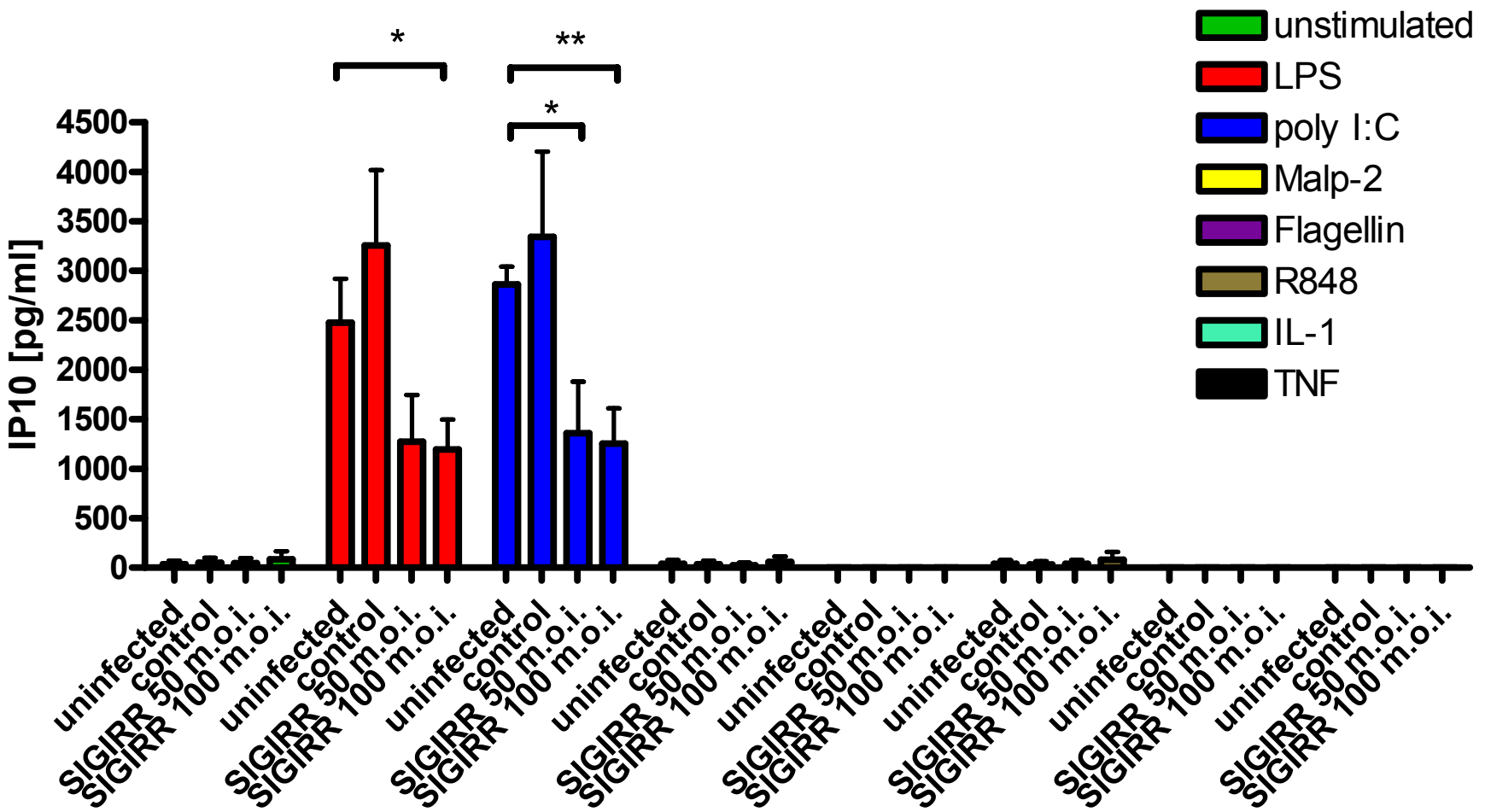


Figure 4.9: SIGIRR wt inhibits TLR4, TLR2, TLR5, TLR-7/8 and IL-1R signalling in primary human monocyte derived macrophages. Cytokine induction was assayed in culture supernatant by ELISA (A, TNFα; B, IL-10; C, IP10). Macrophages were used at day 4 of *in vitro* differentiation with 100 ng/ml M-CSF in RPMI 5% FCS. Cells were left uninfected, or infected (50:1 or 100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 24 h post infection cells were stimulated with 10 ng/ml LPS, 20 µg/ml poly I:C, 10 ng/ml MALP-2, 10 ng/ml Flagellin, 100 ng/ml R848, 20 ng/ml IL-1α or 20 ng/ml TNFα. After further 18 h supernatants were collected and TNFα, IL-10 and IP10 induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4, data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

4.2.6 DCs, but not M-CSF macrophages, show reduced cell viability after AMAXA transfection

So far this study relied on the over-expression of proteins in order to investigate protein function. The use of siRNA allows the specific knock down of genes and is therefore a suitable alternative to study the role of particular proteins. Until recently siRNA knock down in primary human cells has been difficult due to low transfection efficiencies in those cells. The AMAXA transfection system provides cell type specific protocols to transfect plasmids as well as oligo-nucleotides into primary human cells. The monocytes used in this study were isolated from PBMCs and transfected using the AMAXA transfection protocol for primary human monocytes (see chapter 2 section 2.3.11) to knock down SIGIRR. Using fluorescently labelled oligos, a transfection efficiency of around 50% was reached (Figure 4.10). Following transfection, the monocytes were left in culture containing the appropriate differentiation cytokines for 48 h in order to obtain either macrophages or DCs before experiments were performed (Figure 4.10).

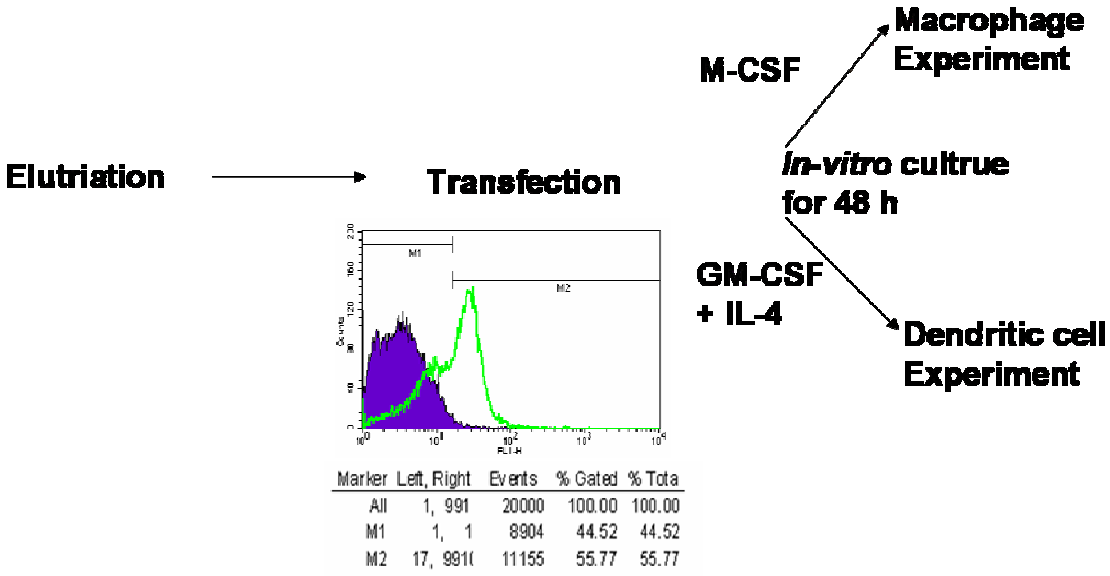


Figure 4.10: The transfection efficiency of monocytes, using fluorescently labelled oligos and the AMAXA transfection system, reaches 50%. PBMC isolated monocytes were transfected using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were left for 24 h before cells were washed and amount of transfected cells measured by Flow cytometry. Blue: cells cultured in the presence of oligo but untransfected; Green: oligo transfected cells.

In order to establish whether cell vitality is affected by the transfection procedure, MTT assays were performed 66 h after transfection (Figure 4.11 and Figure 4.12).

Monocytes transfected with SIGIRR siRNA and differentiated into DCs using GM-CSF and IL-4, showed reduced cell viability of up to 50%, 66 h after transfection (Figure 4.11). This loss of viability seems not to be a SIGIRR siRNA specific effect since cells transfected with scrambled

control oligos also showed reduced cell vitality (Figure 4.11). It rather seems to be due to the transfection procedure of AMAXA systems. However, monocytes transfected with scrambled control oligos or SIGIRR siRNA and differentiated into macrophages using M-CSF, showed no loss of viability (Figure 4.12).

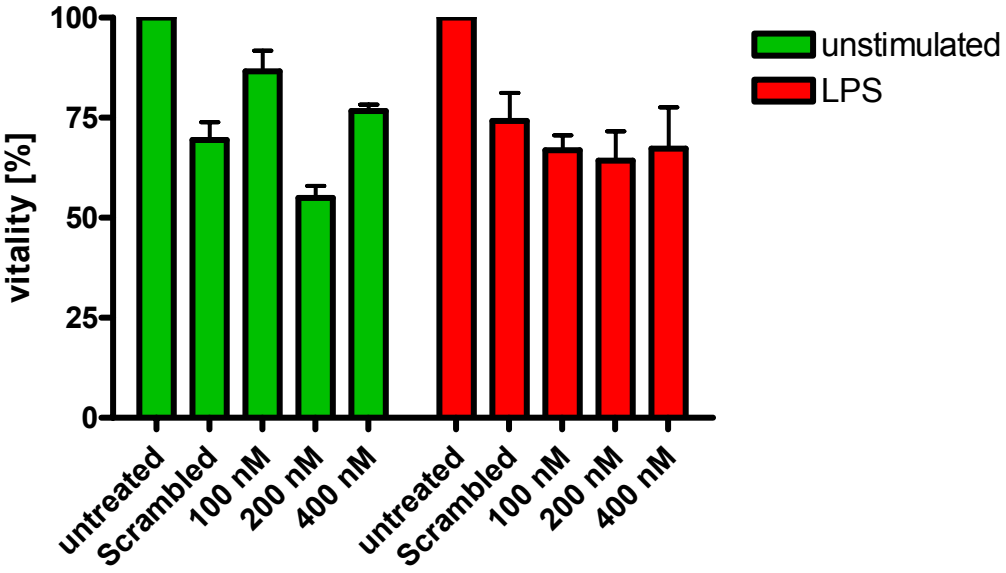


Figure 4.11:GM-CSF and IL-4 differentiated DCs show reduced vitality after siRNA transfection. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated for 48 h with 50 ng/ml of GM-CSF and 10 ng/ml of IL-4 before being stimulated with 10 ng/ml of LPS or left un-stimulated. Cells were incubated for 18 h before cells were incubated with MTT over night, lysis performed and OD574 measured. Data are normalised to untreated cells and expressed as mean $\pm$ SD. n = 2, data is 2 independent experiments pooled**.**

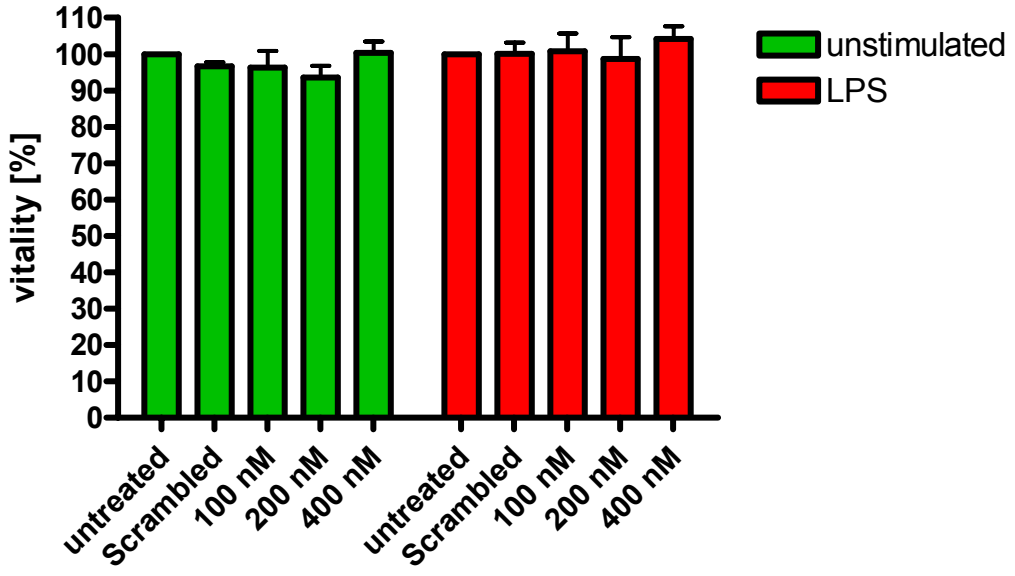


Figure 4.12: M-CSF differentiated macrophages show no reduced vitality after siRNA transfection. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled control oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stimulated with 10 ng/ml of LPS or left unstimulated. Cells were incubated for 18 h before cells were incubated with MTT over night, lysis performed and OD574 measured. Data are normalised to untreated cells and expressed as mean ± SEM. n = 4, data is 4 independent experiments pooled.

Due to the loss of cell vitality in GM-CSF and IL-4 differentiated DCs transfected with the AMAXA transfection system only M-CSF differentiated macrophages were used in this study for further functional experiments.

4.2.7 SIGIRR siRNA reduces SIGIRR mRNA and Protein expression in M-CSF differentiated macrophages

For further investigations the kinetics and extent of mRNA knock down had to be examined. Therefore, SIGIRR mRNA levels were measured using real-time PCR (Figure 4.13). Monocytes were transfected with a titration of SIGIRR siRNA ranging from 100 nM to 400 nM, before being incubated between 24 h and 96 h (Figure 4.13).

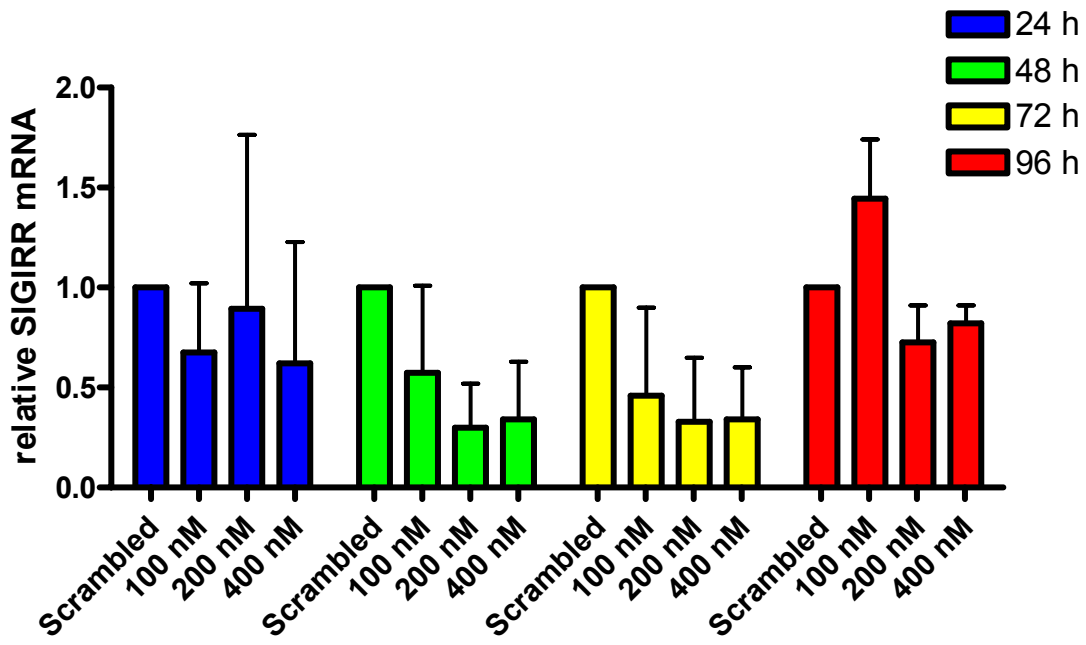


Figure 4.13: SIGIRR mRNA knock down occurs between 48 h and 72 h after transfection. PBMC isolated monocytes were transfected with 400 nM of scrambled control oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 24 h, 48 h, 72 h and 96 h before mRNA from $5x10^6$ cells was extracted. Taqman PCR was performed and data was normalised to GAPDH expression. Data is expressed relative to scrambled control oligo transfected cells. Data are expressed as mean $\pm$ SD. n = 2, this data is 2 independent experiments pooled.

Monocytes transfected with SIGIRR siRNA and differentiated using M-CSF, showed a reduction of SIGIRR mRNA at 48 h and 72 h after transfection (Figure 4.13). The levels of SIGIRR mRNA return to basal levels 96 h after transfection (Figure 4.13).

Besides showing the knock down of mRNA, it is important to examine that protein levels are also affected by reduced mRNA levels at this time point. Moreover, it is important to examine off target effects of the siRNA oligos. Therefore, SIGIRR siRNA transfected monocytes were stained for SIGIRR and TLR4 48 h after transfection (Figure 4.14). Monocytes transfected with 100 nM, 200 nM and 400 nM of SIGIRR siRNA show a significant and titratable reduction of SIGIRR protein. At the same concentrations TLR4 expression is unaffected (Figure 4.14). At 400 nM of SIGIRR siRNA the reduction of SIGIRR protein amounts to 50%.

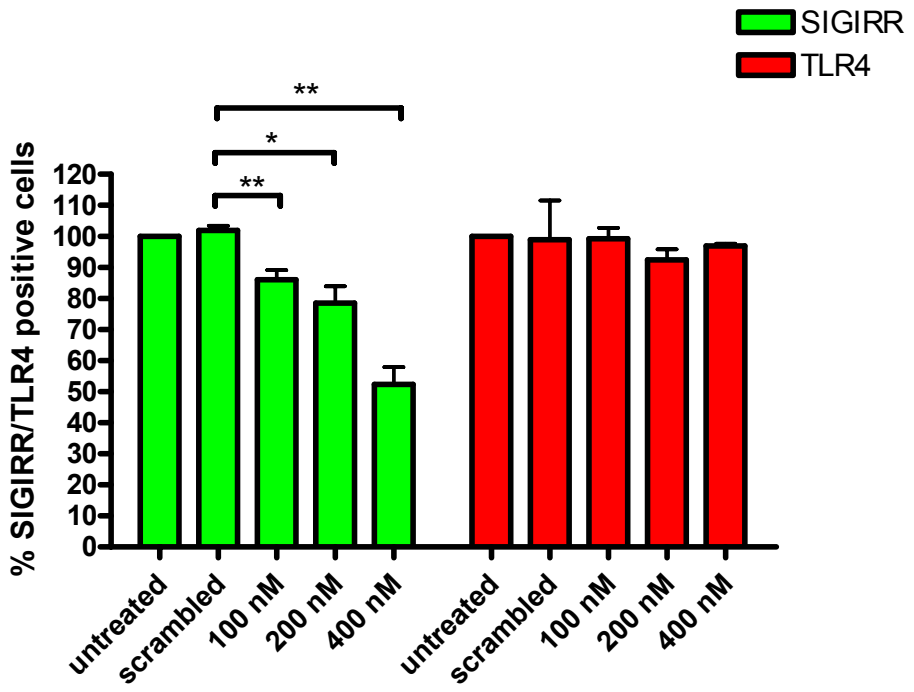


Figure 4.14: SIGIRR Protein is knocked down 48 h after transfection of human monocytes. PBMC isolated monocytes were transfected with 400 nM of scrambled control oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stained using BAF990 anti-SIGIRR antibody (R&D) or anti-TLR4 antibody (BD). $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

The use of SIGIRR siRNA in macrophages results in a reliable knock down of SIGIRR mRNA and protein of 50% at a concentration of 400 nM siRNA without affecting TLR4 protein. The following investigations were performed in transfected monocytes, differentiated using M-CSF for 48 h after transfection.

4.2.8 SIGIRR siRNA knock down super-induces TLR4, TLR3, TLR7/8 and IL-1R dependent cytokine production

In order to confirm the role of SIGIRR as an inhibitor of TLR signalling, as shown by over-expression of SIGIRR wt in DCs and macrophages (Section 4.2.4 and 4.2.5), SIGIRR knock down macrophages were stimulated with TLR4, TLR3 and TLR7/8 ligands (Figure 4.15).

Knock down of SIGIRR in macrophages and subsequent stimulation with LPS, R848 and IL-1 results in a significant increase of TNFα (A, Figure 4.15) and IL-10 production (B, Figure 4.15). This increase in cytokine production is titratable dependent on the concentration of siRNA used in accordance with the level of SIGIRR protein knock down shown in Figure 4.14. While SIGIRR knock down in macrophages results in super-induction of IL-10 in response to LPS, R848 and IL-1α it has no effect on TNFα signalling, thereby indicating its specificity (B, Figure 4.15). No significant levels of TNFα or IL-10 were produced by macrophages stimulated with poly I:C.

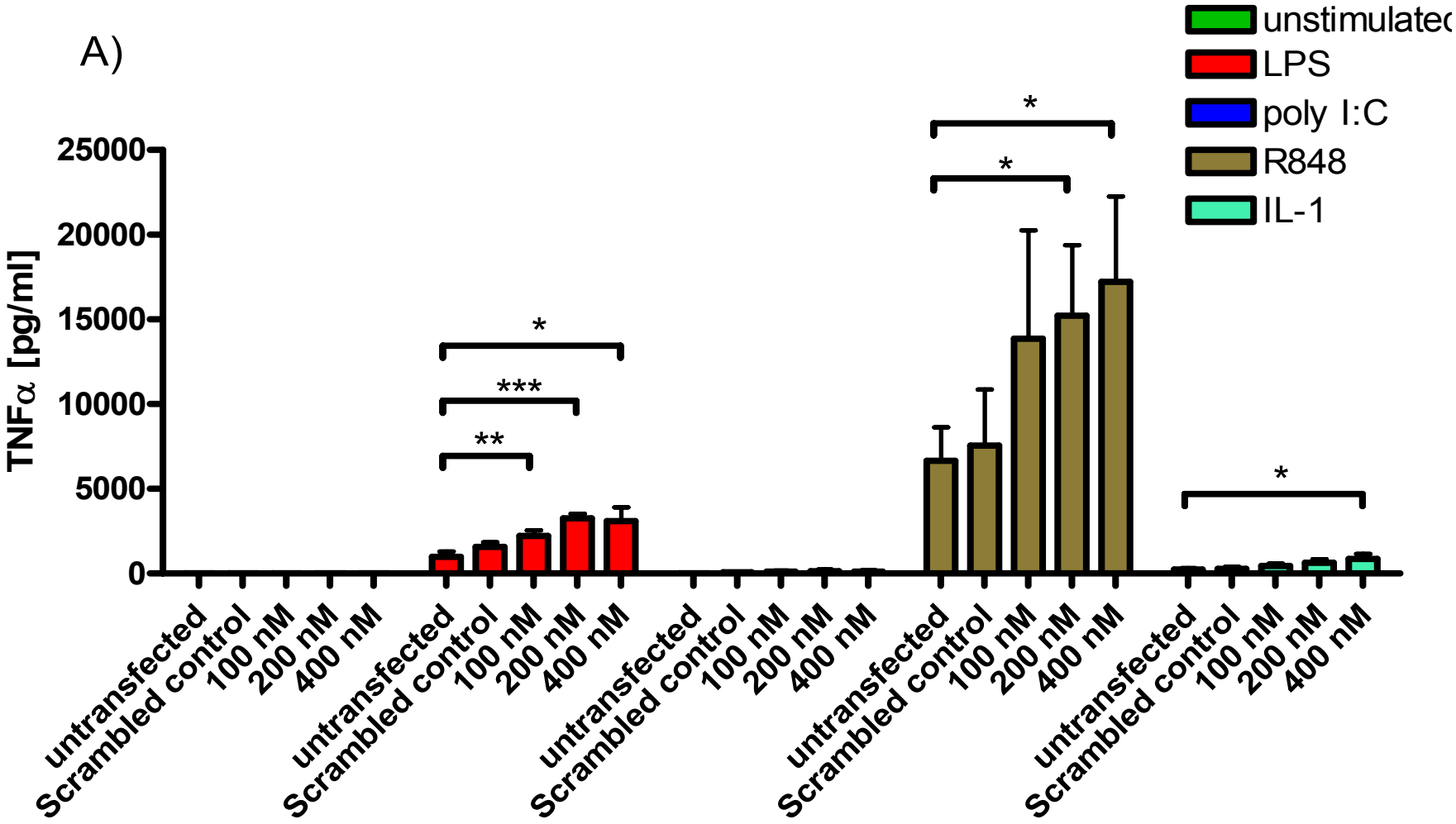


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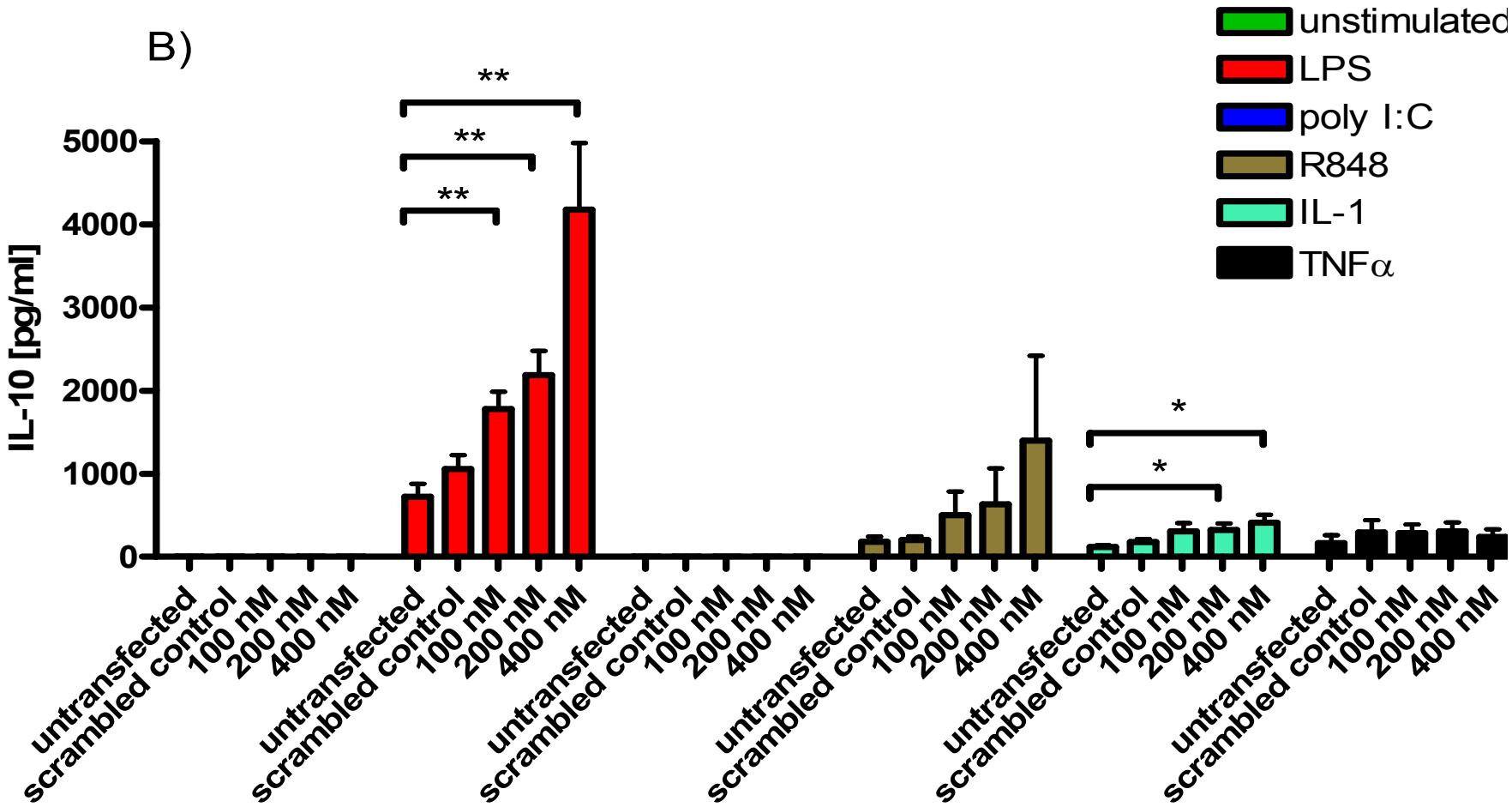


Figure 4.15: TNFα (A) and IL-10 (B) are super-induced in SIGIRR knock down, M-CSF differentiated macrophages stimulated with TLR ligands. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled control oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stimulated with 10 ng/ml LPS, 20 µg/ml poly I:C, 100 ng/ml R848, 20 ng/ml IL-1α or 20 ng/ml TNFα. After further 18 h supernatants were collected and TNFα (A) and IL-10 (B) induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4, data is 4 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

Beside the investigation of the effect of SIGIRR knock down on TNFα and IL-10 production, which are both dependent on MyD88 transduced signalling, this study also examined the production of IP10 (Figure 4.16). In response to LPS as well as poly I:C the expression of IP10 was significantly increased in SIGIRR knock down macrophages (Figure 4.16). In the case of TLR3 signalling this increase, similar to TNFα and IL-10 induction, is titratable dependent on the amount of siRNA transfected into the cells. No detectable levels of IP10 were produced in response to TLR7/8 and IL-1R signalling, which do not utilise the TRIF dependent signalling pathway (Figure 4.16).

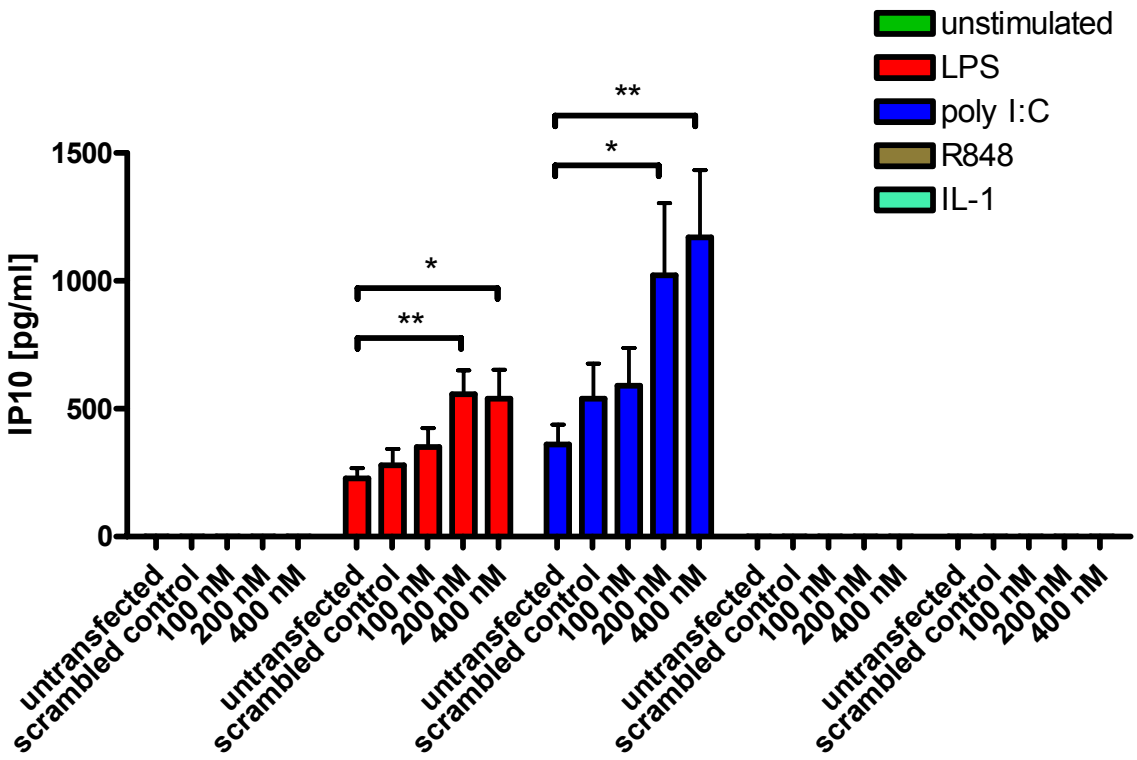


Figure 4.16: IP10 is super-induced in SIGIRR knock down, M-CSF differentiated macrophages stimulated with TLR ligands. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled control oligo, 100 nM SIGIRR siRNA, 200 nM SIGIRR siRNA or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stimulated with 10 ng/ml LPS, 20 µg/ml poly I:C, 100 ng/ml R848, 20 ng/ml IL-1α or 20 ng/ml TNFα. After further 18 h supernatants were collected and TNFα (A) and IL-10 (B) induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 8, data is 8 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

The use of siRNA to knock down SIGIRR in macrophages suggests a role of SIGIRR as an inhibitor of signalling pathways utilising TIR domains for signal transduction. TLR as well as IL-1R signalling are affected by SIGIRR knock down in these cells while TNFα signalling is unaffected.

4.2.9 SIGIRR knock down super-induces NF-κB, MAPKs and IRF3

While the induction of cytokine production is a general indication of TLR activation, the activation of specific signal transducers allows a more detailed examination of the TLR signalling pathways. Therefore, further investigations of the signalling pathways affected by the inhibition of SIGIRR were performed by examining the activation of NF-κB, the MAPKs JNK, ERK1/2 and p38 MAPK as well as IRF3 in SIGIRR knock down macrophages. Therefore, SIGIRR knock down macrophages were stimulated over a time course of 120 min and the phosphorylation of IκBα, as an indication for NF-κB activation, as well as the phosphorylation of the MAPKs were examined by Western Blot (Figure 4.17).

Treatment of macrophages with scrambled oligo control resulted in an activation of NF-κB, JNK, ERK and p38 MAPK similar to untreated cells as was shown before in our laboratory. The knock down of SIGIRR in macrophages resulted in an increased phosphorylation of IκBα, in particular, after 5 min of LPS stimulation but also after 40 min and 60 min of stimulation compared to macrophages transfected with a scrambled control oligo. However, while JNK and ERK also showed increased phosphorylation in response to LPS in SIGIRR knock down cells, p38 MAPK seems to be unaffected by SIGIRR knock down (Figure 4.17). The increase in phosphorylation of IκBα, JNK and ERK1/2 was not accompanied by a change in the kinetics of phosphorylation of these proteins (Figure 4.17).

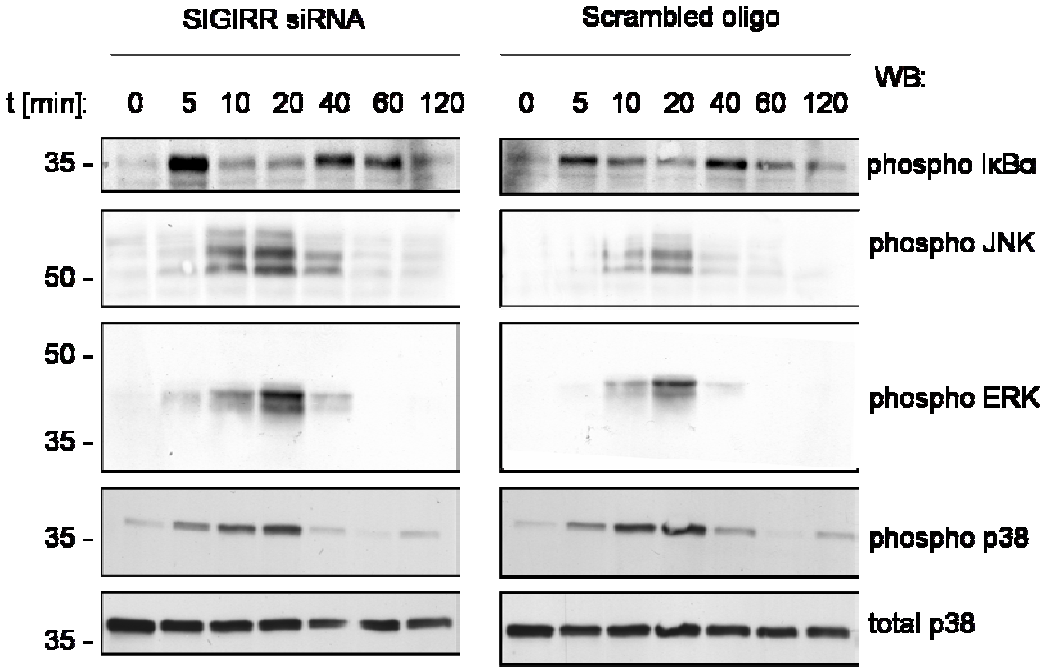


Figure 4.17: NF-κB, JNK and ERK1/2 are super-activated in response to LPS in SIGIRR knock down macrophages. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled control oligo or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stimulated with 10 ng/ml LPS over a time course of 120 min. Thereafter, cells were lysed in 1% Triton X 100 and lysate separated by SDS-PAGE. Specific protein was detected by blotting with ani-phospho IκBα- (New England Biolabs), anti-phospho JNK-(New England Biolabs), anti-phospho ERK1/2 (New England Biolabs) or anti-phospho p38 antibody (Cell Signalling Technology, Beverly, MA, USA).As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa). Membrane was stripped and counter-blotted using stripping buffer. As a loading control anti-total p38 was used (made in house). Experiment shown is representative of 2 independent experiments.

The super-induction of IP10 by LPS and poly I:C in SIGIRR knock down cells indicates, that the TRIF dependent pathway is also regulated by SIGIRR. However, it is still unclear, whether IP10 production is a secondary effect resulting from type I interferons produced in response to LPS and poly I:C or directly induced by the TRIF pathway (Honda and Taniguchi, 2006). Therefore, the activation of IRF3, the main transcription factor directly activated by the TRIF pathway was further examined. IRF3 becomes phosphorylated, dimerises and consequently translocates to the nucleus (Honda and Taniguchi, 2006). In order to asses IRF3 activation in response to LPS, nuclear proteins were extracted and nuclear IRF3 detected by western blot over a time period of 30 min (Figure 4.18). After 20 min of LPS stimulation IRF3 was detectable in the nucleus, which further increased 30 min after LPS stimulation (Figure 4.18). In SIGIRR knock down cells there was an additional increase in the amount of IRF3 translocated to the nucleus at the 20 min and 30 min time points compared to scrambled control

oligo transfected cells (Figure 4.18). This would indicate an increased activation of IRF3. As a loading control βActin and αTubulin were used. The nuclear extraction blot stains negative for αTubulin, excluding possible contaminations with cytosolic proteins (Figure 4.18).

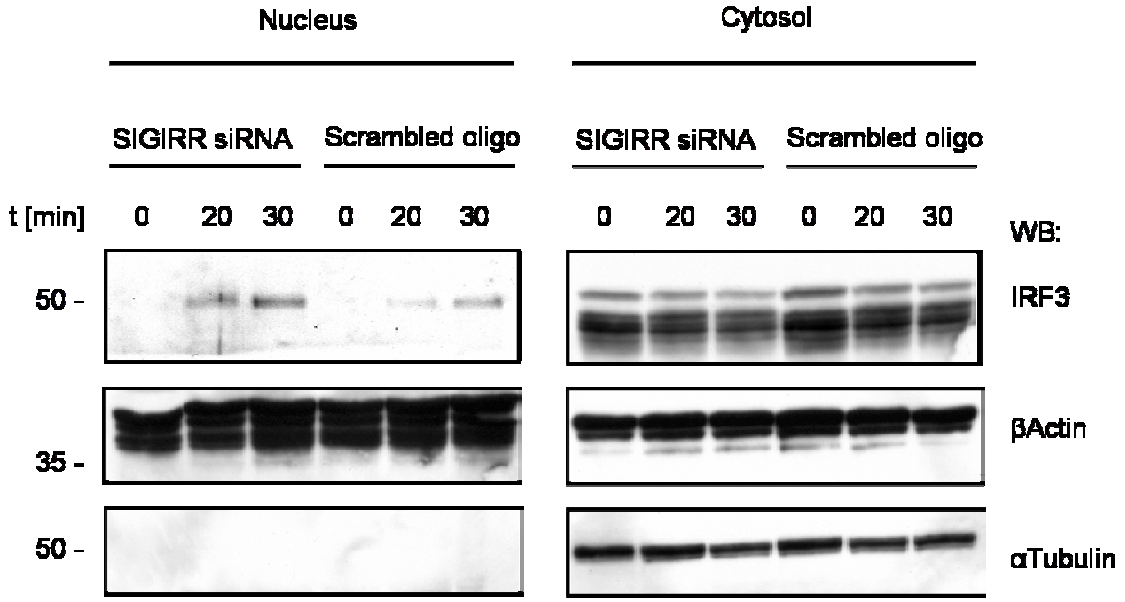


Figure 4.18: Increased IRF3 nuclear translocation in response to LPS in SIGIRR knock down macrophages. PBMC isolated monocytes were left untransfected or transfected with 400 nM of scrambled control oligo or 400 nM SIGIRR siRNA using the AMAXA protocol for primary human monocytes in accordance with the manufactures instructions. Cells were incubated with 100 ng/ml of M-CSF for 48 h before being stimulated with 10 ng/ml LPS over a time course of 30 min. Thereafter, nuclear- and cytosolic cell compartments were lysed and proteins separated by SDS-PAGE. Specific protein was detected by blotting with ani-IRF3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA USA). As a marker the Full range Rainbow molecular weight marker RPN800 from Amersham was used (scale in kDa). Membrane was stripped and counter-blotted using stripping buffer. As a loading control anti-βActin and anti-αTubulin were used (Sigma, Poole, Dorset, UK). Experiment shown is representative of 2 independent experiments.

These results indicate, that SIGIRR inhibition acts upstream of IκBα, JNK and ERK phosphorylation as well as IRF3 activation. SIGIRR has already been shown to interact with TLR proximal signal transducing molecules, including MyD88, in HEK293 cells in a transient manner after LPS stimulation (Qin et al., 2005). Based on the results obtained in HEK293 cells, SIGIRR has been proposed to act as a blocking receptor.

4.2.10 SIGIRR wt interacts with MyD88 wt in un-stimulated human DCs

In order to investigate whether SIGIRR interacts with MyD88 in primary human DCs and to investigate the character of this interaction, co-immunoprecipitation studies of SIGIRR and MyD88 were performed. Therefore, DCs were infected with SIGIRR and either human MyD88 wt, human MyD88 Pro220His or human MyD88 DD (Chapter 3 Figure 3.15). After 24 h of over-expression SIGIRR was immunoprecipitated using an anti-Flag antibody. The proteins were separated by SDS-PAGE and MyD88 constructs detected by western blot using an anti-HA antibody (Figure 4.19).

While MyD88 wt co-immunoprecipitated with SIGIRR the interaction was significantly reduced with MyD88 Pro200His, which contains a point mutation in its TIR domain (Figure 4.19). MyD88 DD, which is TIR domain deleted, completely lost the ability to interact with SIGIRR (Figure 4.19).

These results suggest that SIGIRR interacts with MyD88 through TIR domain interactions in primary human DCs. This interaction is not dependent on any stimuli, which would suggest that a constitutive interaction occurs between SIGIRR and MyD88 in primary human DCs. This would strengthen the hypothesis of SIGIRR acting as a sink and thereby inhibiting TLR and IL-1R signalling.

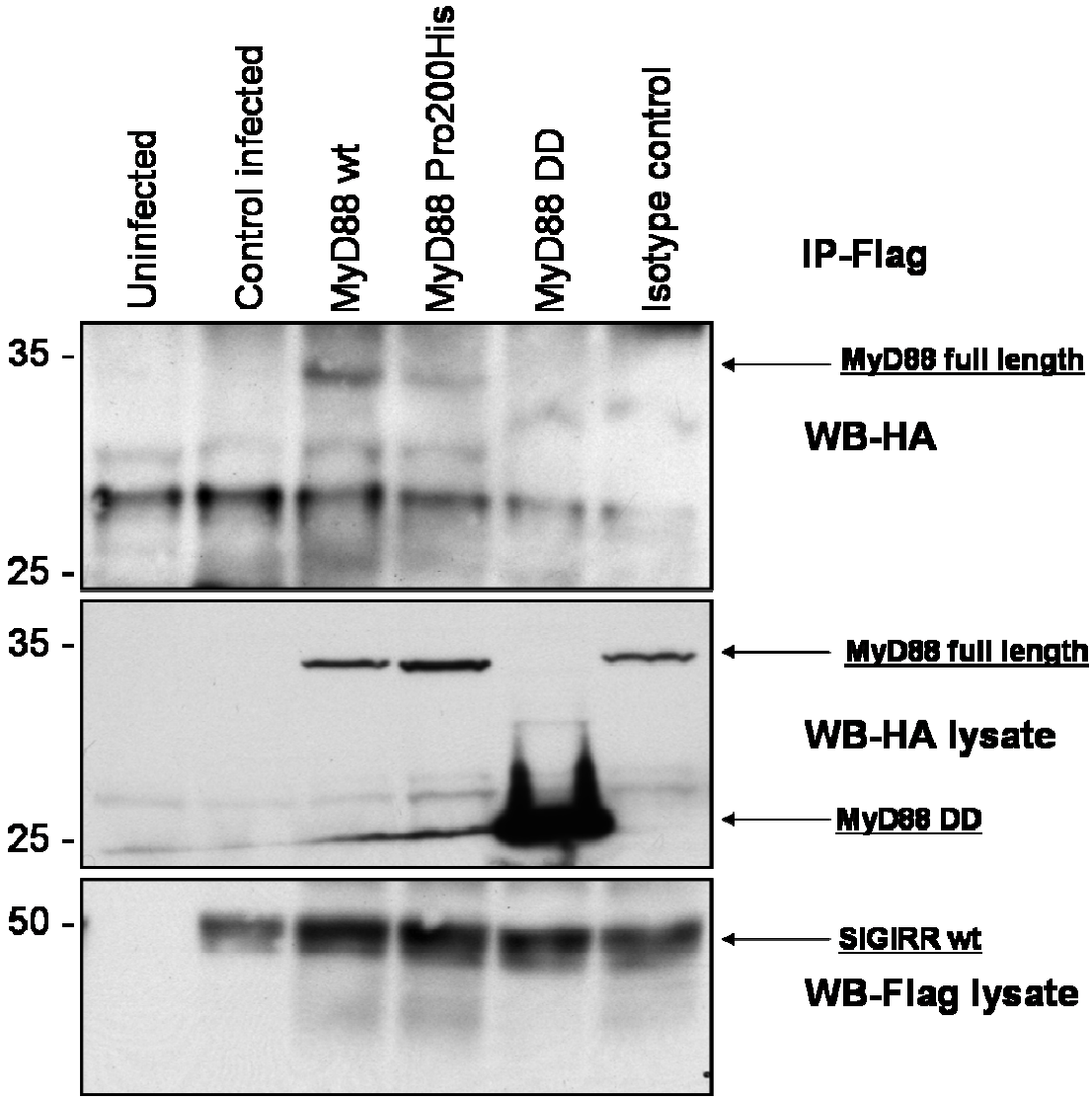


Figure 4.19: SIGIRR interacts with MyD88 through TIR domain interactions in un-stimulated primary human DCs. DCs were used at day 4 of *in vitro* differentiation with 50 ng/ml of GM-CSF and 10 ng/ml of IL-4 in RPMI 5% FCS. Cells were left uninfected, or double infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing SIGIRR wt and GFP (control), human MyD88 wt, human MyD88 Pro200His (point mutation of AS200 Pro to His) or human MyD88 DD (TIR domain deleted. Cells were left over-expressing for 24 h before being lysed. SIGIRR was immuno-precipitated using an anti-Flag antibody (Sigma, Poole, Dorset, UK) and MyD88 constructs were detected by western blot using an anti-HA antibody (Covance, California, USA). Experiment shown is representative of 3 independent experiments.

4.3 Conclusions

The observation of DC activation by the over-expression of TIR domain, made in chapter 3, would suggest a DC specific inhibitory mechanism. This mechanism would require a protein, which is expressed in DCs but not in M-CSF differentiated macrophages and shows inhibitory functions. Furthermore, since a TIR domain is required for the activation of DCs and the mechanism proposed in chapter 3 is dependent on MyD88 recruitment to the inhibitory receptor, the elusive protein should contain a TIR domain of its own.

In recent years, a multitude of TLR inhibitory molecules have been described, however, the requirements stated above restrict the candidates to three: MyD88s, a short splice version of MyD88, ST2 and SIGIRR, both members of the IL-1R family (Lang and Mansell, 2007). Of these three molecules only SIGIRR has been shown before in mice and humans to have a restricted mRNA expression pattern, being highly expressed in immature DCs but not in mature DCs and macrophages (Polentarutti et al., 2003). This observation makes SIGIRR a promising candidate for the hypothesis proposed in chapter 3.

In this study real-time PCR data could confirm the restricted expression pattern of SIGIRR, being expressed in monocytes, M-CSF differentiated macrophages and immature DCs but being expressed at only low levels in GM-CSF differentiated macrophages and fibroblasts (Figure 4.1). It is intriguing that different macrophage subtypes show a selective expression of SIGIRR mRNA. This observation is reflected in the current literature. In mice, freshly isolated macrophages do not express SIGIRR while bone marrow derived macrophages start expressing it after 5 days in M-CSF containing L929 cell-conditioned media culture and even up-regulate it after 7 days (Polentarutti et al., 2003). The same investigation has shown that while the macrophage cell line GG2EE does express SIGIRR mRNA, another macrophage cell line (J774) does not (Polentarutti et al., 2003). Studies performed in humans used macrophages differentiated from monocytes in the presence of human serum. These macrophages showed no mRNA expression of SIGIRR (Garlanda et al., 2004). However, the use of human serum neither allows the adjustment of accurate culture conditions with differentiation factors nor is it clear, which differentiation factors are present. Therefore, the results obtained in human macrophages are difficult to interpret. Yet, the results obtained in mice and cell lines support the observations made in this study that SIGIRR can be expressed in macrophages dependent on the differentiation factor used. However, since M-CSF differentiated macrophages do express SIGIRR but are not activated by the over-expression of a TIR domain, SIGIRR might be excluded as a possible candidate for the mechanism proposed in chapter 3.

Current literature does not describe protein expression patterns of SIGIRR in mice or humans. Since the expression of mRNA can differ significantly from the actual expression of protein it is important to assess SIGIRR protein expression in human cells. However, the antibodies for SIGIRR currently available are not able to specifically detect SIGIRR by western blot. One reason for the difficulty to obtain a specific anti-SIGIRR antibody might be due to posttranslational modifications of the protein, which might interfere with epitope recognition. Previous studies performed in murine kidney cells have shown that SIGIRR is highly glycosylated (Lech et al., 2007). Furthermore, the Centre for Biological Sequence Analysis (CBS) software predicts 6 N-glycosylation sites and 1 O-glycosylation site for human SIGIRR (URL: http://www.cbs.dtu.dk/services/NetOGlyc/). Therefore, another method to detect protein has been used in this study. Flow cytometry showed that SIGIRR is expressed in DCs as well as monocytes and M-CSF differentiated macrophages but not GM-CSF differentiated macrophages, thereby confirming the mRNA expression pattern. Interestingly, it seems that SIGIRR is expressed intracellularly in those cells. While these results might exclude SIGIRR as a DC specific inhibitor, it would be important to investigate if the TLR and IL-1R inhibitory role of SIGIRR, observed in mice and cell lines, can also be observed in primary human cells. Moreover, the intracellular expression of SIGIRR could have differential influences on TLRs expressed on the surface (e.g. TLR4) compared to TLRs expressed intracellularly (e.g. TLR7/8). Examples for such TLR specific mechanism is provided by the endosomal protein UC93B, which specifically regulates TLR7, TLR8 and TLR9 signalling (Kim et al., 2008). Since an adenovirus encoding SIGIRR, to over-express it in DCs and M-CSF macrophages, was available these studies were pursued.

The over-expression of SIGIRR in primary human DCs as well as M-CSF differentiated macrophages resulted in an inhibition of TNFα and IL-10 production in response to TLR2/6, TLR4, TLR5, TLR7/8 and IL-1R stimuli but not TNFα. In these cells, SIGIRR does not seem to have a differential effect on TLR signalling. The results rather suggest that SIGIRR functions as a general inhibitor of TIR domain dependent signalling. This is further indicated by the inhibition of IP10 production following TLR4 as well as TLR3 stimulation, which suggests that SIGIRR does inhibit the MyD88 dependent as well as the TRIF dependent TLR signalling pathway in human DCs and macrophages. So far, studies have investigated the inhibitory role of SIGIRR in SIGIRR knock out mice as well as cell lines (Garlanda et al., 2004; Qin et al., 2005; Wald et al., 2003). *In vivo*, SIGIRR null mice have been shown to have a reduced threshold to lethal endotoxin challenge with LPS (Wald et al., 2003). Investigating specific cell types more closely *ex vivo*, it has been found that BMDCs from SIGIRR knock out mice produce increased amounts of IL-6 as well as IP10 in response to LPS (TLR4) or CpG (TLR9) but not poly I:C (TLR3) or *Candida albicans* (stimulating TLR2 as well as Dectin-1) (Garlanda

et al., 2004), suggesting TLR specificity of SIGIRR. This has also been shown in HEK293 and Hela 229 cell lines using NF-κB luciferase assays, which were inhibited by SIGIRR over-expression in response to IL-1, LPS (TLR4) or Flagellin (TLR5) but not poly I:C (TLR3) or TNFα (Qin et al., 2005). However, in contrast to those reports a TLR specific inhibition has not been observed in SIGIRR deficient spleen derived monocytes, which produce increased amounts of CCL2 in response to Pam_3Cys (TLR2/1), poly I:C (TLR3), LPS (TLR4) and CpG (TLR9) (Lech et al., 2007). Results obtained in this study do not support differential effects of SIGIRR on TLRs as observed before in mice and cell lines. They are, however, in accordance with previous reports suggesting that SIGIRR inhibition is specific to TIR domain signal transduction (Wald et al., 2003).

An alternative approach of studying the function of SIGIRR is by utilising siRNA. While siRNA knock down of proteins is commonly used in cell lines, it is still difficult to exploit this method in primary human cells due to low transfection efficiencies. Therefore, I evaluated and established the AMAXA transfection system for primary human monocytes, which reliably allows the transfection of plasmids and oligo nucleotides into primary human cells with transfection efficiencies higher than 50%. However, this method includes the electrical pulsing of cells, which might influence their vitality, especially in long term cultures. While transfection of monocytes and consequent culture of the cells using M-CSF showed no effect on cell vitality, cell differentiation using GM-CSF and IL-4 resulted in a loss of vitality of up to 50%. The loss in vitality of DCs might be a result of the long term experiment performed in this study. Other studies have already described the sensitivity of DCs to electroporation comparing short term cultures (less then 24 h) with long term cultures of more than 24 h (Lenz et al., 2003). As a consequence of these results only M-CSF differentiated macrophages were used for further functional experiments.

The kinetics of siRNA knock down differs from gene to gene and depends on the amount of gene expression as well as the half life of mRNA as well as protein. Real-time PCR results of a time course of SIGIRR knock down show that SIGIRR mRNA levels are reduced by approximately 50% at an siRNA concentration of 400 nM, 48 h and 72 h after transfection compared to scrambled control oligos. Similar to the reduction in mRNA levels, there is also a 50% reduced expression of SIGIRR protein at an oligo concentration of 400 nM 48 h after transfection. The partial knock down of SIGIRR protein might be explained by the fact that the transfection efficiency of the AMAXA system is around 50%. Another possible shortcoming of siRNA are off-target effects on structurally related proteins. The intra-cellular region of SIGIRR shows high sequence homology to TLR4 (Thomassen et al., 1999) but no effects on the TLR4 expression could be observed as a result of SIGIRR knock down.

The SIGIRR knock down macrophages show super-induced production of TNFα and IL-10 in response to LPS, R848 and IL-1α but not TNFα stimulation compared to untransfected cells and scrambled control oligo transfected cells. The super-induction observed in SIGIRR knock down macrophages is titratable dependent on the amount of siRNA used. Moreover, IP10 expression is also super-induced in LPS or poly I:C stimulated SIGIRR knock down macrophages. These results are in accordance with the over-expression studies and suggest a general role of SIGIRR in inhibiting TLR- as well as IL-1R signalling. Therefore, this is the first report showing functional inhibition of SIGIRR in primary human cells in response to a variety of TLR stimuli. Moreover, it is the first time that siRNA knock down is being utilised to examine the function of SIGIRR in TLR signalling.

The outcome of TLR signalling is the induction of NF-κB, MAPKs and IRF3. Compared to scrambled control oligo transfected cells, SIGIRR knock down macrophages show an increased phosphorylation of IκBα at 5 min as well as 40 min to 60 min following LPS stimulation, while the kinetics of phosphorylation remain the same. Similar to the increased phosphorylation of IκBα there is also an increase in the phosphorylation of JNK and ERK at 20 min after LPS stimulation. Interestingly, the phosphorylation of p38 MAPK seems to be unaffected by the knock down of SIGIRR in macrophages. While this observation is difficult to explain, one reason could be that the partial knock down of SIGIRR of 50% in human macrophages is not enough to observe an effect on p38 MAPK phosphorylation. However, it is also intriguing that so far, no publication on SIGIRR has presented data on the effect of SIGIRR on p38 MAPK activation while its inhibitory role of JNK activation is well documented in mice and cell lines (Qin et al., 2005; Wald et al., 2003; Xiao et al., 2007). Current literature suggests that JNK as well as p38 MAPK activation is a result of the similar TLR signalling cascades (Ashwell, 2006; Lee and Kim, 2007). After TLR stimulation TRAF6 becomes ubiquinated, which results in the recruitment of TAK1 (Ashwell, 2006; Lee and Kim, 2007). Consequently, TAK1 activates the NF-κB cascade as well as MKK6, which results in the phosphorylation of JNK and p38 MAPK. Results in our lab suggest that while the activation of p38 MAPK, following LPS stimulation, is Btk dependent the activation of JNK and ERK are not (unpublished data, Dr. Maria Smolinska). This observation indicates separate pathways leading to the activation of MAPKs, which might be responsible for the differential effect of SIGIRR on MAPK phosphorylation. However, the mechanisms that account for the activation of specific MAPKs in response to receptor engagement through distinct MAP3Ks are far from being understood. Therefore, the observation of a specific involvement of SIGIRR in MAPK activation is still part of ongoing investigation in our laboratory.

Following the observation of a regulation of IP10 production by SIGIRR the activation of IRF3 in SIGIRR knock down macrophages was further investigated. As a result of TLR4 or TLR3

stimulation, TRIF recruits TBK1 and IKKi, which leads to IRF3 phosphorylation (Lee and Kim, 2007). Subsequently, IRF3 dimerises and translocates to the nucleus (Honda and Taniguchi, 2006). SIGIRR knock down macrophages show increased IRF3 nuclear translocation 20 min and 30 min after LPS stimulation compared to scrambled control oligo transfected macrophages. Currently, it is still unclear whether IP10 is a secondary product of LPS or poly I:C induced type I interferon expression or a direct result of TLR stimulation (Honda and Taniguchi, 2006). The increased activation of IRF3 in SIGIRR knock down macrophages, however, suggests that SIGIRR inhibits the TRIF signalling pathway directly following TLR4 stimulation.

Taken together these results suggest an involvement of SIGIRR in the MyD88 dependent as well as MyD88 independent TLR signalling pathway. Moreover, they indicate that SIGIRR acts upstream of the activation of NF-κB, the MAPKs and IRF3. Previous studies in cell lines suggest that SIGIRR functions at receptor proximal signalling events, possibly as a blocking receptor (Qin et al., 2005). The transient interaction of SIGIRR with TLR4 as well as MyD88, IRAK1 and TRAF6, following LPS stimulation, has been previously shown in HEK293 cells (Qin et al., 2005). In immature human DCs, SIGIRR interacted with wild type MyD88. This interaction was nearly completely lost by co-expression of SIGIRR with MyD88 Pro200His, which contains a point mutation in its TIR domain. No interaction could be detected when SIGIRR was co-expressed with MyD88 DD, which is TIR domain deleted. Thus, these results suggest that a TIR domain is necessary for SIGIRR to interact with MyD88. Moreover, the interaction between SIGIRR and MyD88 might be constitutive as it does not depend on the stimulation of the DCs. Although, it has to be taken into account that SIGIRR and MyD88 were over-expressed in these studies. Nonetheless, SIGIRR possibly acts as a sink, sequestering MyD88, and thereby inhibiting TLR as well as IL-1R signalling. Since, this mechanism would depend on TIR domain interactions it could explain why TNFα signalling as well as IFNγ signalling (Wald et al., 2003) is not regulated by SIGIRR.

This is the first report describing an involvement of SIGIRR in TLR signalling regulation in primary human myeloid cells. It describes a specific expression pattern in human macrophages dependent on the differentiation factor used. Moreover, using adenovirus vectors to over-express SIGIRR as well as siRNA to knock down SIGIRR in M-CSF differentiated macrophages reveals a general regulation of TLR signalling by SIGIRR. This regulation might be due to SIGIRR acting as a sink, thereby sequestering TLR adaptor molecules. TLR signalling is a strong inducer of an inflammatory response. In recent years, it was also implicated in the induction and progression of inflammatory diseases including rheumatoid arthritis (Drexler et al., 2006; Marshak-Rothstein, 2006). Since this study shows that SIGIRR is a potent inhibitor of TLR signalling in cells relevant to inflammatory diseases, further

investigations were performed to examine the role of SIGIRR in RA disease models *in vivo* as well as *in vitro*.

CHAPTER 5

ROLES FOR SIGIRR IN THE REGULATION OF RHEUMATOID ARTHRITIS

5.1 Introduction

In recent years increasing evidence emerged that TLRs are not only able to detect pathogens but also endogenous danger signals (Brentano et al., 2005a; Drexler et al., 2006; Marshak-Rothstein, 2006; Marshak-Rothstein and Rifkin, 2007; Rifkin et al., 2005; Tsan and Gao, 2004). These danger signals have been shown to be present in the RA synovial fluid (Schett et al., 1998; Scott et al., 1981; Taniguchi et al., 2003; Yu et al., 1997). Therefore, the release of endogenous TLR-ligands during inflammation may activate TLRs and perpetuate the disease. Evidence in mice as well as humans, support a role of TLR activation by endogenous ligands in the destructive process observed in RA (Brentano et al., 2005b; Kawane et al., 2006; Sacre et al., 2007a). TLR9 was shown to be activated by endogenous DNA released during the process of cell necrosis. Therefore, the escape of genomic DNA during cell death might lead to the induction of inflammatory disease. That this might be the case has been demonstrated in DNase II $^{-/-}$ mice, which develop chronic polyarthritis that resembles human rheumatoid arthritis (Kawane et al., 2006). Furthermore, a study using human synovial membranes from RA patients has shown that the spontaneous release of cytokines and MMPs by those cells can be inhibited through over-expression of dominant negative constructs of MyD88 or Mal. Furthermore, it has been shown that conditioned cell media from RA synovial membranes is able to activate human monocyte derived macrophages in a MyD88 and Mal dependent manner (Sacre et al., 2007a), thereby indicating the presence of an endogenous TLR ligand in RA synovial cell supernatants.

The role of SIGIRR as an inhibitor of TLR and IL-1R signalling and thereby inhibitor of inflammation has been well established. SIGIRR$^{-/-}$ mice have more severe inflammation in the DSS-induced colitis model (Garlanda et al., 2004; Xiao et al., 2007) and are more susceptible to lethal endotoxin challenges (Wald et al., 2003). Given the potential of TLRs in driving RA, I further investigated the role of SIGIRR in suppressing signalling by the TLR and IL-1R families in RA disease models.

This study characterises SIGIRR as a suppressor of spontaneous cytokine release in human RA membranes. Therefore the data presented here provides further evidence, which suggests a role of TLRs in driving the inflammatory process observed during the progression stage of RA. Moreover, this investigation demonstrates the involvement of SIGIRR in regulating the adaptive immune response, which could be observed during the induction of CIA in SIGIRR deficient animals.

5.2 Results

5.2.1 SIGIRR inhibits spontaneous release of cytokines by human RA synovial membranes

The human RA *in vitro* model, developed at the Kennedy Institute of Rheumatology, utilises RA synovial membranes obtained from patients undergoing joint replacement (Hermann et al., 1998; Katsikis et al., 1994). This system has been used to establish the potent role of TNFα in RA pathogenesis (Brennan et al., 1989a; Brennan et al., 1989b). The RA synovial membranes are treated with collagenase to obtain a single cell culture, which consist of synovial fibroblasts, lymphocytes as well as macrophages and DCs. The resulting cell cultures spontaneously produce cytokines and MMPs without further stimulation, which can be measured as a read out in functional assays. Therefore, the function of SIGIRR in an established inflammatory environment was investigated using these human RA synovial membrane cultures.

5.2.1.1 SIGIRR is expressed by macrophages in the human RA synovial membrane

The expression of SIGIRR in human RA synovium was examined by flow cytometry staining of synovial membrane cultures (Figure 5.1). RA synovial membranes were co-stained for CD3 (T cells) or CD14 (monocytes/macrophages) and SIGIRR. However, the cells obtained from synovial membranes did show a high degree of unspecific binding, as observed using an isotype control antibody (Figure 5.1). Therefore, it is difficult to interpret whether synovial macrophages or synovial resident T cells do express SIGIRR. Although it seems that $CD14^+$ macrophages did stain positive for SIGIRR, this was not reflected in the percentages (Figure 5.1). The experiment shown in Figure 5.1 is a representative of three donors. Different procedures using a range of different blocking buffers did not improve the quality of the staining. Ultimately, other methods to detect SIGIRR expression e.g. flow cytometry on sorted cells, real-time PCR or histology, will need to be utilised.

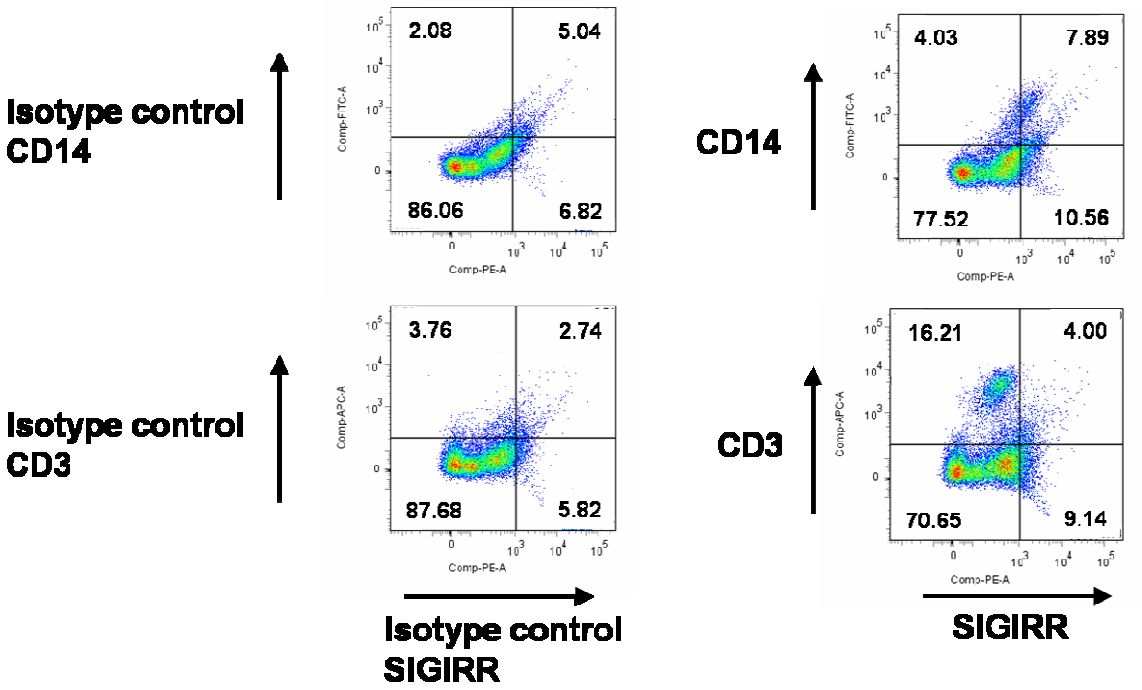


Figure 5.1: SIGIRR expression by synovial resident and T cells is difficult to interpret due to a high degree of unspecific staining. RA synovial membrane cells from the same donor were examined by intracellular flow cytometry staining for the expression of SIGIRR expression. A representative histogram plot of 3 donors is shown. Permeabilized cells were incubated with isotype control (BD Bioscience) or anti-SIGIRR antibody (BAF990, R&D) and co-stained with either anti-CD3 (BD Bioscience) or anti-CD14 (BD Bioscience).

5.2.1.2 SIGIRR over-expression inhibits the spontaneous release of cytokines by RA synovial membrane cultures

Adenoviral infection of RA synovial cells allows the investigation of the function of SIGIRR in an established human inflammatory environment. The protocol of RA synovial cell infection by adenoviruses has been established in our laboratory and results in an infection efficiency of above 90% (Foxwell et al., 1998). Moreover, the viral infection of RA synovial cells has no significant influence on cell vitality as shown in Figure 5.2.

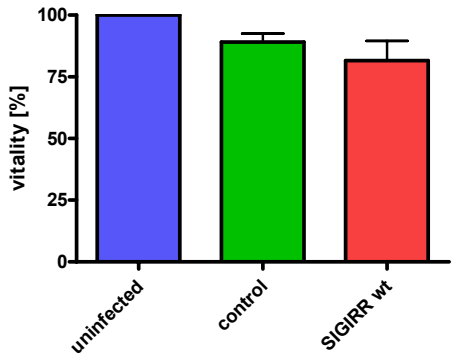


Figure 5.2: SIGIRR wt over-expression does not significantly affect the cell vitality of RA synovial membranes. RA synovial membrane cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 48 h post infection cells were incubated with MTT over night, lysis performed and OD574 measured. Data are normalised to uninfected cells and expressed as mean $\pm$ SEM. n = 4, data is 4 independent experiments pooled.

RA synovial cells spontaneously produce cytokines without further stimulation (Sacre et al., 2007a). Therefore, the effect of SIGIRR on the spontaneous release of cytokines was measured by ELISA (Figure 5.3). Over-expression of SIGIRR led to a significant suppression of the release of IL-6 and TNFα (Figure 5.3 A and B respectively) as well as a reduction in IL-10 release (C, Figure 5.3). However, no effect on IL-8 production was observed by SIGIRR over-expression (D, Figure 5.3). These data would suggest that IL-6, TNFα and IL-10 production in the RA synovial membrane is driven by IL-1R and/or TLR signalling.

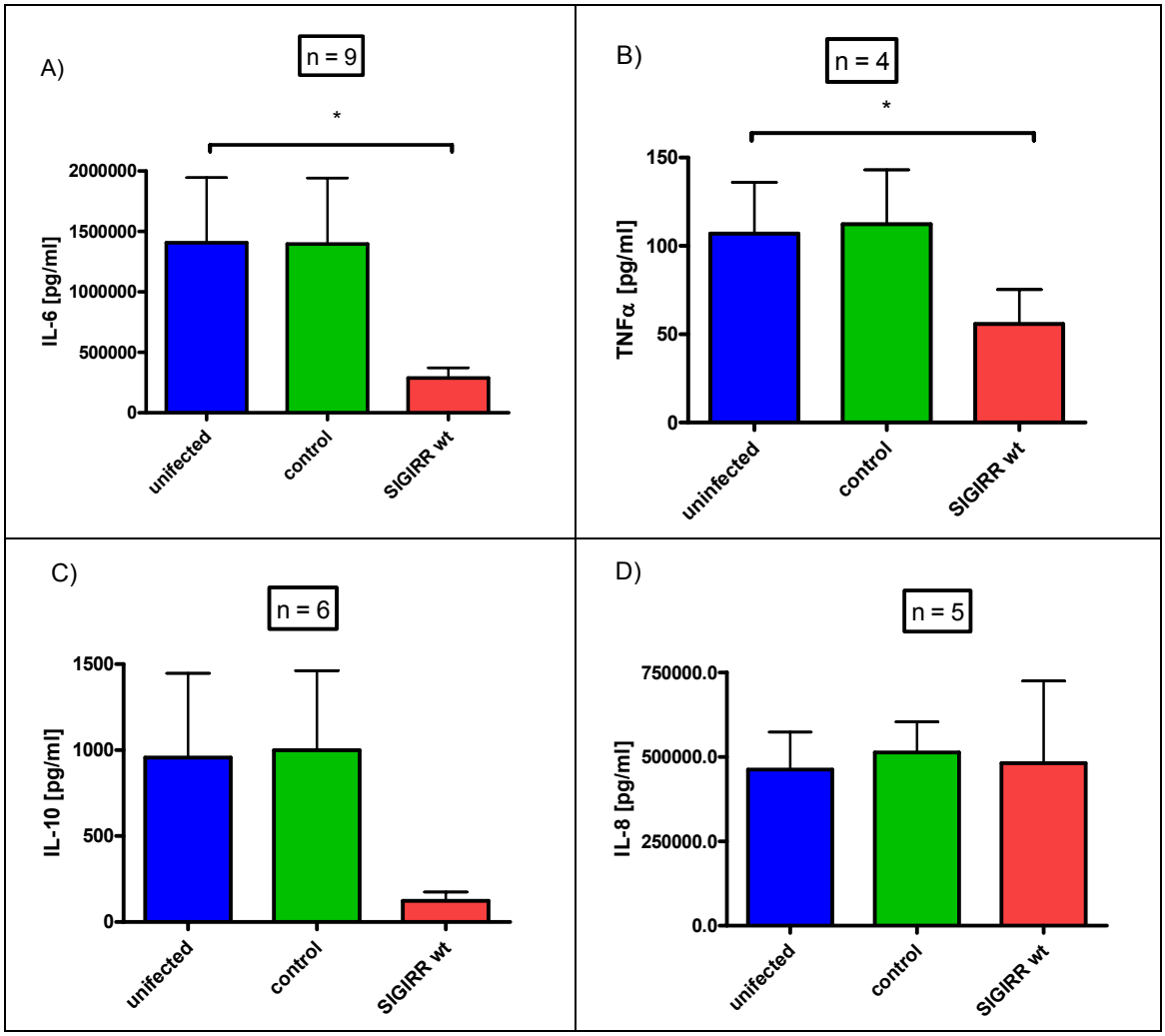


Figure 5.3: SIGIRR wt over-expression inhibits the spontaneous release of IL-6 (p = 0.02), TNFα (p = 0.021) and IL-10 (p = 0.059) but not IL-8 by RA synovial membranes. RA synovial membrane cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control) or SIGIRR wt. 48 h post infection supernatants were collected and A) IL-6, B) TNFα C), IL-10 and D) IL-8 levels measured using ELISA. Data are expressed as mean ± SEM. Data is n independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

5.2.2 SIGIRR deficient mice show reduced disease incidence following CIA induction

Given that SIGIRR was shown in this study to inhibit TLR signalling in primary human macrophages and DCs as well as to inhibit the spontaneous release of cytokines by RA synovial membranes *in vitro*, I further examined the function of SIGIRR in an *in vivo* model of RA, collagen induced arthritis. In accordance with the UK licensing law for animal experiments the mice were immunised by Dr. Julia Inglis. All other experimental procedures were performed by me.

5.2.2.1 SIGIRR deficient BMDCs super induce cytokine production in response to TLR stimulation

Previous studies on SIGIRR deficient BMDCs have shown that those cells hyper-respond to TLR stimulation by super-inducing cytokine production (Garlanda et al., 2004). In order to confirm these data in our laboratory BMDCs were stimulated with LPS and Malp-2 and subsequent TNFα production was measured by ELISA (Figure 5.4). BMDCs from SIGIRR deficient mice produce more TNFα in response to LPS and Malp-2 compared to WT animals. Also a gene dosage effect can be observed as SIGIRR$^{+/-}$ mice produce intermediate amounts of TNFα in response to LPS and Malp-2 compared to WT and SIGIRR$^{-/-}$ animals (Figure 5.4).

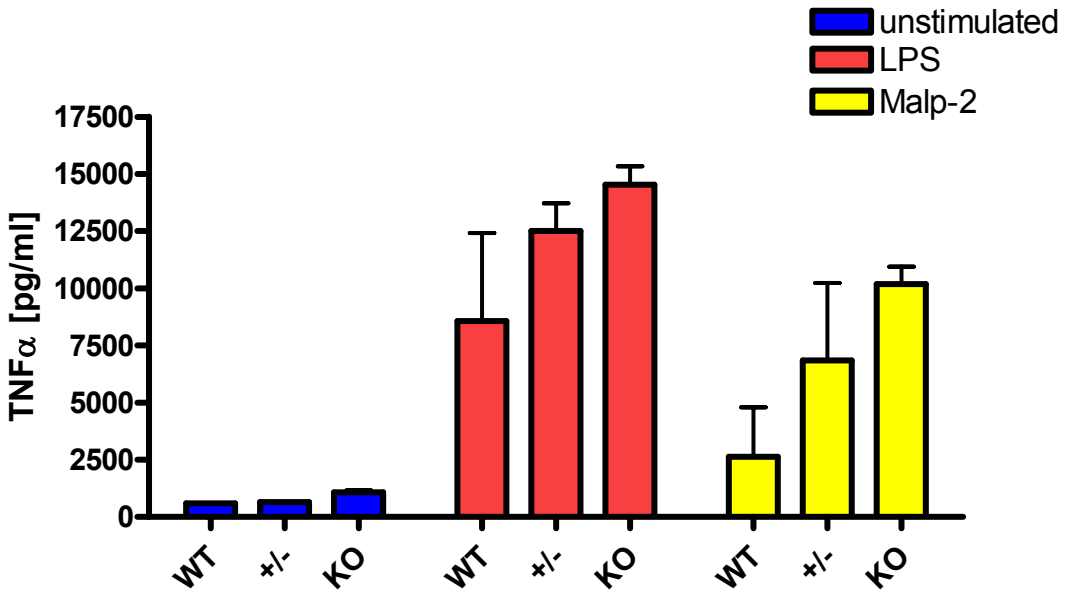


Figure 5.4: Bone marrow derived DCs of SIGIRR$^{-/-}$ mice produce increased amounts of TNFα compared to WT mice following TLR stimulation. TNFα induction was assayed in culture supernatant by ELISA. BMDCs were used at day 6 of *in vitro* differentiation with 100 ng/ml GM-CSF in RPMI 5% FCS. Cells were stimulated with 100 ng/ml LPS, 20 µg/ml poly I:C, 10 ng/ml Malp-2. After further 18 h supernatants were collected and TNFα induction assayed by ELISA. Data are expressed as mean ± SD. n = 2, data is 2 independent experiments pooled.

5.2.2.2 SIGIRR$^{-/-}$ mice show reduced incidence and disease severity following CIA immunisation

CIA is a widely-used animal model of human rheumatoid arthritis, which utilises type II collagen, expressed exclusively in the articular cartilage of the joint, as an auto antigen (Cho et al., 2007). Induction of CIA depends on the proliferation of CD4^{+} T cells 3 to 5 weeks after immunisation (Cho et al., 2007). The SIGIRR$^{-/-}$ mouse used in this study is on the C57Bl/6 background, which can be immunised using chicken type II collagen and Complete Freund´s Adjuvant (CFA) to induce CIA as described before (Inglis et al., 2007). Although, the C57/Bl6 mice show a milder disease phenotype compared to DBA/1 mice, the disease is more chronic with a more pronounced and persistent T cell response (Inglis et al., 2007). Moreover, unlike DBA/1 mice, C57/Bl6 mice develop an arthritic disease, which more closely correlates with the human disease as they respond to methotrexate treatment, which is a classical treatment in humans (Inglis et al., 2007).

The immunised mice began showing signs of the disease, e.g. paw swelling and paw redness, between day 16 and day 20 after immunisation (Figure 5.5). All mice that reached a minimum clinical score of 1.5 were considered to be diseased. 29 days post immunisation WT mice showed a disease incidence of 91.6% (11/12). In contrast, heterozygous- and homozygous SIGIRR$^{-/-}$ mice only showed 77.7% (14/18) and 50% (4/8) incidence respectively (Figure 5.5). 40 days after immunisation the trial had to be terminated due to the severity of the disease in WT mice. By then all mice showing signs of disease reached a minimum of 11 days past disease onset (Figure 5.5).

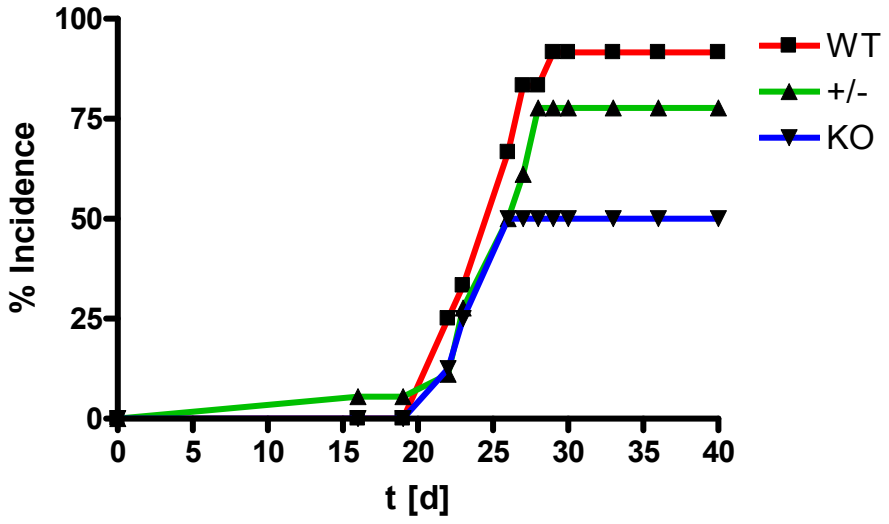


Figure 5.5: SIGIRR$^{-/-}$ mice show a lower CIA incidence compared to heterozygous SIGIRR deficient mice and WT mice. Mice were immunised with type II collagen in complete Freund's adjuvant (n = 12 WT mice, n = 18 SIGIRR$^{+/-}$ mice, n = 8 SIGIRR$^{-/-}$ mice). Mice with a minimum clinical score of 1.5 were considered diseased and incidence was recorded. 91.6% of WT mice (11/12), 77.8% of SIGIRR$^{+/-}$ mice (14/18) and 50% of SIGIRR$^{-/-}$ mice (4/8) showed disease 29 days post immunisation.

Over the period of 11 days the diseased WT animals had significantly increased paw swelling as well as clinical score compared to diseased heterozygous- and homozygous SIGIRR$^{-/-}$ mice (A and B Figure 5.6). A gene-dosage effect was observed as mice heterozygote for SIGIRR developed disease of an intermediate severity between homozygous SIGIRR$^{-/-}$ and WT mice as measured by paw swelling and clinical score (A and B Figure 5.6). Paw swelling was observable in multiple paws of the WT mice, whereas in most heterozygous and homozygous SIGIRR$^{-/-}$ mice only one paw per animal was affected by the disease (Figure 5.7). Histological examination of the joints showed only minor signs of inflammation in the joints of SIGIRR$^{-/-}$ mice, while there was major cell infiltration into the joint space (JS) and synovium (S) as well as cartilage (C) and bone (B) destruction in joints from SIGIRR$^{+/-}$ and WT animals (Figure 5.8).

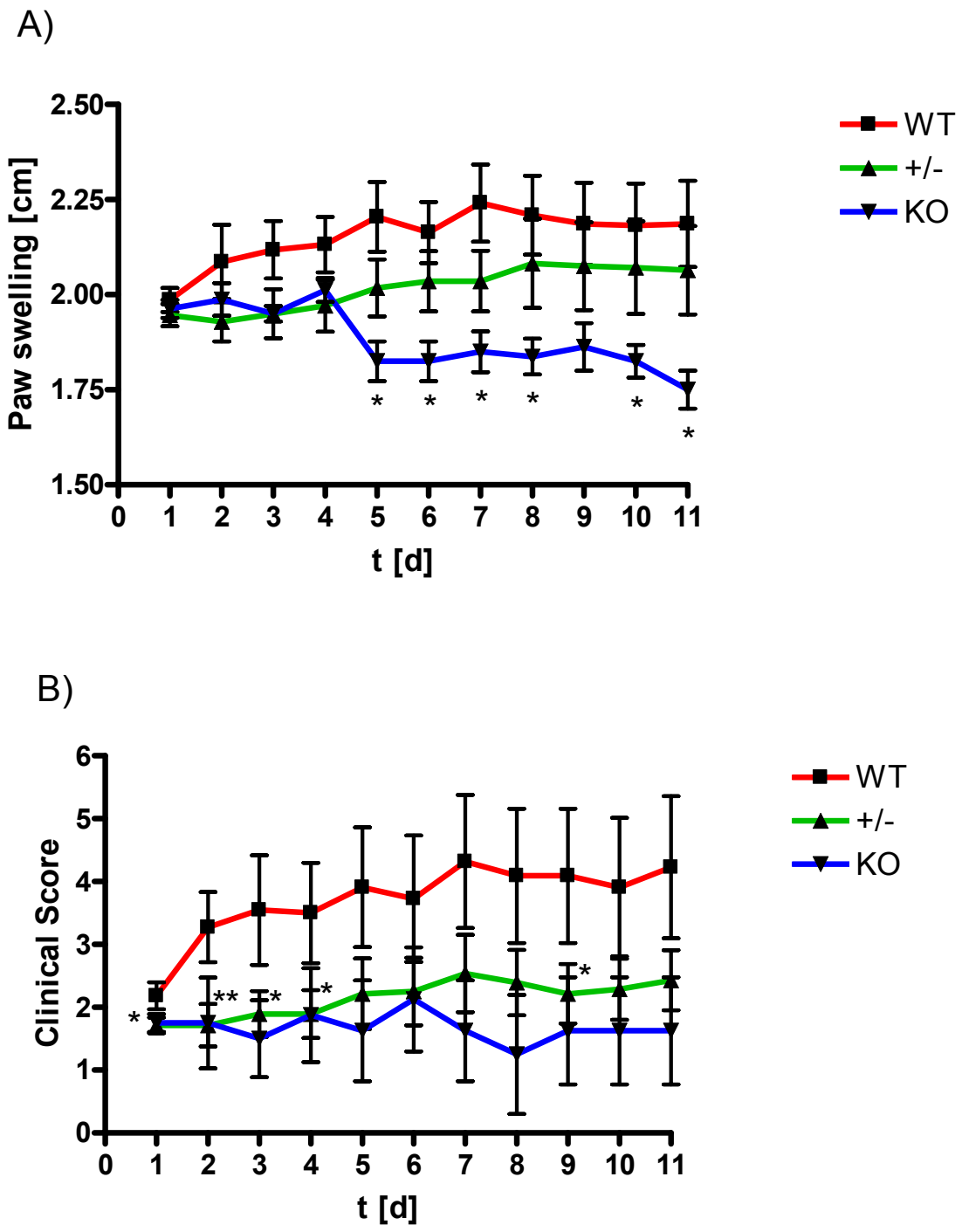


Figure 5.6: SIGIRR$^{-/-}$ mice show a lower clinical score and paw swelling compared to heterozygous SIGIRR deficient mice and WT mice during 11 days of CIA. (A) Paw diameter as an average of the 4 paws and (B) clinical score of the diseased mice were measured for 11 days post disease onset (n = 11 WT arthritic mice, n = 14 SIGIRR$^{+/-}$ arthritic mice, n = 4 SIGIRR$^{-/-}$ arthritic mice). SIGIRR$^{-/-}$ mice showed reduced paw swelling and a lower clinical score compared to SIGIRR$^{+/-}$ mice and WT animals. Data are expressed as mean $\pm$ SEM. Data is n animals pooled. . $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

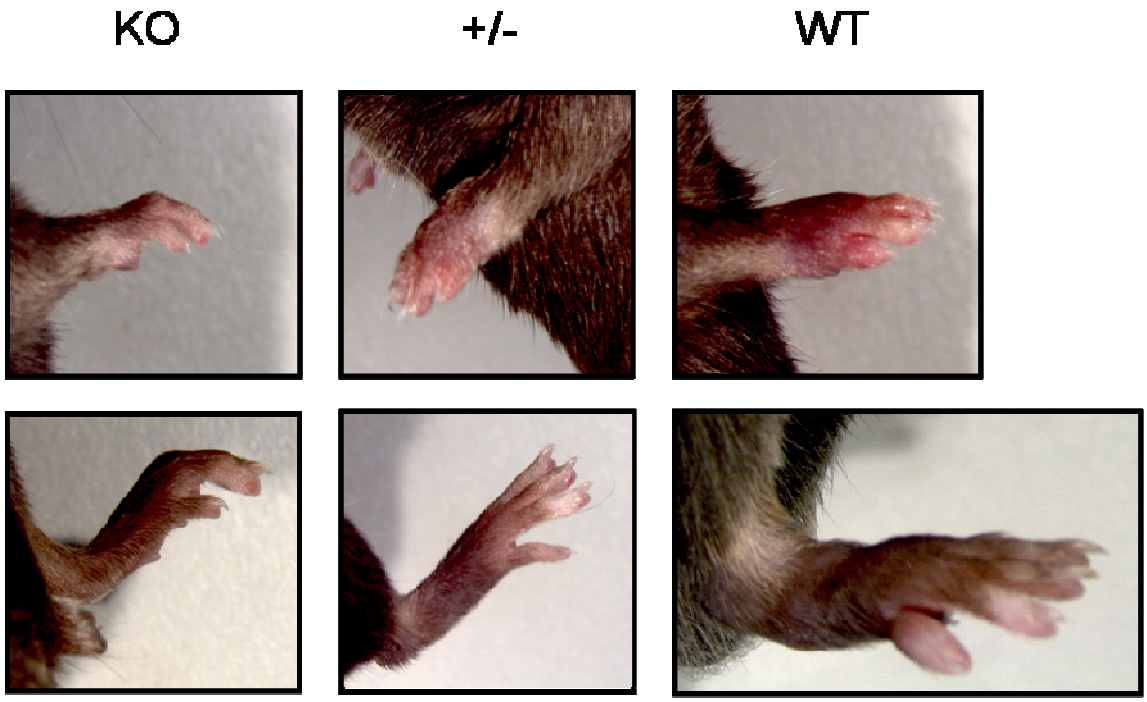


Figure 5.7: CIA induction leads to severe swelling of multiple paws in WT mice whereas heterozygous- and homozygous SIGIRR deficient mice have only single paws affected with milder swelling.

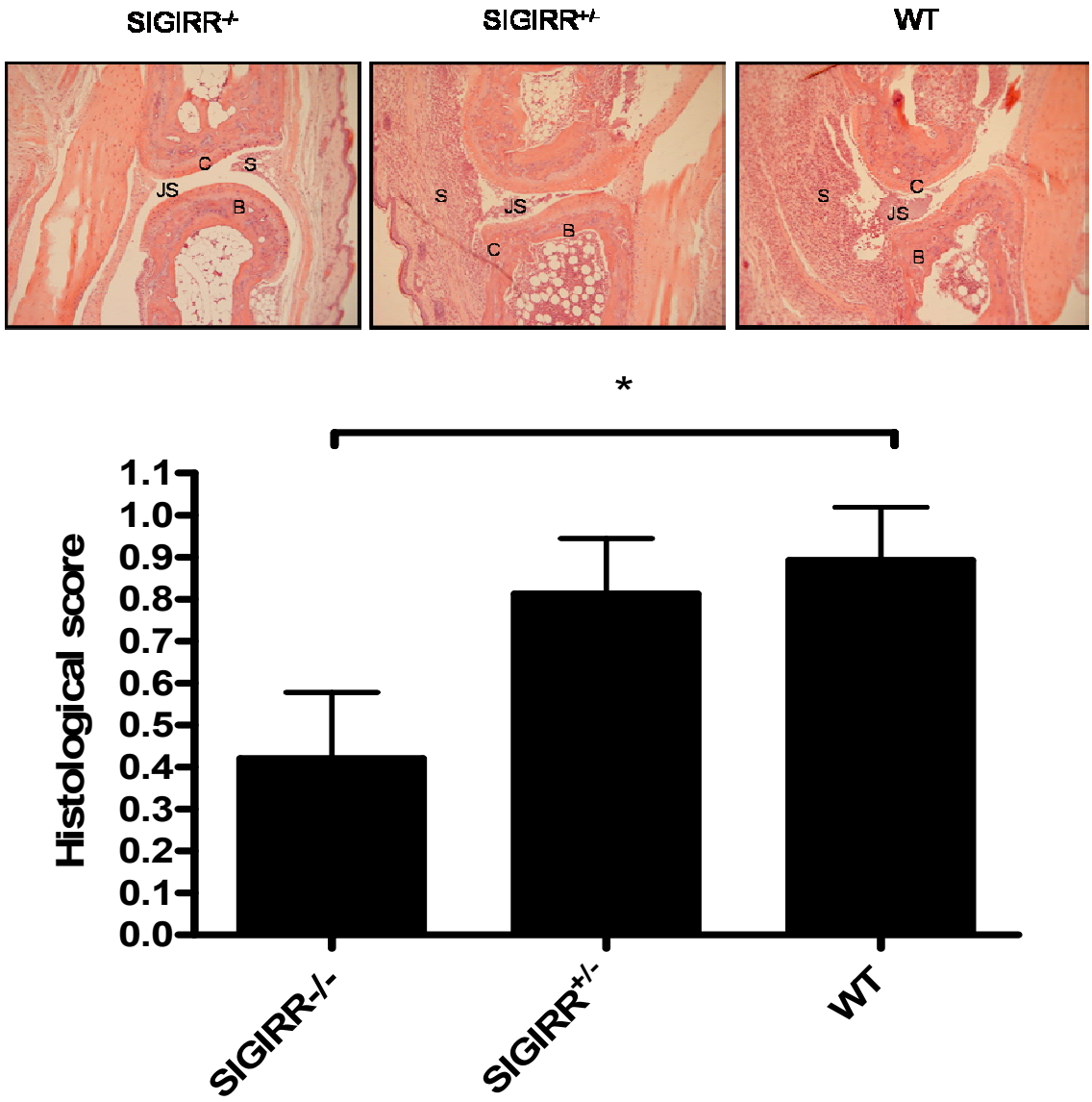


Figure 5.8: SIGIRR$^{-/-}$ mice show a significant lower histological score compared to heterozygous SIGIRR deficient mice and WT mice during 11 days of CIA (p = 0.0314). Figure shows proximal interphalongeal joint (magnification x20). Arthritic mice were sacrificed 11 days post disease onset. Joints were decalcified, paraffin embedded, and sections (10 µm) stained with haematoxylin and eosin by David Essex (Department of Histopahtology, Charing Cross Hospital). Joints were classified according to the presence or absence of inflammatory cell infiltrates (defined as focal accumulations of leukocytes). Histological analysis was performed in a blinded fashion by Dr Richard Williams (KIR). Homozygous SIGIRR deficient animals showed low grade inflammation, while SIGIRR$^{+/-}$ and WT animal joints showed massive infiltration of mononuclear cells into the joint space as well as cartilage and bone erosion. Abbreviations: joint space (JS), cartilage (C), bone (B), synovium (S). Data are expressed as mean $\pm$ SEM. Data is n animals pooled (n = 11 WT arthritic mice, n = 14 SIGIRR$^{+/-}$ arthritic mice, n = 4 SIGIRR$^{-/-}$ arthritic mice). $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

5.2.2.3 SIGIRR$^{-/-}$ mice produce less anti-collagen class IgG2a/c antibodies compared to WT animals following CIA induction

The production of anti-type II collagen antibodies following immunisation is required for the development of CIA (Cho et al., 2007). In order to investigate the reason for the less severe pathology in SIGIRR$^{-/-}$ mice, antibody production was measured in the serum of mice 11 days after disease onset (Figure 5.9). WT mice as well as heterozygote- and homozygous SIGIRR deficient mice contained equal titres of IgG1 anti-collagen antibody in their serum (A, Figure 5.9). In contrast, homozygous SIGIRR$^{-/-}$ mice produced significantly reduced amounts of anti-chicken collagen IgG2a/c 11 days after disease onset (A, Figure 5.9). Consequently, SIGIRR$^{-/-}$ mice showed a decreased ratio of IgG2a/c to IgG1 antibody production, which may indicate a reduced Th1- and polarised Th2 response (B, Figure 5.9).

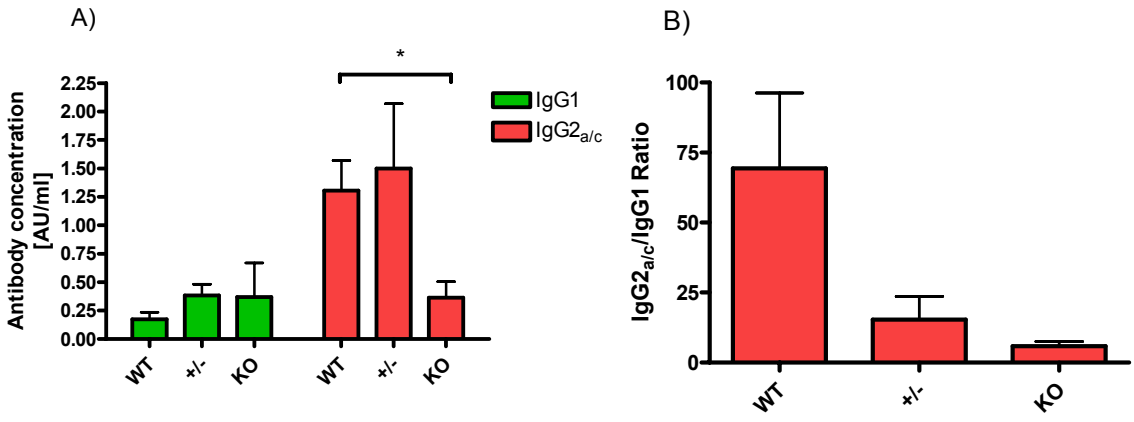


Figure 5.9: SIGIRR$^{-/-}$ mice produce significantly less IgG2a/c ant-collagen antibodies compared to WT mice (p = 0.0303) and show a reduced Th1/Th2 polarised immune response following CIA immunisation. Only mice showing signs of disease were included in this figure. 11 days post disease onset mice were sacrificed and their serum levels of IgG1 and IgG2a/c anti-collagen antibody measured relative to a pooled standard of serum from CIA induced C57Bl/6 mice. (A) WT as well as SIGIRR$^{+/-}$ animals showed an antibody concentration of IgG1 and IgG2a/c characteristic for C57Bl/6 mice immunised with chicken collagen, as shown before (Inglis et al., 2007). SIGIRR$^{-/-}$ mice, however, show significantly lower levels of IgG2a/c anti-collagen antibodies compared to WT mice (n = 11 WT arthritic mice, n = 14 SIGIRR$^{+/-}$ arthritic mice, n = 4 SIGIRR$^{-/-}$ arthritic mice). (B) Consequently, SIGIRR-/- mice also show a lower ratio of IgG2a/c/IgG1 compared to WT animals, suggesting a Th2 polarisation in those mice. Data are expressed as mean $\pm$ SEM. Data is n animals pooled (n = 11 WT arthritic mice, n = 14 SIGIRR$^{+/-}$ arthritic mice, n = 4 SIGIRR$^{-/-}$ arthritic mice). $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

The reduced antibody production in SIGIRR$^{-/-}$ mice does not seem to be due to a general defect in B cell activation since only IgG2a/c production is affected. However, to further exclude the possibility of an intrinsic defect in B cell activation, naive purified B cells from SIGIRR$^{-/-}$ mice were stimulated with IgM or LPS and their proliferation measured (Figure 5.10).

Purified spleen B cells from naive SIGIRR deficient mice significantly hyper proliferated following stimulation with IgM or LPS compared to WT B cells (Figure 5.10). In the case of LPS this is to be expected, given the role of SIGIRR as a TLR4 inhibitor. Therefore, an intrinsic defect in the activation of B cells from SIGIRR deficient mice might be excluded as a reason for reduced IgG2a/c anti-chicken collagen antibody production. This implicates that the reduction in antibody production observed in SIGIRR$^{-/-}$ mice following CIA immunisation may be due to the nature of the T helper cell immune response.

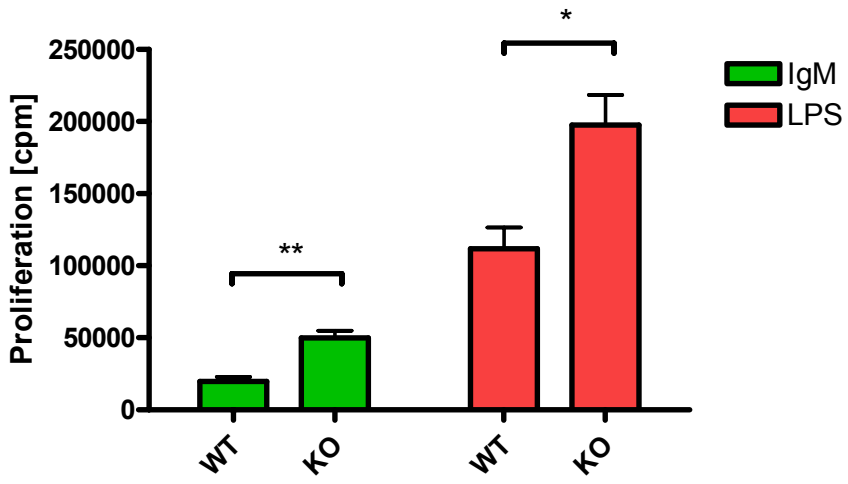


Figure 5.10: Purified naïve B cells from SIGIRR$^{-/-}$ mice hyper proliferate in response to IgM (p = 0.0066) and LPS (p = 0.0281) stimulation. A single cell suspension was prepared from spleens. B cells were positively enriched using anti-IgM MACS micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was assessed by flow cytometric analysis (B cells >90% CD19^{+}). Cells were cultured at 1 x 10^5 cells/ml in 200µl complete RPMI in a flat bottom 96-well plate and cultured for 72 h. B cells were stimulated with IgM (10 µg/ml; BD) or LPS (10 µg/ml, Alexis). For proliferation assays, 48 hours after stimulation cells were pulsed with 1 µCi ^{3}H thymidine per well for 18 h. Cells were then harvested and plates assessed for thymidine incorporation. Data are expressed as mean ± SEM. Data is n = 3 independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

5.2.2.4 SIGIRR$^{-/-}$ lymph node cells and splenocytes induce a Th2 associated cytokine profile following CIA induction

The activation of auto reactive T cells and consequently the stimulation of B cells to produce auto-antibodies are essential for the onset of CIA. Furthermore, the type of T helper cells induced plays a major part in CIA, with Th1 as well as Th17 cells strongly implicated in disease onset and progression (Furuzawa-Carballeda et al., 2007).

In order to investigate the nature of Th cell response induced in SIGIRR$^{-/-}$ mice following CIA induction, cytokines produced by lymph node cells and splenocytes were measured 16 days after immunisation (Figure 5.11). Lymph nodes and spleens were excised from mice 14 days after immunisation and cells cultured *in vitro* un-stimulated or in the presence of type II

collagen or anti-CD3 for 48 h, before supernatants were analysed for cytokine levels (Figure 5.11).

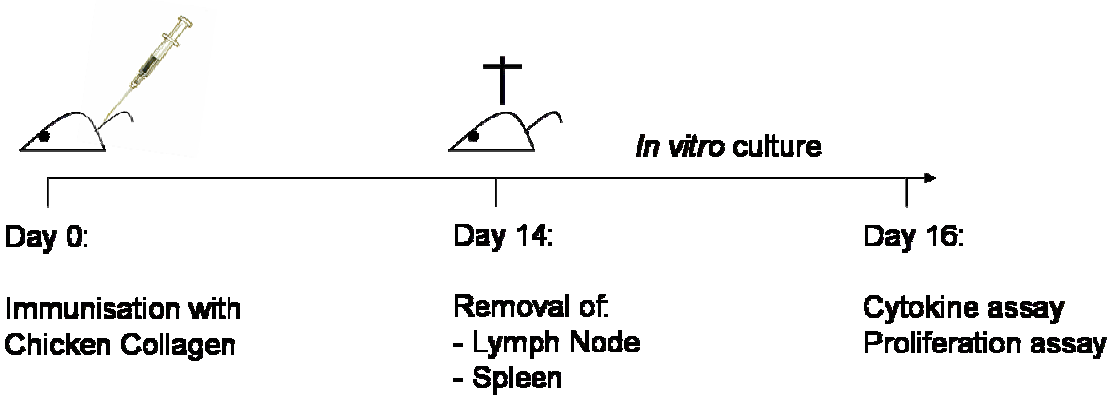


Figure 5.11: Examination of Collagen induced arthritis in SIGIRR$^{-/-}$ mice. Mice were immunised by injecting CFA and chicken collagen at the base of the tale. 14 days after immunisation the mice were sacrificed and lymph nodes as well as spleens removed. Following a 2 day *in vitro* culture of lymph node cells and splenocytes in the presence of anti-CD3 or chicken collagen, cytokine levels in cell supernatants were measured by ELISA and proliferation assays performed.

Lymph node cells and splenocytes from SIGIRR$^{-/-}$ mice showed decreased production of IFNγ compared to WT cells, indicating a reduced Th1 immune response (A and B, Figure 5.12). Moreover, SIGIRR$^{-/-}$ lymph node cells as well as splenocytes produced significantly reduced amounts of IL-17 in the presence of type II collagen and anti-CD3 (A and B, Figure 5.13). By contrast, the amounts of the traditionally Th2 associated cytokines IL-5 and IL-10 produced by SIGIRR$^{-/-}$ lymph node cells and splenocytes showed a reversed pattern (A, B and C, D respectively, Figure 5.14). The cytokine profile observed suggest a Th2 polarised immune response with a reduction in Th1 as well as Th17 cells present in SIGIRR$^{-/-}$ mice induced with CIA.

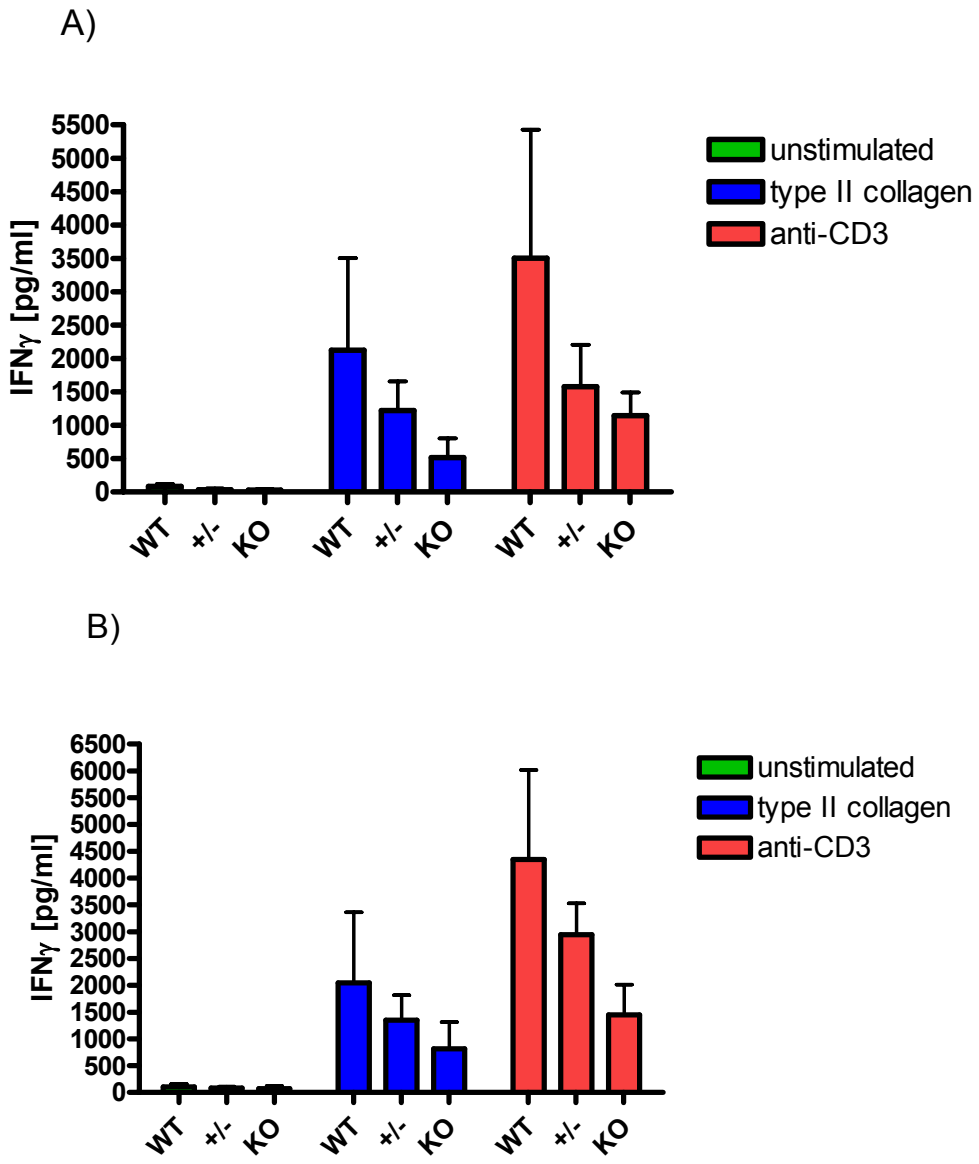


Figure 5.12: Lymph node cells and splenocytes of SIGIRR$^{-/-}$ mice produce less IFNγ compared to WT mice after induction of CIA. WT, heterozygous (+/-) and KO littermate mice were immunised with CFA and chicken collagen. After 14 days lymph nodes and spleens were removed and cells cultured either un-stimulated or in the presence of type II collagen or anti-CD3. After 48 h supernatants from A) lymph node cells and B) splenocytes were removed and IFNγ levels measured by ELISA. Data are expressed as mean $\pm$ SEM. n = 6, data is 6 mice per group pooled.

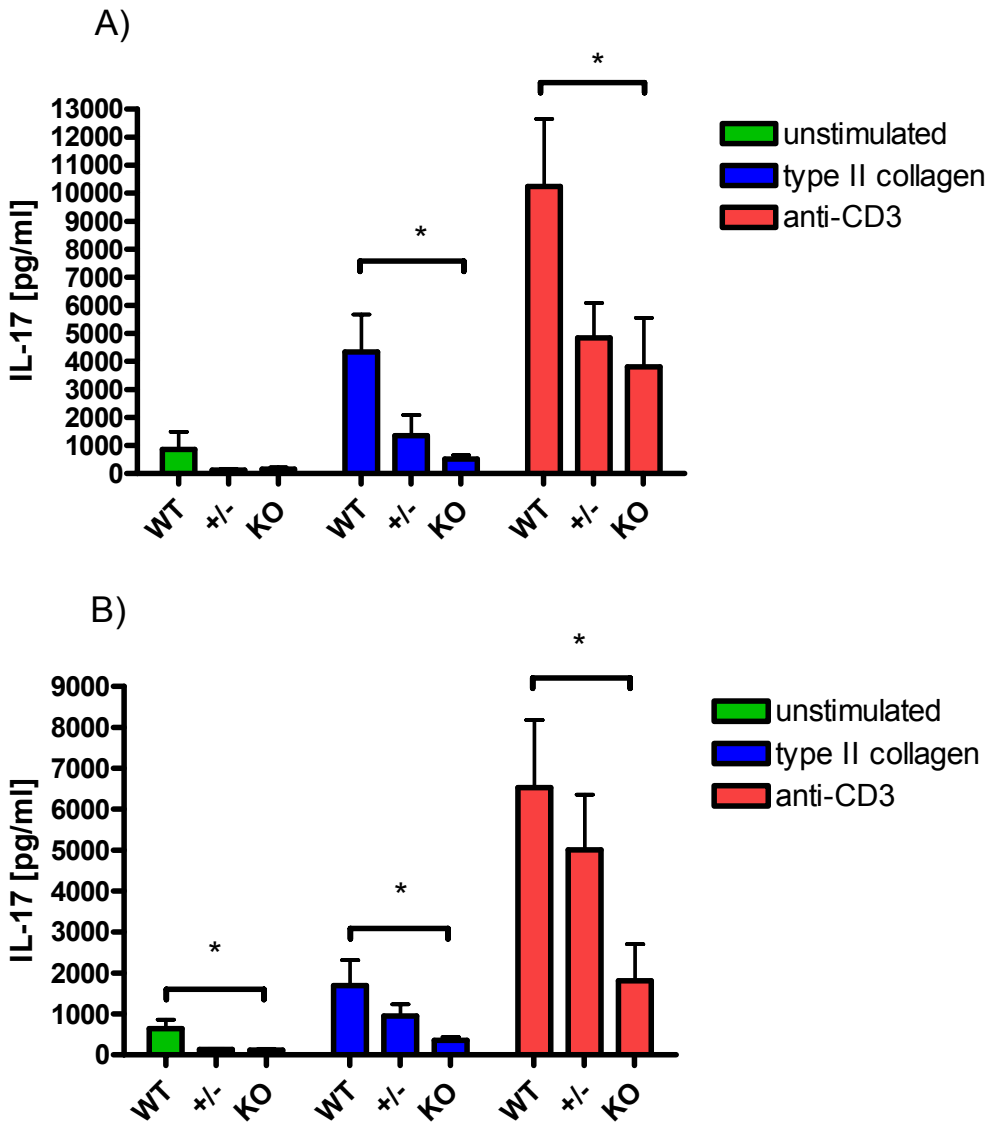


Figure 5.13: Lymph node cells and splenocytes of SIGIRR$^{-/-}$ mice produce less IL-17 compared to WT mice after induction of CIA. WT, heterozygous (+/-) and KO littermate mice were immunised with CFA and chicken collagen. After 14 days lymph nodes and spleens were removed and cells cultured either un-stimulated or in the presence of type II collagen or anti-CD3. After 48 h supernatants from A) lymph node cells and B) splenocytes were removed and IL-17 levels measured by ELISA. Data are expressed as mean $\pm$ SEM. n = 6, data is 6 mice per group pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

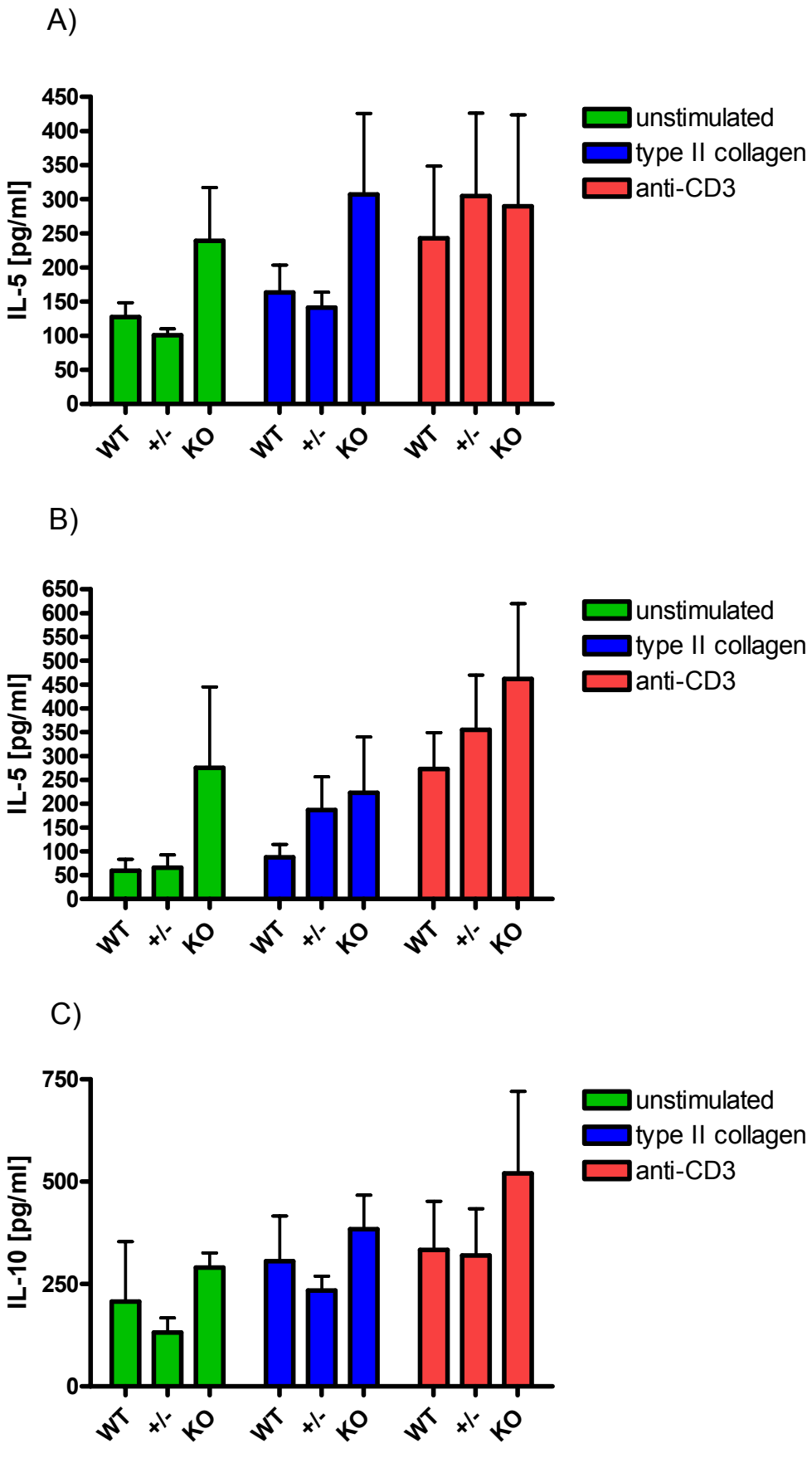


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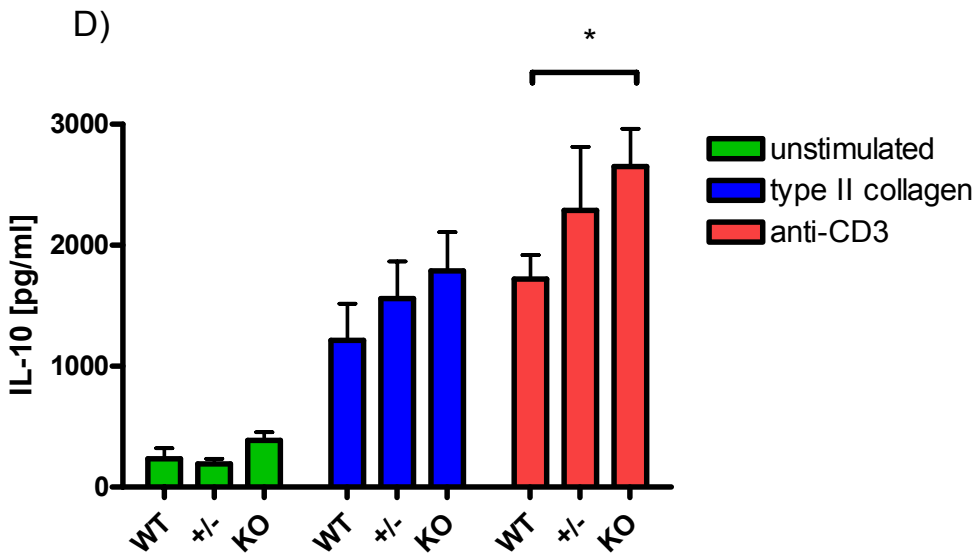


Figure 5.14: Lymph node cells and splenocytes of SIGIRR$^{-/-}$ mice produce more IL-5 (A and B) and IL-10 (C and D) compared to WT mice after induction of CIA. WT, heterozygous (+/-) and KO littermate mice were immunised with CFA and chicken collagen. After 14 days lymph nodes and spleens were removed and cells cultured either un-stimulated or in the presence of type II collagen or anti-CD3. After 48 h supernatants from A and C) lymph node cells and B and C) splenocytes were removed and IL-5 (A and B) as well as IL-10 (C and D) levels measured by ELISA. Data are expressed as mean $\pm$ SEM. n = 6, data is 6 mice per group pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

TNFα is a cytokine produced by T cells as well as macrophages and dendritic cells. Its expression depends on the activation of NF-κB. Previous studies have shown increased NF-κB activation in spleen macrophages from SIGIRR$^{-/-}$ mice in response to LPS (Lech et al., 2007). The levels of TNFα produced by SIGIRR$^{-/-}$ lymph node cells and splenocytes in this study were significantly increased compared to WT cells, thereby confirming previous studies (A and B, Figure 5.15). This further strengthens the observation of a specific influence of SIGIRR on the Th1/Th17/Th2 balance after induction of CIA.

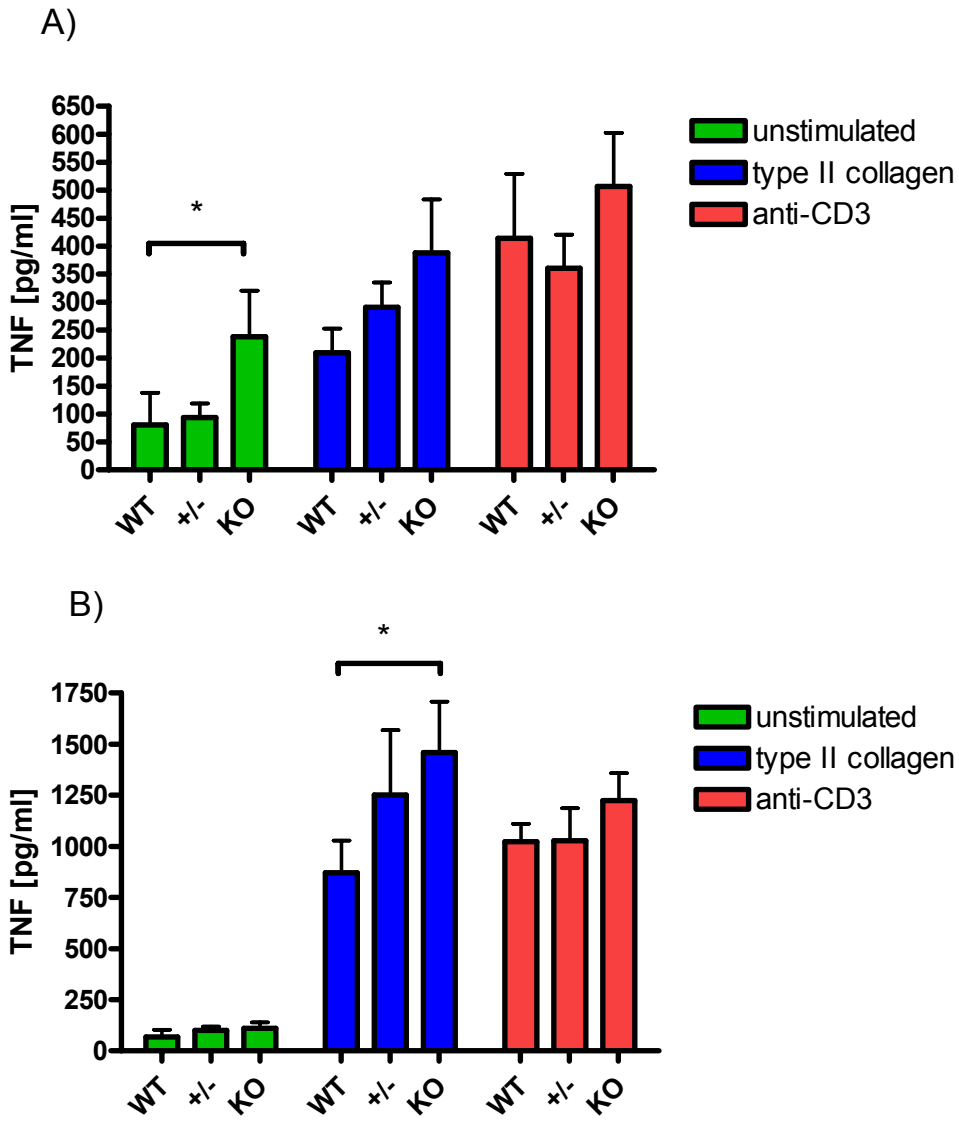


Figure 5.15: Lymph node cells and splenocytes of SIGIRR$^{-/-}$ mice produce more TNFα (A and B) compared to WT mice after induction of CIA. WT, heterozygous (+/-) and KO littermate mice were immunised with CFA and chicken collagen. After 14 days lymph nodes and spleens were removed and cells cultured either un-stimulated or in the presence of type II collagen or anti-CD3. After 48 h supernatants from A) lymph node cells and B) splenocytes were removed and TNFα (A and B) levels measured by ELISA. Data are expressed as mean $\pm$ SEM. n = 6, data is 6 mice per group pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

Following these unexpected results, the proliferation of lymph node cells and splenocytes was measured (Figure 5.16). 16 days post immunisation lymph node cells as well as splenocytes from SIGIRR deficient mice did not show differences in their proliferation in response to chicken collagen or anti-CD3 compared to SIGIRR$^{+/-}$ or WT mice.

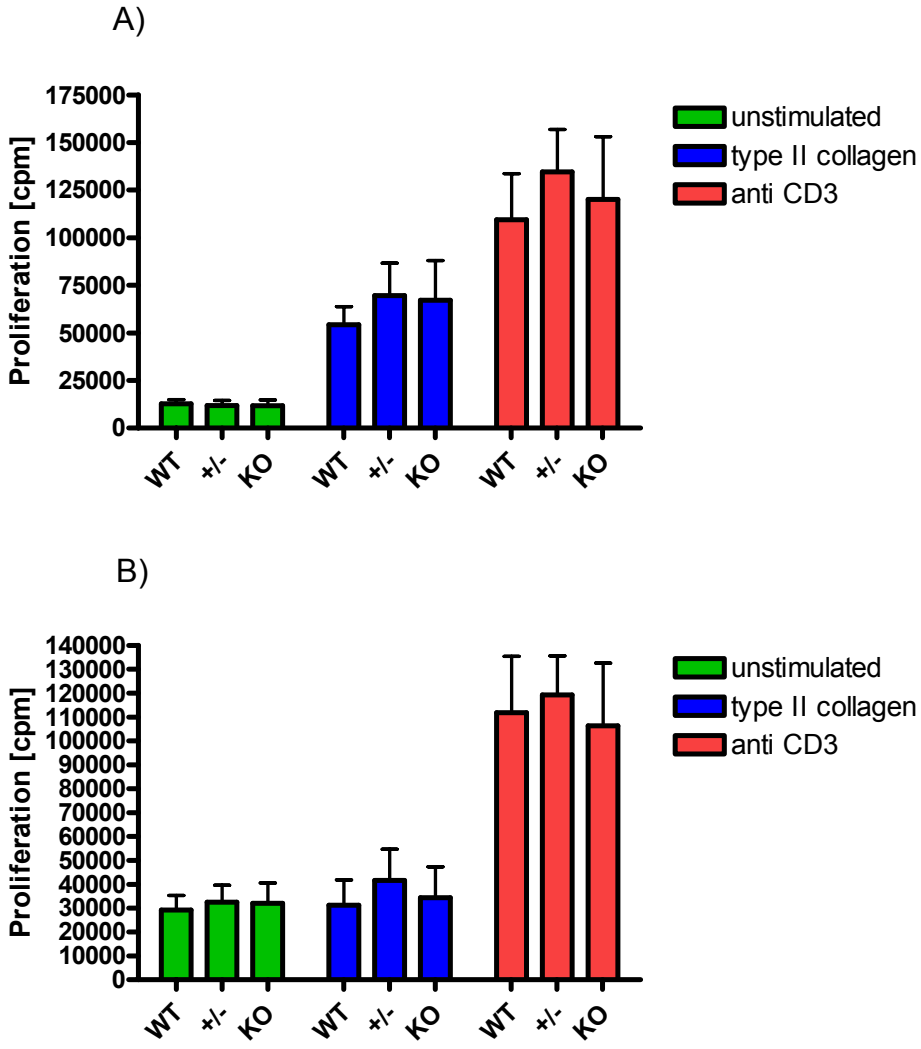


Figure 5.16: Lymph node cells and splenocytes of SIGIRR$^{-/-}$ mice show no differences in proliferation compared to WT mice after induction of CIA. WT, heterozygous (+/-) and KO littermate mice were immunised with CFA and chicken collagen. After 14 days(A) lymph nodes and (B) spleens were removed and cells cultured either un-stimulated or in the presence of type II collagen or anti-CD3. After 48 h the proliferation of A) lymph node cells and B) splenocytes was assessed by 3H incorporation. Data are expressed as mean $\pm$ SEM. n = 6, data is 6 mice per group pooled.

5.2.2.5 SIGIRR inhibits ST2 signalling

Based on the unexpected result of less severe CIA in SIGIRR deficient mice, the question arises as to how SIGIRR can influence the T cell response, since it has not been implicated in T cell signalling to this date. The IgG2a/c/IgG1 ratio observed following CIA progression in SIGIRR deficient mice may suggest a Th2 polarisation (Figure 5.9). This is supported by the cytokine profile induced by lymph node cells and splenocytes from CIA immunised SIGIRR$^{-/-}$ mice (Figure 5.12 to Figure 5.15). Therefore, this study further examined the role of SIGIRR on ST2 signalling. Similar to SIGIRR, ST2 belongs to the IL-1R/TLR family. It is expressed on murine

Th2 but not Th1 cells and was shown to be important in the differentiation to and activation of Th2 cells (Coyle et al., 1999; Lohning et al., 1999; Lohning et al., 1998; Townsend et al., 2000; Xu et al., 1998). More recently, its ligand was described to be the IL-1 family member IL-33 (Schmitz et al., 2005). The administration of IL-33 *in vitro* as well as *in vivo* leads to the production of Th2 associated cytokines IL-5 and IL-13 (Schmitz et al., 2005).

To examine whether SIGIRR is involved in regulating ST2 signalling, purified T cells from naïve SIGIRR$^{-/-}$ mice were stimulated with anti-CD28 and anti-CD3 in the absence or presence of IL-33. Subsequently, their cytokine production as well as the proliferation of T cells was examined (Figure 5.17). IL-33 induced the production of the Th2 cytokine IL-5 by 2 folds in WT T cells. SIGIRR$^{-/-}$ T cells, on the other hand, displayed a significant exaggerated induction of IL-5 compared to WT T cells (A, Figure 5.17). No differences in IFNγ or IL-17 induction were observed between SIGIRR$^{-/-}$ and WT T cells (A, Figure 5.18). Priming of αCD3/αCD28 stimulated T cells with IL-1α also showed no significant differences between the two groups (A, Figure 5.17). The proliferative responses of αCD3/αCD28 stimulated T cells were unaffected by either IL-33 or IL-1α in SIGIRR$^{-/-}$ compared to WT cells (B, Figure 5.17). These data indicate that the absence of SIGIRR increases ST2 signalling, thereby boosting the Th2 response. As a Th2-shift would be suppressive on the Th1/Th17 development, this observation may account for the *in vivo* findings described earlier.

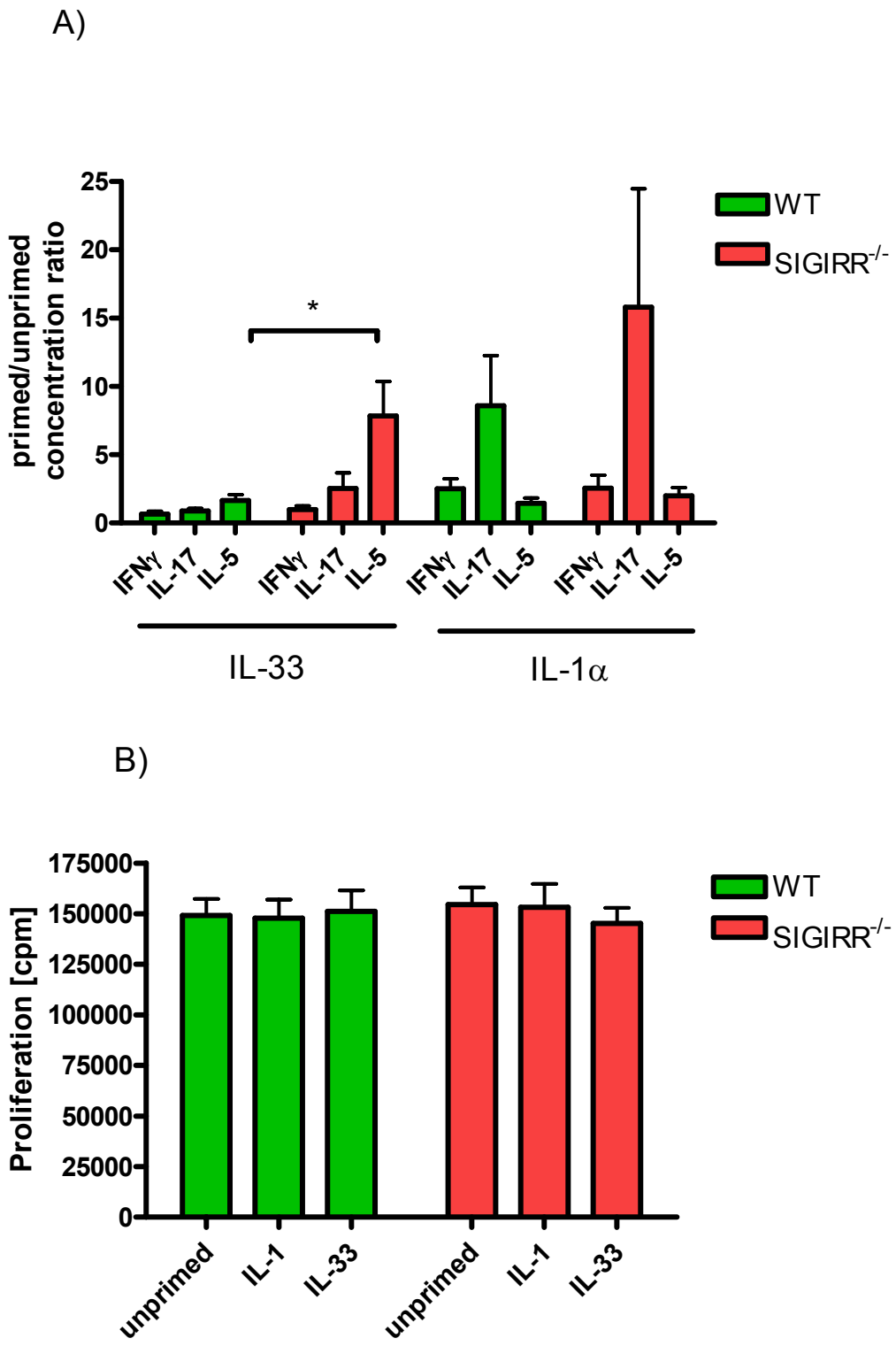


Figure 5.17: Purified Lymph node T cells from naïve SIGIRR$^{-/-}$ mice show A) a significant increase in the production of IL-5 in response to IL-33 compared to WT cells B) while there is no difference in proliferation. Lymph node T cells from naive WT and KO littermate mice were isolated by positive selection for CD4. The isolated T cells were cultured for 2 days in the presence of anti-CD28, anti-CD3 and left unprimed or primed with IL-1α or IL-33. After 48 h A) supernatants were collected and IFNγ, IL-17 and IL-5 levels measured by ELISA and B) the proliferation of the T cells assessed by ^{3}H incorporation. Cytokine concentration data has been normalised to unprimed T cells, which were stimulated in the absence of IL-1 or IL-33. Data are expressed as mean $\pm$ SEM. n = 9, data is 9 mice per group pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

In mast cells it has been shown that ST2 signalling depends on MyD88 (Schmitz et al., 2005). In order to further investigate a possible mechanism of how SIGIRR regulates ST2 signalling, MyD88 deficient purified T cells were stimulated with anti-CD28 and anti-CD3 in the absence and presence of IL-33. Subsequently the cytokine production of those cells was measured (Figure 5.18). While WT T cells increased the expression of IL-5 by 2 folds in the presence of IL-33, this up-regulation was lost in MyD88 deficient T cells (Figure 5.18).

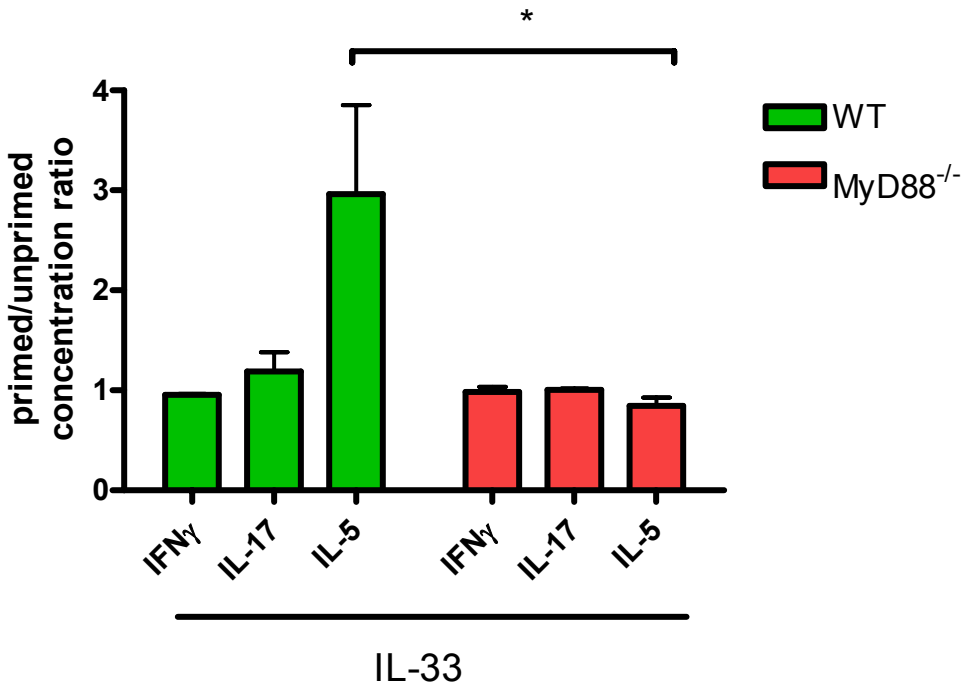


Figure 5.18: Purified Lymph node T cells from naïve MyD88$^{-/-}$ mice do not up regulate the expression of IL-5 in response to IL-33. Lymph node T cells from naive WT and KO littermate mice were isolated by positive selection for CD4. The isolated T cells were cultured for 2 days in the presence of anti-CD28 and anti-CD3 and left unprimed or primed with IL-33. After 48 h supernatants were collected and IFNγ, IL-17 and IL-5 levels measured by ELISA. Cytokine concentration data has been normalised to unprimed T cells, which were stimulated in the absence of IL-1 or IL-33. Data are expressed as mean $\pm$ SEM. n = 3, data is 3 mice per group pooled. $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.005$ ***

5.3 Conclusions

The production of TNFα is a major factor in driving RA pathogenesis (Feldmann et al., 1996). Therefore, great interest exists to identify the pathways leading to TNFα production in RA models. TLR signalling is one pathway, which became implicated in this process. This study presents data, which indicates SIGIRR as a negative regulator of spontaneously released cytokines produced by RA synovial membranes. This is most likely due to the inhibition of IL-1R and/or TLR signalling by SIGIRR. Furthermore, while the function of SIGIRR as an inflammatory inhibitor has been extensively studied in murine cells and human cell lines, no data is so far available on the function of SIGIRR in the adaptive immune response (Garlanda et al., 2007a; Garlanda et al., 2004; Garlanda et al., 2007b; Qin et al., 2005; Xiao et al., 2007). Date from this study implicates SIGIRR in the regulation of $CD4^+$ T cells differentiation (Figure 5.19).

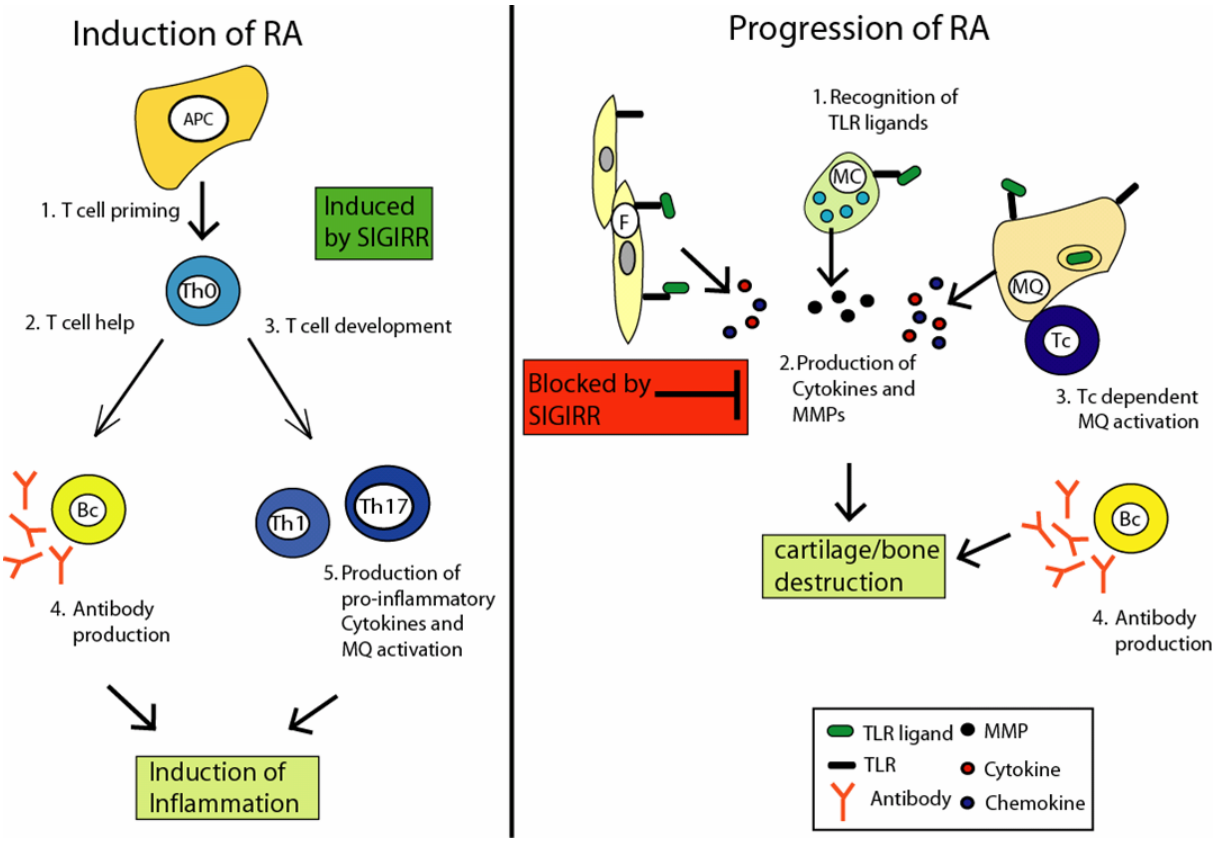


Figure 5.19: Simplified overview of mechanisms involved in RA induction and RA progression and the possible differential effects of SIGIRR on those disease stages. During the induction of RA the development of Th17 and/or Th1 cells from naïve CD4+ T cells as well as the production of auto-antibodies by B cells are essential mechanisms. At later stages of the disease, the production of pro-inflammatory cytokines and MMPs by innate immune cells, synovial fibroblasts and T cells drive the progression of tissue destruction and chronic inflammation. While SIGIRR is able to block the spontaneous release of cytokines by human RA synovial cells it seems to be necessary for Th17 development and antibody production during the induction of CIA. Abbreviations: DC (dendritic cell), F (Fibroblast), MC (monocyte), MØ (macrophage), Tc (T cell), TLR (toll-like receptor), Bc (B cell), MMP (matrix metalloproteinase), Th (T helper cell).

Given the role of SIGIRR in regulating inflammation this study further investigated its function in RA. Although, the aetiology of RA has not yet been fully described, the important role of T cells and the subsequent induction of a pathogenic B cell response in initiating the disease has been extensively shown (Bugatti et al., 2007; Furuzawa-Carballeda et al., 2007). During the progression of RA the continuous production of chemokines, cytokines and MMPs by neutrophils, macrophages and synovial fibroblasts drive the chronic inflammation in the joint as well as joint destruction (Feldmann et al., 1996). Several studies implicate endogenous TLR ligands, so called danger signals released from necrotic cells, in the chronic inflammation and joint destruction observed in RA (reviewed in (Drexler et al., 2006; Roelofs et al., 2008)). Previous results published on SIGIRR would, therefore, predict a role for it in attenuating the chronic inflammation in RA joints.

In order to investigate the function of SIGIRR in a human disease model, RA synovial membranes obtained from patients undergoing joint replacement were used. Expression data of SIGIRR, obtained from flow cytometry analysis, are difficult to interpret due to the high amount of unspecific binding by a unspecific isotype control. A reason for the high degree of unspecific staining of RA membranes could include the treatment of the membranes with collagenase in order to obtain a single cell culture. As the expression pattern of SIGIRR in RA membranes is of great interest for future research it needs to be further examined by different methods, e.g. real-time PCR, or different protocols, e.g. cell sorting. Moreover, future experiments need to compare the level of expression between RA- and OA joints.

Functional studies of SIGIRR by over-expressing it, showed a significant inhibition of IL-6 and TNFα, which is spontaneously produced by RA synovial cells. A similar trend was observed for IL-10 although it did not reach significance. In contrast, IL-8 was not inhibited by SIGIRR over-expression, which may indicate a degree of specificity of SIGIRR. Since SIGIRR has been shown to inhibit IL-1R and TLR signalling (Garlanda et al., 2004; Qin et al., 2005; Wald et al., 2003) the suppression of spontaneous cytokine production by RA membranes, resulting from SIGIRR over-expression, is most likely due to the inhibition of IL-1R and/or TLR signalling. Sacre *et al.* already demonstrated, that the spontaneous release of cytokines by RA-membranes is dependent on the TLR adaptor molecules MyD88 and Mal (Sacre et al., 2007a). Therefore, it is reasonable to assume that SIGIRR inhibits TLR signalling in RA synovial cells by either blocking access for MyD88 to the TLR, as was already shown in other models, or by sequestering MyD88 (Qin et al., 2005). How SIGIRR accomplishes its specificity, inhibiting IL-6 and TNFα but not IL-8, is part of an ongoing investigation. One possible explanation for this observation could be the specific regulation of MAPKs by SIGIRR shown in chapter 4.

To further confirm the attenuating role of SIGIRR in the pathology of human RA this study used an *in vivo* model of RA, CIA. However, SIGIRR deficient mice showed resistance to CIA. Since the incidence of disease as well as its severity was lower in SIGIRR deficient mice, these unexpected results would implicate an involvement of SIGIRR in the initiation of CIA.

The use of anti-CD20 monoclonal antibodies (Rituximab) and CTLA4-Ig fusion proteins implicate a role for activated B cells and T cells in the pathology of RA (Edwards et al., 2004). In the case of CIA the anti-type II collagen antibodies produced are mainly of the IgG2a/c subclass, which have been shown to be increased at the peak of the disease (Cho et al., 2007). Furthermore, several reports have shown that the subclass of antibody produced during CIA can determine the outcome of the disease (Cho et al., 2007). Since results in this study showed a decreased incidence in SIGIRR$^{-/-}$ mice, the anti- type II collagen antibody levels were measured. The amounts of IgG1 and IgG2a/c antibodies produced in WT animals is characteristic for C57Bl/6 mice immunised with chicken collagen, as has been shown before (Inglis et al., 2007). However, SIGIRR$^{-/-}$ mice produce significantly less class IgG2a/c but similar levels of class IgG1 antibodies compared to WT mice 11 days after disease onset. Moreover, the decreased ratio of IgG2a/c to IgG1 implicates a swing from a Th1 to a Th2 response in SIGIRR deficient animals, which could be responsible for a reduced incidence and severity of CIA observed in these animals. As the development of CIA is related to IgG2a/c levels (Cho et al., 2007) the less severe disease in SIGIRR deficient animals might be explained by the lower titres of this antibody subclass. A general defect in B cell activation does not seem to be the reason for the loss in IgG2a/c antibody production as purified B cells from naïve SIGIRR$^{-/-}$ mice show a significant increase in proliferation in response to IgM as well as LPS compared to WT mice.

Since an intrinsic defect in the activation of B cells appeared unlikely and the fact that IgG2a/c production are a characteristic of Th1 activity, the nature of the T helper response in SIGIRR$^{-/-}$ mice was further investigated. SIGIRR deficient mice showed a reduction in IFNγ as well as IL-17 production but increased levels of IL-5 and IL-10 compared to WT cells. This cytokine profile may suggest a Th2 polarised immune response and at the same time a reduction in Th1 and Th17 immunity. CIA is clearly considered to be a Th1 and/or Th17 cell driven disease (Cho et al., 2007). It is still under debate; however, whether Th1 and/or Th17 cells are the driving force in initiating RA. The importance of Th17 cells in RA pathology has been demonstrated in studies using IL-17 deficient mice as well as IL-17 blocking antibodies, which showed suppressed induction and severity in murine arthritis models (Koenders et al., 2005; Lubberts et al., 2004; Lubberts et al., 2005; Nakae et al., 2003). Similarly, mice lacking IL-23, which is necessary for Th17 development, are also protected from developing CIA (Murphy et al., 2003). Moreover, Th17 cells have been shown to be preferentially recruited to the inflamed

joint of mice and humans through the production of CCL20 by synovial fibroblasts in the arthritic joint (Hirota et al., 2007). However, other studies could not confirm an increase in Th17 cells in the joints of RA patients compared to healthy donors, while at the same time showing increased recruitment of Th1 cells to the inflamed joint (Yamada et al., 2007). These differences may as well be due to the model used and/or to the different disease stages investigated. In contrast to a Th1 and Th17 immune response, Th2 cells are considered to be protective for RA initiation and progression (Cho et al., 2007). The Th2 cytokines IL-4 and IL-10 inhibit osteoclastogenesis and thereby the destruction of the bone while at the same time IL-17 enhances osteoclastogenesis (Abu-Amer, 2001; Hong et al., 2000; Kotake et al., 1999). Moreover, the anti-inflammatory properties of IL-10 through suppressing pro-inflammatory cytokine production have been shown in CIA as well as other inflammatory diseases (Walmsley et al., 1996; Williams et al., 2004). Therefore, a reduction in Th1 as well as Th17 immunity together with the induction of a Th2 polarised immune response might explain the reduced incidence and severity of CIA in SIGIRR deficient mice.

This raises the question of why such an unexpected alteration in the adaptive immune response should occur, especially as SIGIRR has not been previously implicated in T cell function. To date SIGIRR is seen as a repressive molecule that inhibits TLR and IL-1R signalling by blocking access of the signalling adaptor MyD88 to the receptor (Qin et al., 2005). Our present understanding of the impact on the T cell compartment of the TLR/IL-1R family is still sparse. But what is known would suggest that de-repression of signalling would be pro-inflammatory: IL-18 drives IFNγ production (Okamura et al., 1995), whereas TLR8 (in humans) is suppressive to regulatory T cell function (Peng et al., 2005). However, recent advances in the understanding of ST2, another molecule belonging to the same family as SIGIRR, may provide an explanation. Similar to SIGIRR, ST2 is a family member of the TIR domain containing receptor family of IL-1Rs and TLRs (Li and Qin, 2005). ST2 is expressed as a soluble version, which is secreted, and as a membrane bound receptor, due to alternative splicing (Yanagisawa et al., 1993). It has been originally described as an orphan receptor specifically expressed on Th2 but not Th1 or Th17 cells and has been shown to be essential for Th2 development and function (Coyle et al., 1999; Lohning et al., 1999; Lohning et al., 1998; Townsend et al., 2000; Xu et al., 1998). Subsequently, its ligand, IL-33, has been described, which has the capability to drive Th2 associated cytokine expression *in vivo* as well as *in vitro* through initiating the recruitment of MyD88, IRAK1, IRAK4 and TRAF6 to ST2 (Schmitz et al., 2005). Results in this study showed that purified T cells from SIGIRR deficient mice increased the production of IL-5 in the presence of IL-33 compared to WT T cells. This effect is specific to IL-33 since its family member IL-1α does not induce increased T cell cytokine expression in SIGIRR deficient T cells. Furthermore, the induction of IL-5 production by IL-33 is dependent on MyD88 as T

cells lacking MyD88 do not increase their IL-5 expression in the presence of IL-33. Therefore, these results implicate SIGIRR as an inhibitor of IL-33 induced ST2 signalling, which is dependent on MyD88. As a consequence mice lacking SIGIRR show increased ST2 signalling, thereby skewing the T cell response towards Th2.

In addition to its role as a Th2 marker, membrane bound ST2 has been described to inhibit TLR signalling in murine macrophages and HEK293 cells (Brint et al., 2004). Interestingly, the soluble version of ST2 has already been examined as a therapy in the CIA model (Leung et al., 2004). It showed significant efficacy in reducing CIA after disease onset, which was ascribed to blocking macrophage activation through an unknown mechanism (Leung et al., 2004). However, its effect on T cells was not further investigated. In a murine asthma model treatment with soluble ST2 reduced the production of IL-4, IL-5 and IL-13 by IL-33 stimulated splenocytes (Hayakawa et al., 2007). It could be demonstrated that soluble ST2 binds IL-33, thereby acting as an antagonist to the membrane bound ST2 (Hayakawa et al., 2007). These results imply that soluble ST2 is an inhibitor of IL-33 signalling in T cells as well as TLR signalling in macrophages through different mechanisms. Interestingly, IL-33 signalling has recently been shown to be protective in other inflammatory conditions. Administration of IL-33 to atherosclerotic $ApoE^{-/-}$ mice has been shown to reduce disease severity (Miller et al., 2008). Similar to soluble ST2, our results describe SIGIRR as a novel inhibitor of membrane bound ST2 signalling in T cells while at the same time being an inhibitor of TLR signalling in macrophages and DCs. SIGIRR inhibits membrane bound ST2 through blocking access to the receptor for downstream signalling transducers, e.g. MyD88. This inhibitory mechanism resembles the mechanism used by SIGIRR to inhibit IL-1R and TLR signalling (Qin et al., 2005).

The observation of lower incidence and less severe CIA in $SIGIRR^{-/-}$ mice is counterintuitive since SIGIRR is an inhibitor of IL-1R as well as TLR signalling (Garlanda et al., 2004; Wald et al., 2003). This present study demonstrates a pre-eminence of a pro-inflammatory role on the adaptive immune response, through the suppression of Th17 development, over an expected pro-inflammatory phenotype on innate immune cells, by SIGIRR (Figure 5.19). Based on the human *in vitro* data, where SIGIRR over-expression inhibits the spontaneous release of cytokines in an established disease model we predict that if the adaptive immune response is bypassed during CIA induction, a more severe pathology in SIGIRR deficient mice will be observed. Therefore, future work will investigate models of arthritis, which bypass the initiation stage of the disease and directly depend on TLR stimulation, e.g. streptococcal cell wall induced joint inflammation (Joosten et al., 2008). Overall these data highlight the intricacies of the relationship between the adaptive and innate immune systems in the development and progression of a complex disease such as RA.

CHAPTER 6

CONCLUDING DISCUSSION

6.1 Concluding Discussion

RA is a complex chronic inflammatory disease involving the innate as well as the adaptive immune systems. Multiple factors play a role in the initiation and progression of the disease. Genetic risk factors are known to contribute to the susceptibility to RA, as do environmental factors such as smoking, infections and the release of danger signals (Krause et al., 1996; Silman et al., 1996; Stastny, 1978). However, it is still unclear whether these factors contribute to the initiation of the disease or are involved in acceleration of pre-existing conditions leading to disease progression.

The initiation of an inflammatory reaction in response to infection is mediated via the recognition of microbial products by PRRs. TLRs are the most well established PRR family capable of inducing signalling pathways, which ultimately result in the production of pro-inflammatory mediators and the induction of the adaptive immune response. This study was based on the hypothesis that TLRs might participate in the pathogenesis of RA, either through the recognition of exogenous pathogenic material, or the detection of endogenous danger signals released as a consequence of tissue damage, cell necrosis or infections.

Our current understanding of TLR function and signalling pathways has emerged from a combination of research in gene targeted mice and immortalised cell lines. At present our knowledge of the function of TLRs in humans is limited partly as a consequence of multiple genetic differences between humans, which cannot be directly related to well controlled experiments in inbred mice. Evidence for a role of TLRs in human disease is largely based on the analysis of polymorphisms of TLRs themselves or downstream signalling mediators (Davidson et al., 2006; Ku et al., 2007; Puel et al., 2004; Schroder and Schumann, 2005). As I commenced my studies, the details of TLR biology in humans were obscure. Therefore, the aim of this thesis was to examine the function of TLR signalling as well as aspects of its regulation in primary human cells, such as DCs and macrophages, with the goal to translate it into disease relevant systems to assess therapeutic targets.

At the start of this project the four TLR adaptor proteins MyD88, Mal, TRAM and TRIF as well as the signalling pathways induced by them had been characterised in gene targeted mice and cell lines. Experiments performed in our laboratory indicated, however, that there are differences not only between those systems and primary human cells, but also between cells of myeloid- and non-myeloid origin with regards to TLR adaptor usage. For example, while the expression of a MyD88 dominant negative construct in HUVECs and synovial fibroblasts reflected the results obtained in cell lines with the inhibition TLR4 and IL-1R but not TLR3 or TNFα signalling, the expression of this construct in macrophages had no effect on TLR4

signalling. Therefore, the first objective of this study was to assess the utilisation of TLR adaptors in another myeloid cell, the DC. My results in chapter 3 demonstrated that **the expression of a wild-type TIR domain results in the activation of human monocyte derived DCs without further stimuli.** This observation does not seem to be restricted to humans, as BMDCs are also activated by MyD88 dn over-expression. Several other groups have shown that TIR domains are able to block TLR signalling, similar to what I observed in RASF and macrophages, but none of these studies specifically investigated DCs (Cirl et al., 2008; Newman et al., 2006; Stack et al., 2005).

The question still remains of how a TIR domain activates DCs, and why it seems to be specific to DCs. DCs are highly specialised innate immune cells and are able to activate the potent adaptive immune response. Because of this DCs might require special regulation in order to control excessive inflammation. The model of a constitutive inhibitory signal, specific to DCs and dependent on MyD88, I proposed in chapter 3, would provide such a mechanism. This would keep immature DCs inactive until a high enough threshold of pathogens is reached or an infection is not cleared quickly enough. Evidence for such DC specific regulatory mechanisms emerged recently. DC immuno-receptor (Dcir), a C-type lectin family member, the absence of which leads to exacerbated CIA in mice, is mainly expressed in DCs (Fujikado et al., 2008). Furthermore, Dcir deficient mice do show an excessively expanded DC population following CIA immunisation, suggesting that an inhibitory role for Dcir, which would be mainly DC specific may play a similar role. Moreover, pathogens have evolved sophisticated molecular strategies to subvert the host defense (Cirl et al., 2008). One of them is the expression of TIR domain orthologs by certain strains of *Escherichia coli* termed TIR domain containing proteins (Tcp) (Cirl et al., 2008). In order to investigate the mechanism underlying the constitutive inhibitory signal in DCs it will be interesting to test if the administration of Tcps leads to the activation of DCs while having no effect on macrophages. Furthermore, immunoprecipitation studies will be necessary to identify MyD88 interacting proteins, which are specific to DCs.

A possible application for the DC specific activation by TIR domains has recently been shown by a colleague at the KIR. Numerous signal transducing molecules have been studied for their properties as an adjuvant during vaccination. One of those molecules is NF-κB inducing kinase (NIK), which enhances the immune response when over-expressed by activating NF-κB and thereby possibly increases the efficacy of vaccinations (Andreakos et al., 2006). A possible advantage of MyD88 dn as an adjuvant compared to NIK would be its DC specific activation. Mice vaccinated with nucleoprotein and MyD88 dn showed similar viral clearance and weight loss in response to influenza x-31 infection compared to mice vaccinated without MyD88 dn but increased infiltration of $CD8^+$ T cells into the lung. This would suggest that MyD88 dn increases the recruitment of $CD8^+$ T cells possibly through increased DC activation (manuscript

in preparation). Moreover, infiltration of other immune cells into the lung as well as bronchoalveolar spaces was reduced, indicating a specific effect on T cell recruitment. Therefore, using MyD88 dn, or possibly other wild-type TIR domain constructs, as an adjuvant in influenza vaccination has the possible advantage of increasing a T cell response while at the same time reducing inflammation in the lung itself and thereby reducing tissue damage, which is the major concern during influenza infection.

The hypothesis of a DC specific inhibitory signal allowed several predictions: The inhibitory receptor must be expressed in immature DCs but not in macrophages and MyD88 must be bound to the inhibitory receptor in a constitutive manner most likely through TIR-TIR interactions. This would require a receptor containing a TIR domain and a high degree of versatility of MyD88, functioning as a signal transducer as well as inhibitor. Indeed, a recent report described an inhibitory function of MyD88. This investigation showed that MyD88 is S-nitrosylated on a specific cysteine residue in its TIR domain, which results in dysfunctional translocation of MyD88 to the cell membrane and the inhibition of TLR4 signalling (Into et al., 2008). Interestingly, only two other TIR domain containing molecules contain this cysteine residue: Mal and SIGIRR (Into et al., 2008). While there are numerous TLR inhibitors described, only SIGIRR fits all predictions, having been shown to be expressed in DCs and not macrophages and being a TIR domain containing inhibitor of TLRs through binding MyD88. This has been shown in mice and cell lines. Therefore, the role and function of SIGIRR in TLR signalling in human DCs was investigated in chapter 4. However, SIGIRR can be excluded as a DC specific TLR inhibitor as **SIGIRR mRNA and protein is expressed by both human DCs and macrophages**. At the time of this study no signalling functions for SIGIRR has been described and the mechanism of TLR inhibition by SIGIRR has not been conclusively shown in primary human cells. Therefore, SIGIRR was further investigated in human DCs and macrophages. Over-expression of SIGIRR as well as siRNA knock down showed that **SIGIRR is an inhibitor of the MyD88- and TRIF-dependent TLR signalling pathway in human DCs and macrophages.**

The current hypothesis is that SIGIRR inhibits TLR4 signalling by transiently blocking access for downstream signalling transducers, including MyD88 (Qin et al., 2005). While the results in chapter 4 confirm associations between SIGIRR and MyD88 in DCs through TIR domain interactions, these interactions are constitutive without a stimulus present. However, the possibility that the interaction observed in this study may be artificial can not be excluded, since both proteins were over-expressed. Nonetheless, these results suggest that SIGIRR acts as a brake on TLR signalling, thereby increasing the amount of stimuli necessary to activate DCs and macrophages. Another possibility could be that SIGIRR is a signal transducing receptor using MyD88 as its adaptor, similar to its family members ST2, IL-1R and TLRs. Although no

general ligand for SIGIRR has been conclusively described so far, a recent report indicates the existence of a SIGIRR ligand. Costelloe and colleagues described that IL-1F5-induced IL-4 production in the brain is dependent on SIGIRR. However, so far this has not been observed in other systems and needs to be further investigated (Costelloe et al., 2008). The hypothesis of SIGIRR transducing a signal is further supported by similarities with its family member ST2. Similar to SIGIRR, ST2 was described to be an orphan receptor and TLR inhibitor by sequestering MyD88 and Mal in macrophages (Brint et al., 2004). However, subsequently its ligand, IL-33, has been described (Schmitz et al., 2005). Therefore, cell specific functions can not be excluded in the search for the role of SIGIRR. Further investigations will be necessary and will include the development of the SIGIRR ecto-domain as a way to investigate its possible ligand as well as immunoprecipitation of endogenous SIGIRR with specific antibodies to define interacting proteins. Moreover, the effects of post-translational modifications, including nitrosylations and glycosylations, on SIGIRR function need to be further examined by using adenoviral gene transfer of single nucleotide mutated constructs of SIGIRR.

Following on from the observation of SIGIRR being an inhibitor of TLR signalling this study further investigated the role of SIGIRR in RA disease models. Currently, the most widespread and successful RA therapeutics are the biologicals, Infliximab/Enbrel/Humera, (Feldmann and Steinman, 2005), which block TNFα, a cytokine produced by innate immune cells such as macrophages (Feldmann et al., 1996). Consequently, there has been a great deal of interest in elucidating the pathways driving the production of TNFα in RA. An attractive candidate is the TLR family. There is increasing evidence that TLRs can also respond to endogenous ligands produced during tissue damage associated with chronic inflammation (Drexler et al., 2006). This hypothesis is supported by studies showing the upregulated expression of TLRs in human RA synovium (Roelofs et al., 2005) and the presence of endogenous TLR ligands in RA synovial fluid (Brentano et al., 2005a; Drexler et al., 2006; Sacre et al., 2007b). Various murine models of disease have implicated TLR4 (Abdollahi-Roodsaz et al., 2008) or TLR9 (Asagiri et al., 2008; Leadbetter et al., 2002) in RA pathogenesis. Given the potential of TLRs in driving RA, I further investigated the role of SIGIRR in suppressing signalling by the TLR and IL-1R families in RA synovial membranes and CIA.

Previous studies have shown that conditioned media from RA synovial membranes is able to activate human macrophages in a MyD88 and Mal dependent manner. This would support the hypothesis of an endogenous TLR ligand driving cytokine production in that tissue (Sacre et al., 2007a). Furthermore, the dependence of spontaneous cytokine production by RA synovial membranes on MyD88 and Mal has been demonstrated (Sacre et al., 2007a). Therefore, I hypothesise that SIGIRR is able to inhibit spontaneous cytokine release by RA synovial membranes through interfering with TLR signalling pathways. This study shows that **over-**

expression of SIGIRR inhibits the spontaneous production of IL-6 and TNFα in RA synovial membranes, most likely by sequestering MyD88/Mal.

Given this inhibitory function of SIGIRR I wanted to further elucidate the function of SIGIRR *in vivo*. Therefore, SIGIRR deficient mice were immunised with CIA. Contrary to my expectations based on the previous results, **SIGIRR deficient animals show decreased incidence and CIA pathology**. This is most likely due to SIGIRR regulating the T cell response. The cytokine profile observed in SIGIRR deficient mice following CIA induction indicates a reduction in Th1 and Th17 immunity and at the same time a Th2 polarised immune response. CIA is clearly considered to be a Th1 and/or Th17 cell driven disease (Cho et al., 2007). In contrast, Th2 immunity is considered to be protective for RA initiation and progression (Cho et al., 2007). Therefore, a reduction in Th1 as well as Th17 immunity together with an induction of a Th2 polarised immune response leads to a reduction in IgG2a/c antibody production. Taken together these studies might explain the reduced incidence and severity of CIA in SIGIRR deficient mice.

Given this unexpected alteration of the adaptive immune response the question arises as to how SIGIRR can influence T cell function. Present studies indicate a pro-inflammatory role of TLR/IL-1R signalling on the T cell compartment. IFNγ production has been shown to be induced by IL-18 (Okamura et al., 1995) and, more recently, stimulation of human TLR8 has been demonstrated to suppress regulatory T cell function (Peng et al., 2005). Therefore, the attention of this study focused on ST2, which has been known for a long time to drive Th2 differentiation (Coyle et al., 1999; Lohning et al., 1998; Moritz et al., 1998; Townsend et al., 2000; Xu et al., 1998). The IL-1R family member ST2 has been described to be specifically expressed on Th2 but not on Th1 or Th17 cells (Nakae et al., 2007). Data published recently by Miller and colleagues, which show a reduction in atherosclerosis severity as a result of IL-33 administration, further strengthens the hypothesis that altered ST2 signalling can influence the pathogenesis of inflammatory diseases (Miller et al., 2008).

The reduced incidence and severity of CIA in SIGIRR$^{-/-}$ mice is paradoxical since SIGIRR is an inhibitor of IL-1R and TLR signalling (Garlanda et al., 2004; Wald et al., 2003). However, data presented here have uncovered a **role for SIGIRR in regulating the T_{h1}/T_{h17}-T_{h2} balance through inhibiting the IL-33/ST2 signalling pathway**. Since IL-33/ST2 signalling depends on MyD88, the mode of action of SIGIRR inhibiting ST2 signalling might be due to MyD88 sequestration/blocking as observed in case of TLR/IL-1R signalling (Qin et al., 2005). While the priming of $CD4^+$ T cells with IL-33 suggests a direct influence of SIGIRR on Th2 development, other indirect mechanisms may exist. One such possible mechanism includes TSLP (thymic stromal lymphopoetin), an IL-7 like cytokine secreted by epithelial cells that

primes DCs to drive Th2 polarisation (Liu, 2006). A recent study by Humphreys *et al.* demonstrated that IL-33 is a strong inducer of TSLP in epithelial cells (Humphreys et al., 2008 3103). Therefore, SIGIRR could act indirectly on T cell development by regulating IL-33 signalling in epithelial cells and thereby influencing the secretion of mediators such as TSLP. The results presented in this study suggest an inhibitory effect of SIGIRR on Th2 cells. However, SIGIRR may also be necessary for Th17 development. A recent study provides evidence for an involvement of SIGIRR on Th17 polarisation. Bozza and colleagues showed that *Candida albicans* infections in SIGIRR deficient mice elicit an enhanced Th17 response due to increased IL-1 and IL-23 production by DCs (Bozza et al., 2008). These results would suggest that SIGIRR has an inhibitory function on Th17 development by regulating IL-1R signalling in DCs. This may suggest that the regulation of T cell development by SIGIRR and the outcome of T cell development depend on the system used and the predominance of cytokine pathways specific to these systems.

Based on results obtained by over-expression of SIGIRR in RA synovial membranes, I hypothesise that if the adaptive immune response is bypassed during CIA induction, a more severe pathology in SIGIRR deficient mice will be observed. Therefore, streptococcal cell wall induced joint inflammation as well as antibody induced arthritis trials will be necessary to test this hypothesis (Joosten et al., 2008). Furthermore, the regulation of ST2 signalling by SIGIRR needs to be further investigated by administration of IL-33 to CIA induced mice, which should resemble the SIGIRR deficient phenotype. Moreover, *in vitro* experiments, including a time course of IL-33 stimulation of SIGIRR deficient T cells using NFAT as a readout, will be necessary to further elucidate the inhibitory function of SIGIRR on ST2. Furthermore, there is the possibility of other pathways affected by SIGIRR, which have not been explored in this present study.

The results presented in this thesis have revealed the complexities of TLR signal transduction in primary human cells. While these observations have raised the question of why these differences exist between cell types, they highlight the importance of studying signalling pathways in relevant cell types. In order to fully understand the roles TLRs have in the human innate as well as adaptive immune response, it is essential to further investigate these differences. This knowledge could potentially lead to the development of new and more specific approaches for the treatment of inflammatory diseases including RA.

CHAPTER 7

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CHAPTER 8

APPENDIX

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Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems

Anna M. Lundberg,[1] Stefan K. Drexler,[1] Claudia Monaco,[1] Lynn M. Williams,[1] Sandra M. Sacre,[1] Marc Feldmann,[1] and Brian M. Foxwell[1]

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TLR3 recognizes double-stranded RNA, a product associated with viral infections. Many details of TLR3-induced mechanisms have emerged from gene-targeted mice or inhibition studies in transformed cell lines. However, the pathways activated in human immune cells or cells from disease tissue are less well understood. We have investigated TLR3-induced mechanisms of human primary cells of the innate immune system, including dendritic cells (DCs), macrophages (MØs), endothelial cells (ECs), and synovial fibroblasts isolated from rheumatoid arthritis joint tissue (RA-SFs). Here, we report that while these cells all express TLR3, they differ substantially in their response to TLR3 stimulation. The key antiviral response chemokine IP-10 was produced by all cell types, while DCs and MØs failed to produce the proinflammatory cytokines TNFα and IL-6. Unexpectedly, TNFα was found secreted by TLR3-stimulated RA-SF. Furthermore, TLR3 stimulation did not activate NFκB, MAPKs, or IRF-3 in DCs and MØs, but was able to do so in ECs and RA-SF. These findings were specific for human cells, thereby revealing a complexity not previously expected. This is the first report of such cell type– and species-specific response for any TLR stimulation and helps to explain important difficulties in correlating murine models of inflammatory diseases and human inflammation. (Blood. 2007;110: 3245-3252)

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Introduction

The discovery of Toll-like receptors (TLRs) uncovered a key mechanism used by the immune system to detect infections and tissue damage.[1] There are 10 known human TLRs that recognize "molecular patterns" produced as a result of pathogenic infections. TLR3 is a member of this receptor family that is activated by double-stranded RNA (dsRNA),[2] an intermediate formed by most viruses during their replication. The viral dsRNA functions as a danger signal released in the extracellular environment from dying virally infected cells, alerting inflammatory cells and contributing to systemic disease.[3,4] In addition to microbial products, there is growing evidence that TLRs also recognize endogenous ligands found at sites of tissue destruction and cell death,[5] and TLR3 has been shown to recognize mRNA released from dying cells.[6] Although there are concerns that some of the data on endogenous ligands could be a result of contamination with microbial products, the detection of endogenous ligands would be useful in alerting the host of the presence of tissue injury induced by infection or other means. However, there is a potential drawback in that the same endogenous ligands are also found at sites of chronic inflammation and could further drive the inflammatory response in a TLR-dependent manner. There is increasing evidence that TLRs could be associated with chronic inflammatory diseases such as rheumatoid arthritis (RA).[5,7] In this context, TLR3 expression is increased in the RA synovium,[8,9] and the presence of TLR3 ligands might affect several aspects of the disease by acting on cells within the joint. In support of this, dsRNA can induce joint inflammation and cause arthritis in a murine disease model.[10]

Given the importance of TLRs in regulation of both positive and negative aspects of the immune response, there has been much interest in how their activation is controlled. The signaling pathways initiated by most TLRs are regulated by the adaptor protein MyD88 (myeloid differentiation factor 88), with some variations by the additional recruitment of MAL (MyD88 adaptor-like), TRAM (TRIF-related adaptor molecule), and TRIF (TIR-domain–containing adaptor inducing interferon β).[11] However, TLR3 is unique since it does not use MyD88 but instead recruits only TRIF, which mediates the activation of NFκB and IRF-3. Activation of these pathways results in expression of various inflammatory mediators including the cytokines tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) and the chemokines IL-8 and IFN-inducible-10 (IP-10). However, as for most TLRs, little is known about TLR3-induced signaling and cellular responses in human primary cells. Previous findings are mainly the result of studies in murine cells, especially derived from knockout animals, or immortalized cell lines, which are all model systems of the human immune system. Furthermore, since many cell lines are of tumor origin, it is unclear if their responses are representative of the normal innate immune response. We therefore felt that it was important to define responses induced by TLR3 in human cell types, and in particular in cells involved in chronic inflammatory disease such as RA.

The findings presented here challenge the current beliefs that signaling pathways initiated by the viral response molecule dsRNA are mediated by NFκB, MAPK, and IRF-3 in all cell types. Our

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results highlight that responses induced by dsRNA/TLR3 differ between human cells of separate lineages, as well as between mouse and human. These are crucial findings for increasing the understanding of the human immune system and the inflammatory response to infection.

Materials and methods

Ethical approval for the use of human materials was granted by the Riverside Research Ethics Committee, London, United Kingdom.

Reagents

Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was generously provided by Beheringwerke (Marburg, Germany) and human recombinant IL-4 was from Novartis Pharma (Basel, Switzerland). Human recombinant macrophage colony-stimulating factor (M-CSF) and human IL-1α were from Genetics Institute (Madison, NJ). *Escherichia coli (E coli)* LPS was obtained from Sigma (St Louis, MO) and polyinosinic-polycytidylic acid (poly I:C) was from Amersham Life Science (Buckinghamshire, United Kingdom). Mouse serum was purchased from Sigma (St Louis, MO); anti–TLR3-FITC (clone 40C1285.6) (raised against a peptide corresponding to AA 54–71 in the LRR motifs of the TLR3 receptor extracellular domain) was from Imgenex (Cambridge, United Kingdom); and the corresponding IgG1-FITC–labeled isotype control was from Becton Dickinson (Oxford, United Kingdom). Protein inhibitor cocktail and chloroquine were purchased from Sigma (Poole, United Kingdom). The IRF-3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-HA, from Covance (Denver, PA); and anti-Flag, from Sigma (Poole, United Kingdom).

Cell isolation and culture conditions

Human peripheral blood mononuclear cells were isolated and differentiated for 5 to 7 days with 50 ng/mL GM-CSF, and 10 ng/mL IL-4 for dendritic cells (DCs), and for 3 to 4 days with 100 ng/mL M-CSF for macrophages (MØs), as previously described.[12,13] When matured, DCs express the specific marker CD83 and MØs express varied levels of CD14. During assays, both cells were cultured at 10^6 cells/mL in RPMI1640 5% FCS, 1% penicillin/streptomycin. Rheumatoid arthritis synovial fibroblasts (RA-SFs) and human umbilical vein endothelial cells (HUVECs) were isolated as previously described.[14,15] RA-SFs express vimentin at more than 95% and were cultured at 10^5 cells/mL in DMEM 10% FCS, 100 U/mL penicillin/streptomycin, and 200 μg/mL indomethacin (Sigma, Poole, United Kingdom); HUVECs were 99.19% pure[16] and were cultured at 10^5 cells/mL on gelatin in RPMI1640, 10% FCS, 10% newborn calf serum (Gibco, Auckland, NZ), 1% penicillin/streptomycin, 15 μg/mL endothelial growth supplement (Sigma, Poole, United Kingdom), and 50 IU/mL heparin (CD Pharmaceutical, Wrexham, United Kingdom). Murine bone marrow DCs were derived as previously described[17] and cultured at 10^5 cells/mL in DMEM 5% FCS and 1% penicillin/streptomycin.

Reverse transcriptase–polymerase chain reaction

RNA was isolated using the Qiagen RNA Blood isolation kit (Qiagen, Hilden, Germany). Total RNA was reverse transcribed with Superscript II RNase H^- reverse transcriptase (Gibco, Paisley, United Kingdom) and oligo (dT) primer. The primers used were as follows: 5′-GCAAACACAAGCATTCGGAATCT-3′ and 5′-TTGAAGGCTTGGGACCAAGGCA-3′ for TLR3; 5′-TGGATACGTTTCCTTATAAG-3′ and 5′-GAAATGGAGGCACCCCTTC-3′ for TLR4; 5′-GAAGCTCAGAAGCAGTATTGGTC-3′ and 5′-GGTTGGTGTAGGATGACAAACTCC-3′ for MD-2. Amplification was performed in a Dyad polymerase chain reaction (PCR) machine (MJ Instruments, Watertown, MA) and consisted of 35 cycles with the annealing temperature 62°C for TLR3 and 40 cycles with the annealing temperature 54°C for TLR4 and MD-2.

Flow cytometry

Intracellular and extracellular TLR3 expressions were determined using flow cytometry. Cells fixed with 2% paraformaldehyde were either left untreated or permeabilized with 1% saponin. The cells were incubated with mouse antihuman TLR3-FITC or isotype control for 30 minutes on ice in the presence of 1% mouse serum. The samples were analyzed on a Becton Dickinson LSR flow cytometer.

Cytokine analysis by enzyme-linked immunosorbent assay

Supernatants were harvested after 18-hour stimulation with LPS (100 ng/mL) or poly I:C (20 μg/mL). Enzyme-linked immunosorbent assay (ELISA) reagents for measurement of human TNFα and IL-6 were from Pharmingen (San Diego, CA) and human IP-10, murine IL-6, and murine TNFα reagents were obtained from R&D Systems (Abington, United Kingdom). Absorbance of 450 nm was measured on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic, Cambridge, United Kingdom) using the Ascent version 2.4.2 software program (Thermo Labsystems, Cambridge, United Kingdom). Results are expressed as the mean concentration of triplicate cultures plus or minus SD.

Mixed lymphocyte reaction

Human DCs were stimulated and cultured in graded doses with 1×10^5 allogeneic elutriated T cells in quadruplicate. Proliferation was measured on day 5 by thymidine incorporation after a 16-hour pulse with [^{3}H] thymidine (0.5 μCi/well [0.0185 MBq]; Amersham Life Science).

TaqMan real-time PCR

Total RNA was extracted using RNeasy Kit (Qiagen) and PCR was performed using an ABI PRISM 7700 Sequence Detection System and predeveloped assay reagents (PerkinElmer Applied Biosystems, Warrington, United Kingdom). The one-step real-time PCRs were run for 40 cycles, and all quantitations were normalized to GAPDH. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

NFκB luciferase reporter assay

Cells were infected for 1 to 2 hours in serum-free media with a recombinant replication-deficient adenovirus containing an NFκB luciferase reporter gene, which is a modification of the pNFκB-Reporter vector (BD Clontech, Palo Alto, CA) and was kindly provided by Dr Paul McCray (University of Iowa). DCs and MØs were infected at 100:1 multiplicity of infection (moi); ECs, at 100:1 at 60% confluence; and RA-SFs, at 500:1, resulting in more than 90% infection efficiencies, as shown by us and others.[12,13,18,19] The cells were cultured for 24 hours prior to 6-hour stimulation. The luciferase assay was performed as previously described.[20]

Western blotting

Nuclear protein fractions were extracted as previously described,[21] and whole-cell lysates were prepared in 1% Triton X100, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.1 mM Na_3VO_4, 5 mM NaF, 1 mM DTT, and 1 × protein inhibitor cocktail. Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel and transferred to a PVDF membrane for Western blotting.

Limulus assay

The endotoxin content in all reagents was measured with the Limulus assay (BioWhittaker, Wokingham, United Kingdom) according to the manufacturer's instruction. Only batches of poly I:C with no detectable levels of endotoxin were used in this study.

Statistical analyses

Mean and standard deviation (SD) were calculated using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA).

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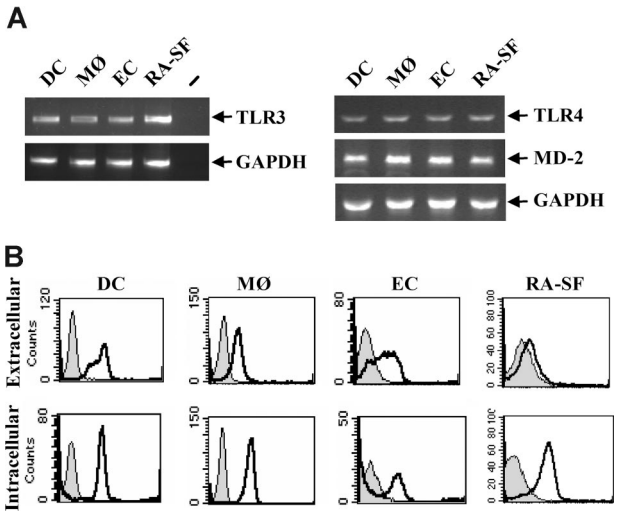


Figure 1. TLR3 is expressed on all cell types used in this study. Human DCs were generated from peripheral blood monocytes after 5- to 7-day culture in the presence of 10 ng/mL IL-4 and 50 ng/mL GM-CSF. MØs were differentiated from peripheral blood monocytes for 3 to 4 days in the presence of 100 ng/mL M-CSF. ECs were isolated from human umbilical veins and were used at passages 2 to 3. Fibroblasts were isolated from synovial tissue from RA patients undergoing joint replacement. (A) Total RNA was isolated from each cell type and 1 μg was subjected to reverse transcriptase-PCR and analyzed for TLR3, TLR4, MD-2, or GAPDH expression with PCR. – indicates PCR-negative control. (B) DCs, MØs, ECs, and RA-SFs were analyzed for both intracellular and membrane-bound TLR3 by flow cytometry. Cells stained with isotype control (filled histograms) or anti–TLR3-FITC antibody (open black histograms) are shown.

Results

Analysis of TLR3 expression on human immune cells

The effects of TLR3 stimulation on human immune cells were analyzed due to their key roles in infectious diseases and acute as well as chronic inflammation. Professional immune cells such as human primary dendritic cells (DCs) and macrophages (MØs), and nonprofessional immune cells such as endothelial cells (ECs) and synovial fibroblasts from RA disease tissue (RA-SFs) were examined. TLR3 expression was detected at the RNA level in all the cell types studied (Figure 1A). By fluorescence-activated cell sorting (FACS), a proportion of the cells was shown to express TLR3 extracellularly, whereas a higher level of TLR3 was found expressed intracellularly in all cell types (Figure 1B).

Differential induction of proinflammatory and interferon response cytokines by TLR3 stimulation

To evaluate the responses of TLR3 stimulation, the effects of poly I:C treatment on cytokine production by the different human immune cells were examined. The TLR4 ligand LPS was used as a control stimulus, and, as shown in Figure 1A, all cell types studied also express TLR4 and MD-2. In murine cells, LPS induces similar pathways as dsRNA, leading to production of proinflammatory cytokines such as IL-6 and TNFα, as well as expression of IFN-regulated genes (IRGs), such as the chemokine IP-10.[2,22] As predicted, poly I:C induced IP-10 production from DCs to similar levels as LPS-stimulated cells (Figure 2A). However unlike LPS, poly I:C did not induce TNFα, IL-6 (Figure 2A), or IL-8 (data not shown) in human DCs. This was not due to production of the anti-inflammatory cytokine IL-10, since it was not induced by poly I:C, whereas IL-10 was produced after LPS stimulation (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The induction of IP-10 but not TNFα or IL-6 was repeated in poly I:C–stimulated MØs (Figure 2A). This effect was specific for human cells, since TNFα or IL-6 was produced by murine DCs after poly I:C stimulation (Figure 2B), confirming previous studies.[2,22] As poly I:C was described by another group to induce TNFα and IL-6 mRNA in human DCs,[23] we investigated whether stimulation with poly I:C could increase TNFα and IL-6 mRNA levels in the absence of increased protein expression. However, studies of mRNA expression confirmed our protein studies with poly I:C inducing very little, if any, TNFα and IL-6 mRNA, whereas LPS caused high mRNA levels of both cytokines (Figure 2C). In addition, we could detect type I IFN from both poly I:C– and LPS-stimulated human DCs (data not shown). Stimulation of human DCs with poly I:C was also previously shown to induce DC activation and functional maturation.[24-26] We could confirm these findings, since poly I:C stimulation of human DCs resulted in increased ability to induce an allogenic MLR (Figure 2D) and up-regulation of the costimulatory markers CD80 and CD86 and the maturation markers HLA-DR and CD83 (Figure 2D).

Given these unexpected findings in human primary DCs and MØs, we examined the responses to poly I:C in ECs, which was previously shown to produce IL-6 mRNA and adhesion molecules after poly I:C stimulation.[27,28] In addition, we analyzed the responses in RA-SFs, which have also been shown to respond to poly I:C treatment.[29-31] Stimulation of ECs with poly I:C induced IP-10 secretion, whereas no substantial production could be observed after LPS treatment (Figure 2E). Furthermore, poly I:C stimulation of ECs led to the production of IL-6 and IL-8, at levels comparable with LPS (Figure 2E and data not shown). Similar results were also observed for poly I:C–stimulated RA-SFs (Figure 2E). An interesting observation made in this study was that poly I:C could induce TNFα secretion by human RA-SFs (Figure 2F). This effect was specific to poly I:C since TNFα levels could not be detected after LPS stimulation (Figure 2F). It was also found specific for RA-SF, since poly I:C–stimulated human skin fibroblasts (HSFs) produced only IL-6 and not TNFα (data not shown). This is the first report describing the ability of a ligand to induce TNFα production from a human fibroblast population. Overall, these results indicated that responses induced by poly I:C in human cells differed depending on the cell lineage and can be divided into response induced by myeloid cells (DCs and MØs) and nonmyeloid cells (ECs and RA-SFs).

Poly I:C stimulation was previously shown to be blocked by inhibitors of endosomal maturation,[32,33] suggesting that the TLR3 signaling is initiated intracellularly. To address this issue, we investigated if the cell type–specific difference was the result of TLR3 activation mediated from different locations in myeloid and nonmyeloid cells. However, in the presence of chloroquine, an inhibitor of endosomal acidification, IP-10 production was inhibited in both DCs and RA-SFs (Figure 3). This suggests that cells of both lineages use the intracellular TLR3 and that the distribution of the receptor is not the cause of these variations.

Cell lineage–specific activation of NFκB and MAPKs upon TLR3 stimulation

The finding that poly I:C induces only proinflammatory cytokines in certain cell types prompted the examination of downstream signaling pathways. NFκB is involved in regulating TNFα and IL-6 expression, and previous studies have claimed that dsRNA stimulation can activate NFκB.[2,34-39] We examined if the cell

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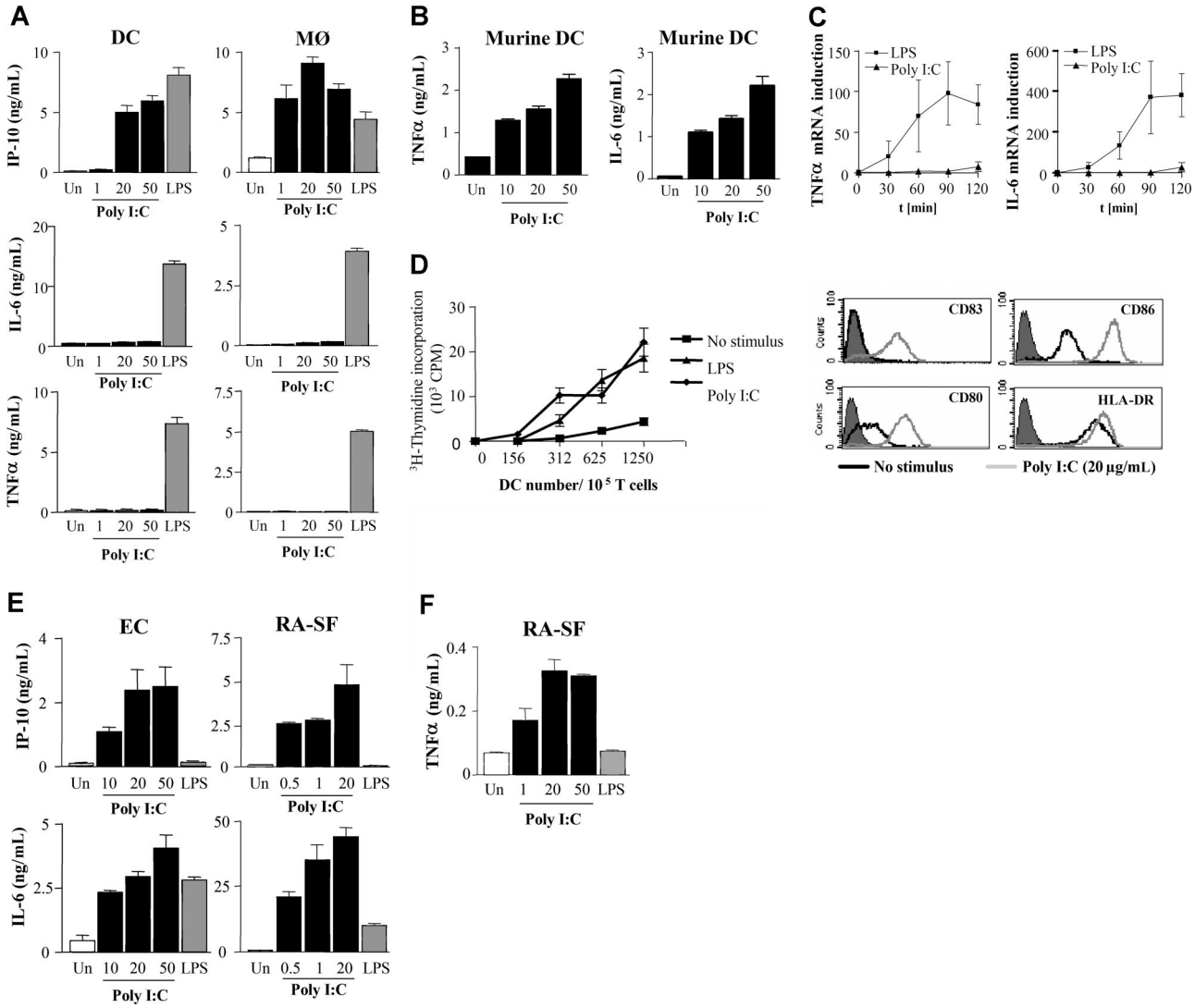


Figure 2. Poly I:C–induced expression of IFN-regulated genes but not proinflammatory cytokines is both cell-type and species specific. (A) Human DCs and MØs, (B) murine DCs, (E) ECs, and (E,F) RA-SFs were stimulated with either 100 ng/mL LPS or poly I:C (in μg/mL) as indicated. The supernatants were analyzed for IP-10, IL-6, and TNFα by ELISA. The data are mean values (± SD) from triplicates and are representative of at least 3 experiments using cells of different donors. (C) Human DCs were stimulated with either 100 ng/mL LPS or 20 μg/mL poly I:C, and total RNA was isolated and assessed for the levels of TNFα and IL-6 mRNA by Taqman PCR. The points correspond to values from 4 different donors (± SD). (D) Human DCs were stimulated with 20 μg/mL poly I:C or 100 ng/mL LPS and cocultured with 10^5 allogeneic T cells in quadruplicate. Proliferation was determined on day 5 using [^{3}H] thymidine. Each point represents the mean (± SEM) from 3 independent experiments on unrelated donors. Human DCs were also examined for cell surface expression of CD80, CD86, HLA-DR, and CD83 by flow cytometry. Unstimulated DCs (black line) and DCs treated with 20 μg/mL poly I:C for 48 hours (gray line) were compared. Cells stained with isotype control (filled histograms) or anti-TLR3 antibody (open black histograms) are shown. One representative of 3 independent experiments is shown.

type–specific differences observed with cytokine production were due to a variation in NFκB regulation. Interestingly, in DCs and MØs, poly I:C was unable to activate NFκB, whereas LPS was a potent activator, as expected (Figure 4A). In contrast, NFκB transcriptional activity in ECs and RA-SFs was induced by poly I:C to levels comparable with LPS stimulation (Figure 4A). Furthermore, the lack of response in the myeloid cells was again shown to be a species-specific effect of human cells since poly I:C could stimulate NFκB activity in murine DCs (Figure 4B). The NFκB results were supported by studies of poly I:C–induced IκBα degradation. In a similar manner, poly I:C did not induce IκBα degradation in either DCs (Figure 5A) or MØs (data not shown). Conversely, poly I:C stimulation of RA-SFs resulted in IκBα degradation, confirming our NFκB results (Figure 5B). The poly I:C–induced IκBα degradation in RA-SFs occurred with a delayed kinetics than LPS stimulation, which induced IκBα degradation in all cell types, as expected.

The production of proinflammatory cytokines can also be regulated by the 3 major MAPKs: p38 MAPK, JNK (p46/p54), and ERK (p42/p44) MAPKs. It was previously reported that poly I:C treatment induced phosphorylation of the MAPKs in murine cells and various cell lines.[2,35,36,40-44] However in our study, poly I:C stimulation did not induce phosphorylation of p38, JNKs, or ERKs at any time point analyzed in either human DCs (Figure 6A) or MØs (data not shown), whereas LPS was found to potently induce all 3 groups of MAPKs, as expected. Conversely, stimulation of RA-SFs by both poly I:C and LPS resulted in activation of all 3 MAPKs, albeit with different kinetics (Figure 6B). To elucidate if the late activation of the MAPK in response to poly I:C was due to an indirect effect, we stimulated RA-SFs in the presence of brefeldin A, an inhibitor of the secretory pathway. Brefeldin A had no effect on poly I:C–induced ERK phosphorylation (Figure S2), indicating that the activation was not mediated by a secreted autocrine mediator.

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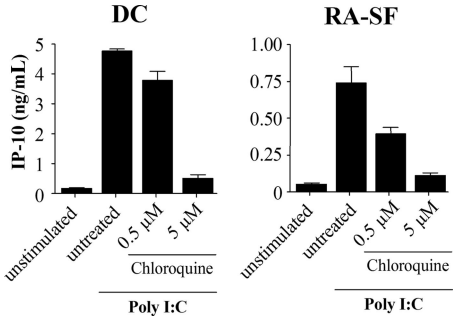


Figure 3. Poly I:C stimulation is dependent on endosomal maturation in both human dendritic cells and RA synovial fibroblasts. Human DCs and RA-SFs were incubated with chloroquine for 1 hour prior to stimulation with 20 μg/mL poly I:C for 18 hours. The supernatants were analyzed by ELISA. The data are mean values (± SD) from triplicates.

IRF-3 activation in response to poly I:C stimulation is restricted to cells of nonmyeloid lineage

IRF-3 has been shown to have a critical role in TLR3-induced responses through the TRIF-dependent pathway leading to IFNs and IRGs, such as IP-10. We aimed to address the question of whether the induction of IRF-3 could explain the poly I:C–induced IP-10 production in DCs and MØs in the absence of NFκB and MAPK activation. IRF-3 is found in the cytoplasm as an inactive monomer and becomes activated by phosphorylation, followed by dimerization and translocation to the nucleus.[45,46] The involvement of IRF-3 in poly I:C signaling was examined by measuring the change in IRF-3 nuclear levels. In this study, we could only detect IRF-3 nuclear translocation after LPS, but not poly I:C treatment of DCs (Figure 7A) and MØs (data not shown). Conversely, in both RA-SFs (Figure 7B) and ECs (data not shown), the results showed enhanced levels of nuclear IRF-3 after only poly I:C and not LPS stimulation. These results further confirmed that signaling events induced by poly I:C are tightly regulated according to the cell type involved, and differ substantially between professional and nonprofessional immune cells.

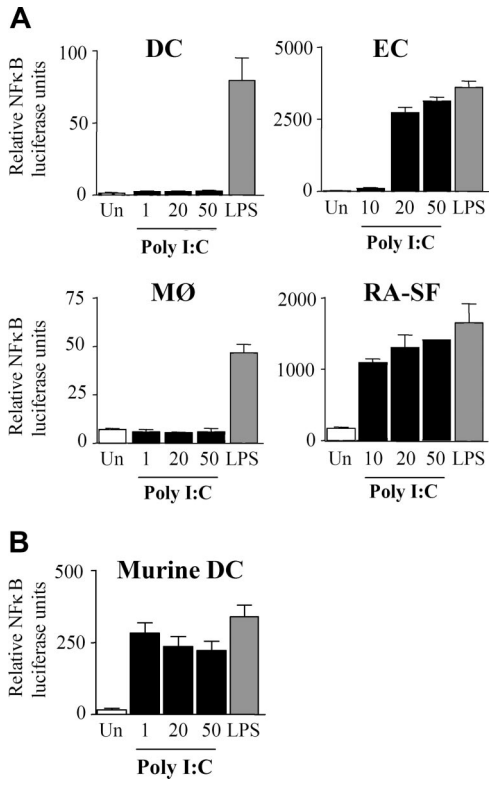


Figure 4. Poly I:C stimulation induces activation of NFκB in endothelial cells and RA synovial fibroblasts but not in dendritic cells and macrophages. (A) Human DCs, MØs, ECs, RA-SFs, and (B) murine DCs were infected for 1 hour with an adenovirus carrying an NFκB luciferase reporter gene. The cells were stimulated for 6 hours with 100 ng/mL LPS or poly I:C (in μg/mL) as indicated and then analyzed for luciferase activity. The data are mean values (± SD) from triplicates and are representative of at least 3 experiments using cells of different donors.

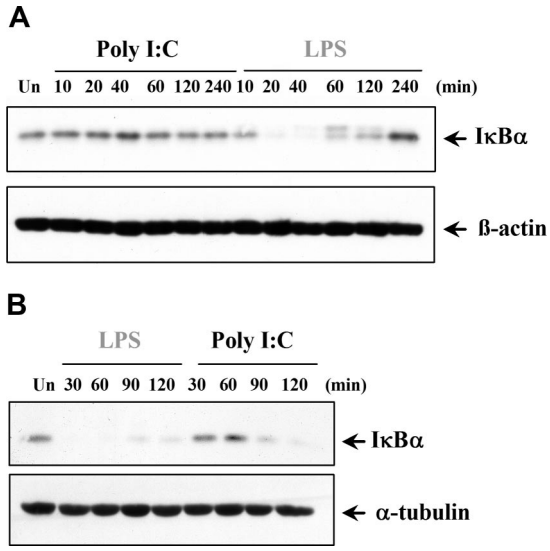


Figure 5. Poly I:C stimulation induces degradation of IκBα in fibroblasts but not in dendritic cells. (A) DCs and (B) RA-SFs were either left untreated (Un) or stimulated with poly I:C (20μg/mL) or LPS (100 ng/mL). The cell lysates were examined for the level of IκBα, β-actin, or α-tubulin by Western blotting. Representatives of 3 independent experiments per cell type are shown.

Discussion

This study investigated and compared the responses induced by TLR3 stimulation of human primary DCs, MØs, ECs, and RA-SFs from disease tissue. These cell types are involved in responses to infection and both acute and chronic inflammation. The results in this study have uncovered a considerable selectivity in the TLR3-induced responses of these cell types, with substantial differences observed between myeloid cells (DCs and MØs) and nonmyeloid cells (ECs and RA-SFs). Surprisingly, DCs and MØs were found to produce the IRG IP-10, but not cytokines such as TNFα, IL-6, or IL-8 after poly I:C stimulation. Studies on mRNA expression of TNFα and IL-6 in human DCs confirmed our protein data showing no poly I:C–induced cytokine production, whereas we could detect IFNβ mRNA, as shown by other groups.[25,26] This contradicts a previously published study that failed to detect IFNβ but showed induction of TNFα and IL-6 mRNA, although no protein studies were performed.[23] Furthermore, we showed that poly I:C stimulation of human DCs induced up-regulation of the maturation markers HLA-DR, CD80, CD86, and CD83, and T-cell stimulatory capacity, all of which have previously been demonstrated by other groups.[24-26] This is the first report showing induction of DC maturation and production of IRGs from human DCs and MØs, but not proinflammatory cytokines, in response to TLR3 stimulation. Furthermore, of great interest, this phenotype was shown to be specific for human myeloid cells since both TNFα and IL-6 could be produced by murine DCs after poly I:C stimulation. Moreover, in contrast to DCs

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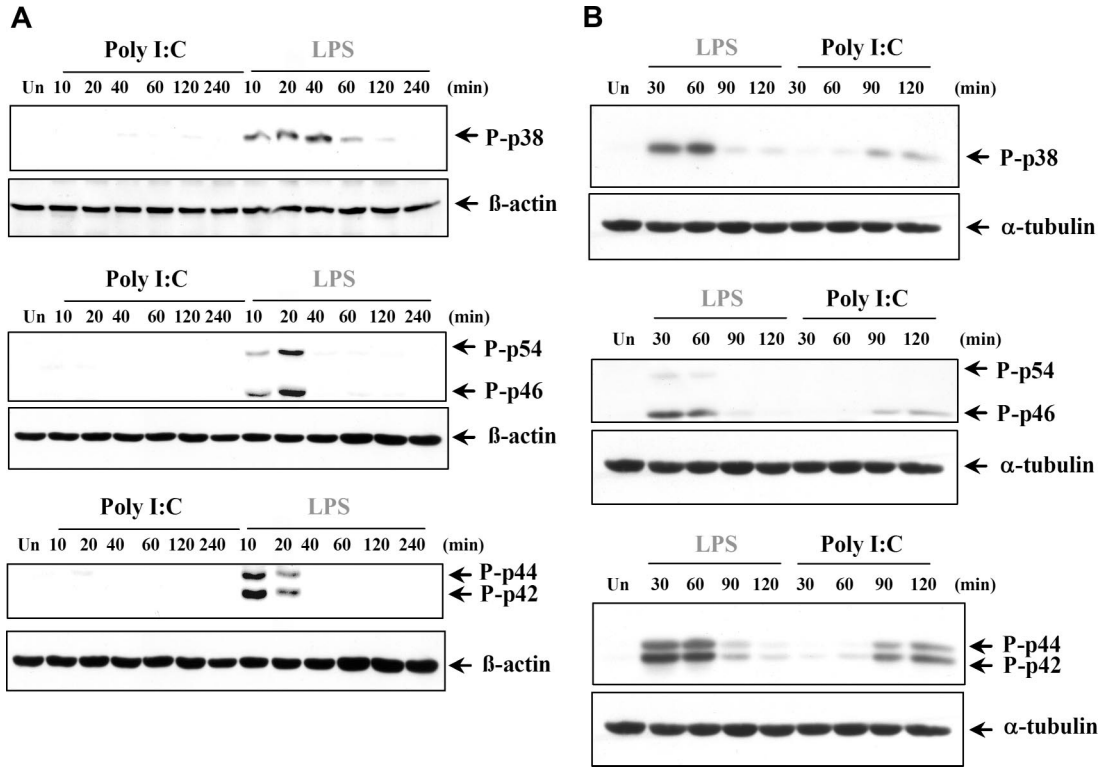


Figure 6. Poly I:C treatment does not induce phosphorylation of p38, JNK, and ERK in human dendritic cells but does so in RA synovial fibroblasts. (A) Human DCs and (B) synovial fibroblasts from RA tissue were either left untreated (Un) or stimulated with LPS (100 ng/mL) or poly I:C (20μg/mL). The cell lysates were examined for phospho-p38, phospho-JNK, phospho-ERK, or β-actin by Western blotting. Representatives of 3 independent experiments are shown.

and MØs, the cells of nonmyeloid origin, ECs and RA-SFs, produced both types of cytokines after poly I:C treatment. This is a major difference in the behavior of TLR3 signaling between human and mouse and could affect how we interpret and extrapolate data between the 2 systems, especially when considering viral disease models, where TLR3 might have a role, or where cell necrosis and release of RNA may be involved.

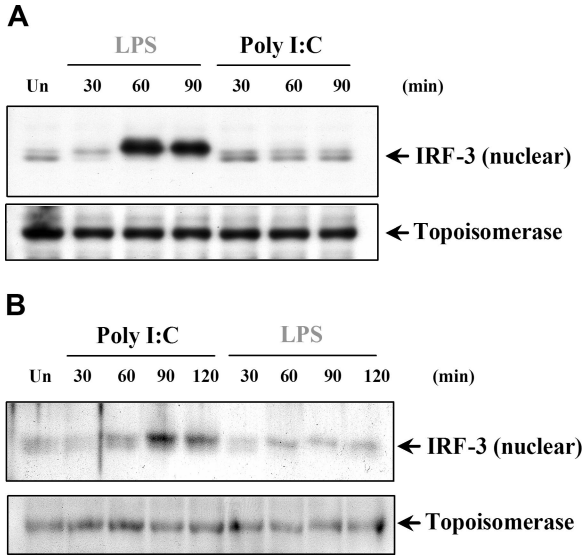


Figure 7. Poly I:C stimulation of RA synovial fibroblasts but not DCs activates nuclear translocation of IRF-3. (A) Human DCs and (B) RA-SFs were either left unstimulated (Un) or stimulated with poly I:C (20 μg/mL) and LPS (100 ng/mL), and the nuclear fractions were examined for nuclear IRF-3 and topoisomerase by Western blotting. Representatives of 3 independent experiments per cell type are shown.

The differences between the myeloid and nonmyeloid cell types were also reflected in the TLR3 signaling pathways. Unexpectedly, poly I:C did not induce activation of NFκB, MAPKs, or IRF3 in DCs and MØs, but was able to do so in ECs and RA-SFs. The inability of poly I:C to activate these pathways in DCs and MØs could explain the failure to produce TNFα, IL-6, and IL-8, since their expressions are regulated by NFκB. Conversely, the activation of NFκB and MAPKs in RA-SFs and ECs would also explain why poly I:C could induce IL-6 and IL-8 in these cells. Our results confirm the previously reported observation that poly I:C stimulation regulates NFκB in human ECs.[27,47]

We have previously shown that there are major differences in the signaling pathways of other TLRs between human cells of myeloid and nonmyeloid lineages.[19,20,48] A possible explanation for our results could be cell type–specific expression of signaling molecules between cell lineages, as was shown with IKKε expression between murine macrophages and fibroblasts.[49] Such differential expression could account for IFNβ and IP-10 production but no NFκB activation or secretion of proinflammatory cytokines in poly I:C–stimulated DCs and MØs. It is possible that these cells lack a signaling molecule similar to *feckless* described by Beutler et al.[50] Cells from mice with a *feckless* mutation fail to activate NFκB or produce TNFα in response to dsRNA/TLR3, although dsRNA is still able to initiate IFNβ production. Another possibility is that additional transcription factors, such as other IRFs, are involved in TLR3 activation in human myeloid cells. As such, we have evidence that poly I:C induces a rapid and transient nuclear accumulation of IRF-1 in DCs (Figure S3). This is an interesting area that we are actively pursuing further.

An interesting finding in this study was that poly I:C stimulation of RA-SFs induced TNFα production, which was specific for poly I:C since LPS treatment failed to induce the same response. The ability of poly I:C to induce TNFα production does not appear to be

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a general property of all fibroblast populations. We were unable to detect TNFα production in HSFs in response to poly I:C even though IL-6 was produced by these cells. This is not the only difference we have observed between RA-SFs and HSFs, as the former[48] unlike the latter are able to respond to LPS. One could therefore hypothesize that RA-SFs have an inflammatory phenotype, possibly due to their environment. It is interesting to note that poly I:C induces TNFα production in cultures of RA joint synovial membranes from which RA-SFs are derived (S.M.S. et al, manuscript in preparation). Furthermore, TNFα production by poly I:C–stimulated RA-SFs is unlikely to be caused by contamination of MØs or T cells, since we could not detect CD14- or CD3-expressing cells in our RA-SF cultures. If MØs were present, we would have observed TNFα expression after LPS and not poly I:C stimulation of these cultures. However, although unlikely, we cannot completely rule out the presence of other contaminating cell types. Historically nonmyeloid cells were not believed to be able to express TNFα, however, supporting observations were recently reported by 2 independent groups in murine embryonic fibroblasts (MEFs).[51,52] They showed that LPS stimulation of MEFs induced low levels of TNFα that was a result of IRF-3 activation. We show in our human cell systems that there is a correlation between TNFα production and IRF-3 activation. As such, LPS stimulation of DCs and MØs induces TNFα production and IRF-3 activation, whereas poly I:C does not. The situation is reversed in RA-SFs and it is poly I:C stimulation and not LPS that induces both TNFα and IRF-3.

We have previously shown that NFκB is required for LPS-induced MHC class II and CD80/86 expression and antigen presentation.[53,54] However, since TLR3-induced up-regulation of these molecules could be observed in the absence of a detectable NFκB activation, their expression appears to be NFκB independent. Furthermore, IRF-3 is thought to be required for TLR3-induced type I IFN and IP-10 production. However, since no IRF-3 activation could be seen in DCs and MØs, it is unclear what regulates the production of IP-10 and type I IFNs in these cell types. This is the subject of ongoing investigations.

One obvious reason for the variations between the cell types is that they have very different functions in the immune system. DCs and MØs are professional antigen-presenting cells involved in T-cell activation, whereas ECs and RA-SFs are nonprofessional immune cells. Thus, given their diverse roles in the immune response, it makes sense that the same ligand would induce individual cell type–specific responses. For example, stimulation of DCs and MØs by TLR3 appeared to selectively induce IP-10, a key chemokine targeting T lymphocytes, hence specifically enhancing recruitment of T cells to the site of inflammation. In contrast, this selectivity in cytokine production was not observed with ECs and RA-SFs. In these cells, TLR3 activation instead induced a broader range of proinflammatory mediators, suggesting participation of these cell types to chronic inflammation. We also show that TLR3 stimulation results in DC maturation, favoring increased T-cell activation. Furthermore, DCs and MØs are capable of inducing cross-presentation,[55,56] and TLR3-stimulated DCs can cross-present antigens from virally infected cells to promote priming of virus-specific T cells.[57-59] It is therefore possible that TLR3-stimulated DCs and MØs are involved in recruitment of T cells and induction of cross-presentation. Interestingly, TLR3 stimulation also leads to a marked increase in cross-priming toward cell-associated antigens.[60] In this way, peripheral tolerance could be overcome by dsRNA and thus trigger an autoimmune organ-specific disease.[60]

The results presented in this study differ from the literature using other cell systems, and they indicate that the signaling pathways induced by poly I:C in human primary cell types and cells from disease tissue are complex. While these observations have raised the question of why differences exist between cell types stimulated with the same ligand, they also highlight the importance of studying signaling pathways in relevant cell types and the danger in directly transferring the results observed in cell lines or murine systems to human systems. By characterizing the pattern of activation induced by each cell type in response to the TLR3 ligand, this study increases the understanding of the possible contributions of each cell to the innate host responses to viral infections or tissue damage. The differences in TLR3 stimulation between the cell types are subject to further investigation, in order to fully understand the specific role of TLR3 in the human immune response.

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Authorship

Contribution: A.M.L. and S.K.D. performed research; C.M., L.M.W., and S.M.S. contributed new reagents/analytic tools; A.M.L, M.F., and B.M.F. designed research, analyzed data, and wrote the paper. B.M.F. and M.F. contributed equally to this work.

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SUPPLEMENT

Could toll-like receptors provide a missing link in chronic inflammation in rheumatoid arthritis? Lessons from a study on human rheumatoid tissue

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In the last decade the development of a number of biological therapies has revolutionised the treatment of rheumatic diseases. The first and most widely used of these approaches, tumour necrosis factor (TNF) blockade (infliximab, entanercept, adalimumab), has now been administered to over a million patients. However, the success of these biological therapies has also highlighted their limitations. None of these treatments has shown a 100% patient response; normally responses are in the 50–70% range. As proteins, these drugs cannot be given orally and they are expensive to produce, a cost ultimately borne by the patient/health provider that can seriously limit the availability of these drugs. Lastly, these treatments, whether involving the systemic neutralisation of a cytokine (eg, TNF or IL6 receptor blockade (tocilizumab)), the ablation of a B cell population (anti-CD20, rituximab), or the potential disruption of important cellular interactions as with CTLA4-Ig (abatacept), can cause major perturbations of the immune system, the long-term effects of which are still unclear. At present, treatments such as TNF blockade can result in an increased infectious risk and the reactivation of tuberculosis can be a major issue in certain populations. As with all therapies, there is an increasing large refractory population over time. Therefore, despite the undoubted success of these therapies, there is room for improvement. Although it might be too much to expect any new treatment to affect a ''cure'' (all the current biological therapies require repeated administrations), there are definite gains to be made in terms of cost, oral bioavailability and a more selective interference with the immune–inflammatory response.

An important consequence of the success of biological therapies has been to validate the role of various cytokines and cells in the pathophysiology of rheumatoid arthritis (RA). The clinical results obtained with TNF blockade has confirmed in many ways the preclinical studies that originally indicated that TNF was a key regulator of the inflammatory cascade in RA. This includes the production of other cytokines such as IL6, angiogenesis, adhesion molecules and consequent cell migration, and the production of matrix metalloproteinases (MMP) and other factors involved in tissue destruction.[1 2]

A consequence of the success of blocking TNF has been to focus attention on finding suitable ''drugable'' targets, either proximal or distal of this cytokine. This quest of ''drugability'' has focused research on the signalling pathways associated with cytokine production or action. So far two enzymes have clearly led the field as potential targets, p38MAPK and IKK2. Both targets have the perceived benefit of being involved in the production and actions of TNF. IKK2 is a key enzyme in the activation of the NF-κB transcription factor that is required for the production of many other inflammatory factors.[3 4] p38MAPK is mainly implicated in the production of TNF through the post-transcription regulation including the stabilisation of mRNA; it is also involved in the regulation of TNF in RA.[5] However, despite both enzymes being identified some time ago, IKK2 in 1997[6] and p38MAPK in 1994,[7] no compound has yet succeeded in the clinic. To our knowledge, the most advanced p38MAPK inhibitors have reached phase II where progress has stopped, usually due to toxicity. Whether this toxicity is target-related or not is unclear, however, deletion of the α isoform of p38MAPK is embryonically lethal in mice.[8] A clear confirmation of the wealth of published data from in vitro studies indicate that this enzyme is very widely distributed and has a role in multiple pathways of physiological importance.[9] Similarly, the deletion of IKK2 from mice also leads to embryonic lethality,[10] this may not be so surprising an observation given that cell survival is among the multiple roles of NF-κB activation. There is the suspicion therefore that systemic inhibition of either of these enzymes with an acceptable toxicity profile may be a difficult goal to achieve.

THE QUEST FOR THE MECHANISMS INDUCING TNF PRODUCTION IN RA, THE BASIS OF DISEASE CHRONICITY

Another major question that has arisen from the biological therapies is that in general they do not achieve a permanent remission of the disease. Anti-TNF approaches require repeated administration as much as twice weekly (entanercept) and once every 8 weeks for the longer-lived antibodies such as infliximab. The factors that drive TNF production would appear to be still active and unaffected by these treatments. Therefore the identification of the pathways driving the production of TNF would not only move our understanding closer to the source of the chronicity of RA, but also potentially provide a better therapeutic target. Inhibiting the inducer(s) of TNF production would provide a more selective suppression of TNF than the systemic neutralisation of the cytokine. This should

Abbreviations: MMP, matrix metalloproteinases; PAMPs, pathogen-associated molecular patterns; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TIR, toll/interleukin 1 receptor; TLR, toll-like receptor; TNF, tumour necrosis factor

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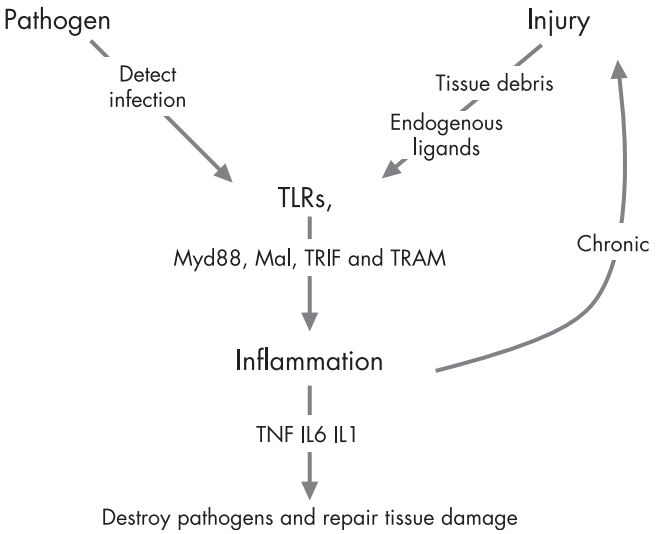


Figure 1 Toll-like receptors: a link between acute and chronic inflammation. TLRs can respond to both endogenous and exogenous ligands leading to the production of inflammatory factors. Damage to cells caused by inflammation may potentially feed back on to the TLRs through generation of endogenous ligands, thus initiating a cyclical pattern that may underlie chronic inflammation.

have benefits in terms of safety. Of course, in RA where there are likely to be multiple complex interactions between various cell types and soluble factors, there may be no single stimulus driving cytokine production in all patients. Previously, studies from this laboratory suggest that cytokine-activated T cells that are generated in the milieu of the cytokine-rich environment of the inflamed joint are capable of inducing TNF production from macrophages.[11] However, other possible candidates are factors that are normally part of the acute inflammatory response against infection. This is even more appealing when one considers that several forms of self-limiting arthritis are induced by infections such as Lyme disease and chlamydia.[12 13]

In the last decade there has been a major leap in our understanding of how the host immune system responds to the challenge of infection with the identification of the toll-like receptor (TLR) family. The toll receptors were first discovered in Drosophila but are now known to be widely expressed in nature and are even present in plants.[14 15] Although the original toll receptor was originally identified in flies as a developmental protein, in adults it has a major role in defence against infection, a function replicated by TLRs in many organisms including mammals. To date 14 mammalian TLRs have been identified, 10 of which are found in man. These receptors have a structural homology of leucine-rich repeats in their extracellular domains, however, they are designed to recognise different ligands, termed PAMPs (pathogen-associated molecular patterns) (fig 1), that is, molecules that would only be present in the host as the result of the presence of a pathogenic infection. The first identified of these was LPS which is recognised by TLR4.[16] Subsequently, TLR2 and the associated TLRs 1 and 6 were shown to recognise proteoglycans, whereas TLR2 in conjunction with the non-TLR molecule Dectin 1 binds the yeast product zymosan.[17] Flagellin is the ligand for TLR5 and TLR9 recognises the non-methylated CpG rich DNA of bacteria. The other TLRs, 3, 7 and 8, appear to be more concerned with recognising viral infections and, unlike the other TLRs except TLR9, are predominately located in the endosomal compartment rather than on the cell surface.[15] To this end, TLR3 and TLR7/8 recognise dsRNA and ssRNA, respectively. The ligand for TLR10 has not been identified. Like LPS, the other TLR ligands are also potent stimulators of inflammation. Normal infection-induced inflammation is self-limiting and resolves, but there are instances as in septic shock, acute respiratory distress syndrome and cerebral malaria where a failure to regulate the inflammatory response leads it to become part of the pathology.

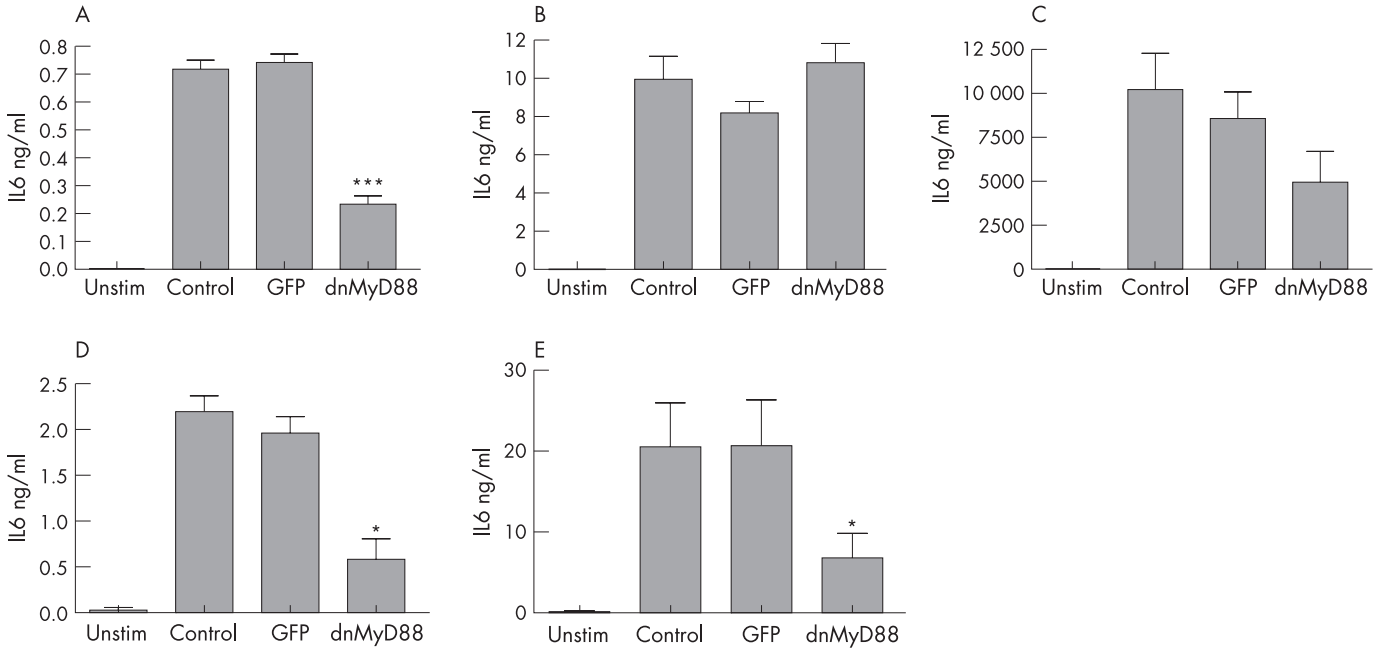


Figure 2 Overexpression of dnMyD88 inhibits TLR2, 5 and 7/8 signalling in human macrophages. M-CSF-derived human macrophages were infected with a dominant negative (dn) MyD88 adenovirus (MOI 100:1) for 1 hour in serum-free media and then cultured for 24 hours in normal media. Cells were then stimulated with media alone or media containing (A) 3 ng/ml Malp-2, (B) 1 ng/ml LPS, (C) 10 ng/ml Pam3Cys, (D) 10 μg/ml Flagellin or (E) 1 μg/ml R-848 for 6 hours. The supernatants were analysed for IL6 by ELISA. Mean cytokine production (±SEM) of triplicate cultures from three unrelated donors is shown. For the statistical analysis, a one-tailed Student t test was used to compare uninfected control cells with recombinant adenovirus-infected cells (*$p<0.05$, ***$p<0.001$).

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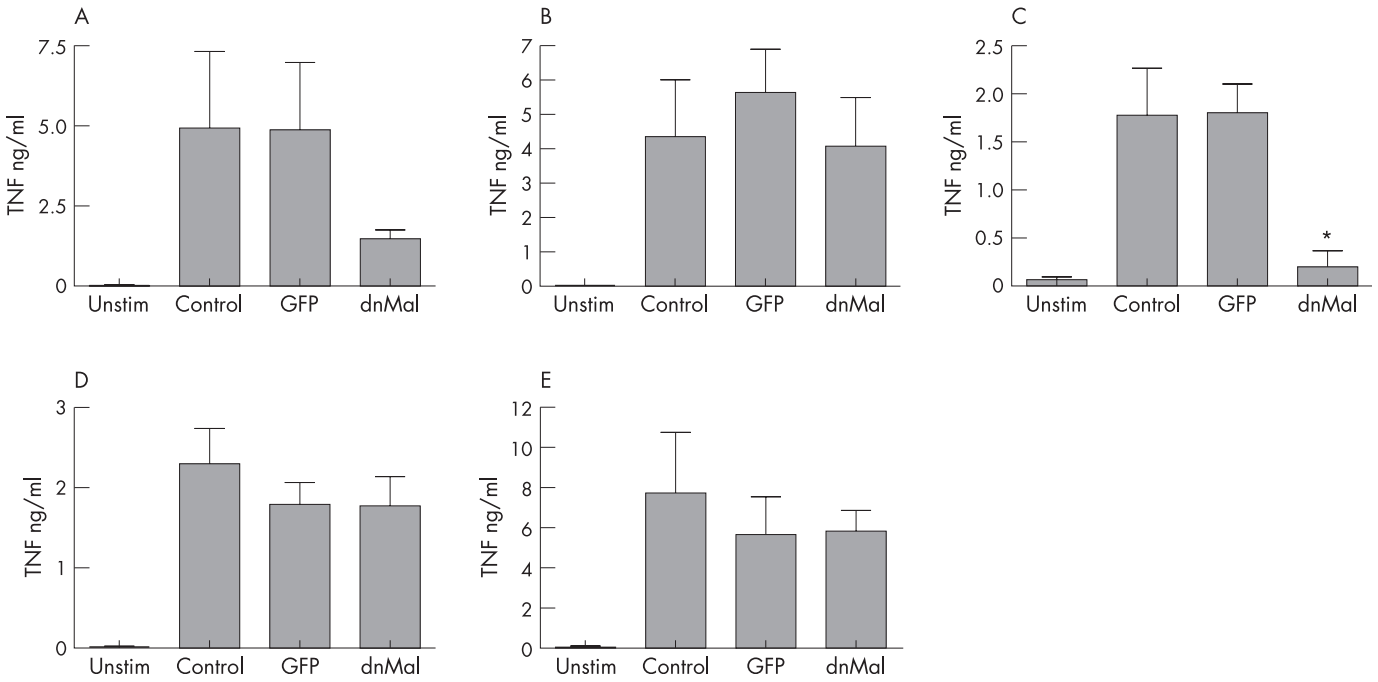


Figure 3 Overexpression of dnMal inhibits TLR2 signalling in human macrophages. M-CSF-derived human macrophages were infected with dominant negative (dn) Mal adenovirus (MOI 100:1) for 1 hour in serum-free media and then cultured for 24 hours in normal media. Cells were then stimulated with media alone or media containing (A) 3 ng/ml Malp-2, (B) 1 ng/ml LPS, (C) 10 ng/ml Pam3Cys, (D) 10 μg/ml Flagellin or (E) 1 μg/ml R-848 for 6 hours. The supernatants were analysed by ELISA for TNF. Mean cytokine production (±SEM) of triplicate cultures from three unrelated donors is shown. For the statistical analysis, a one-tailed Student t test was used to compare uninfected control cells with recombinant adenovirus-infected cells (*$p<0.05$).

COULD TLRS AND THEIR ENDOGENOUS LIGANDS HAVE A ROLE IN RA?

The discovery that the TLRs also recognise endogenous ligands has widened our view of what role these receptors may play in the immune system.[18] Most of the ligands identified to date would be generated as products of tissue injury as cell death (fig 1). The potential benefits of such a complimentary activation of the TLR systems are obvious as it would alert the immune system to breaches of physical barriers such as the skin where infectious organisms might enter. However, there is a major downside in that there is the potential for self-activation of the TLRs. This is possibly the case in sepsis where a major mediator of the exaggerated inflammatory response is the nuclear protein HMGB1.[19] This protein induces macrophage activation and TLR2/TLR4 has been implicated in HGMB1 activity.[20] Also HMGB1 has been recently shown to bind to extracellular DNA and therefore act as co-ligand for TLR9.[21] This potential of endogenous-produced ligands to activate the TLRs presents the scenario that inflammation could be driven independently of any external infection where tissue destruction is occurring for some other reason as in cases of chronic inflammation itself (fig 1). Such a possibility has been proposed in systemic lupus erythematosus (SLE) where there is the formation of HMGB1/DNA complexes that could drive inflammation via TLR9.[21] In addition to TLR9, TLR7 was also shown to be involved in the pathology of SLE through the recognition of RNA immune complexes which leads to B cell activation in synergy with the B cell receptor.[22] Given the amount of tissue destruction that occurs in RA the possibility exists that the self-generation of TLR ligands would perpetuate and amplify the inflammatory process.[23 24]

STUDIES ON TLR SIGNALLING IN RA

To address whether there was a role of endogenous TLR ligands in driving inflammation in RA we utilised the human RA synovial tissue culture model that was originally used to identify the key role of TNF.[25] Rather than attempting to study each individual TLR, we chose to investigate the TLR signalling pathways. Studies in knockout mice have shown that all TLRs, with the exception of TLR3, use the intracellular signalling adaptor molecule Myd88. This protein contains a TIR (Toll/interleukin 1 receptor) domain that forms a homotypic interaction with the TIR domain of the receptor. As the name suggests, the same domain is also found in the IL1 receptor family and, as a consequence, both families use very similar signalling pathways.[26] However, there are certain additional receptor complexities concerning TLR signalling over and above that of IL1. Although TLRs 5, 7, 8 and 9, like IL1 just use Myd88, TLR 1/2/6 and 4 also use a related adaptor molecule, Mal/TIRAP. As a further complexity, TLR4 also engages two further adaptors, TRAM and TRIF, the latter also being the only signalling adaptor used by TLR3.[26] To address the role of TLR signalling in RA membranes, we generated the dominant negative version of Myd88 (dnMyd88) that would neutralise all TLRs (except TLR3) and IL1 signalling, and dominant negative Mal (dnMal) that would allow differentiation from IL1 signalling at least in the case of TLR1, 2, 4 and 6. To deliver these dominant negatives to RA tissue we utilised our adenoviral vectors as used previously in other studies.[27 28] However, before we assessed the role of these molecules in RA, we investigated the activity of the dominant negatives at blocking TLR signalling on populations of normal human monocyte-derived macrophages as the role of these molecules has generally been assessed in various knockout mice. As shown in fig 2, dnMyd88 reduced the production of TNF induced by TLR1/2/6 (MALP2 or Pam3-Cys), TLR5 (Flagellin) and TLR7/8 (R848), and IL1 (not shown). In contrast, dnMal only blocked TLR2 signalling and had no effect on TLR5 or TLR7/8 (fig 3). Unfortunately, these cells do not respond to TLR9 ligands and TLR3 does not induce inflammatory cytokines

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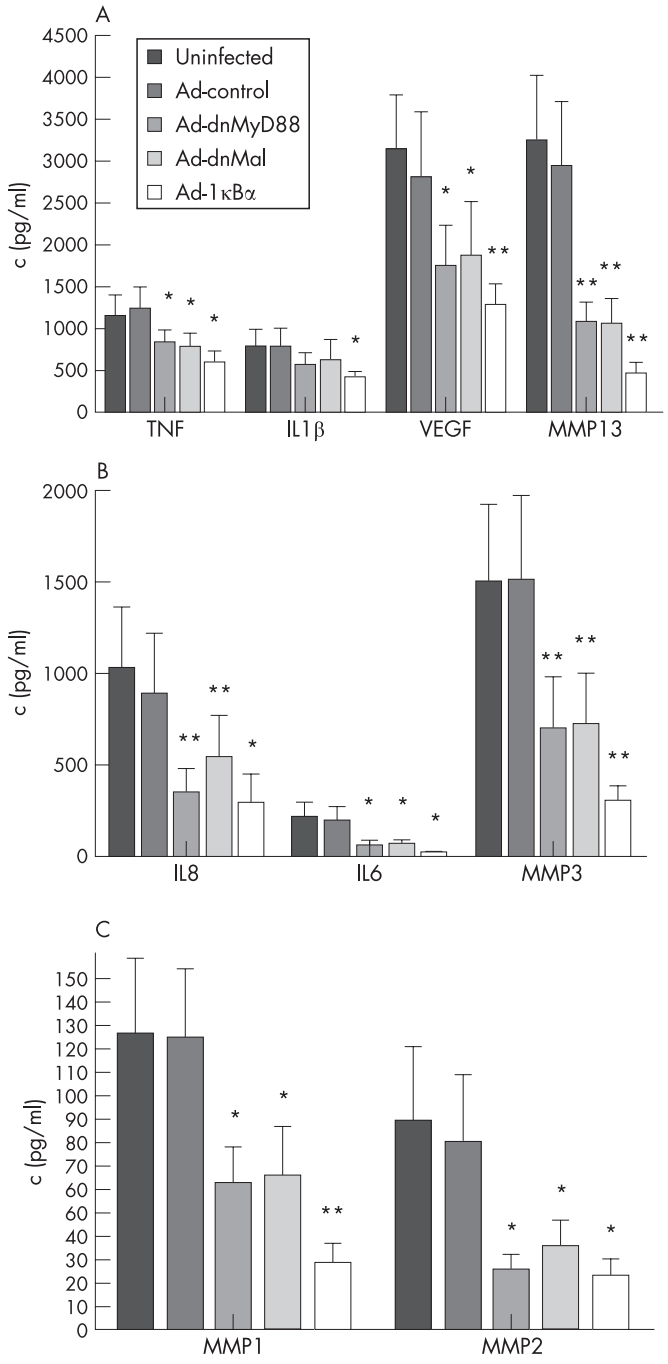


Figure 4 Overexpression of dnMyD88 or dnMal inhibits spontaneous cytokine and MMP production in rheumatoid synovial membranes. Rheumatoid synovial membrane cultures were infected with adenoviruses containing dnMyD88, dnMal and IκBα for 1 hour in serum-free media and the viruses were removed and replaced with normal media. Supernatants were collected after 48 hours and levels of (A) TNFα, IL1, VEGF, MMP13; (B) IL8, IL6, MMP3, and (C) MMP1, MMP2 were measured by ELISA. Concentration of cytokine production from six to eight unrelated patients is shown. For the statistical analysis a one-tailed Student t test was used to compare uninfected control cells with recombinant adenovirus-infected cells (*$p<0.05$, **$p<0.01$).

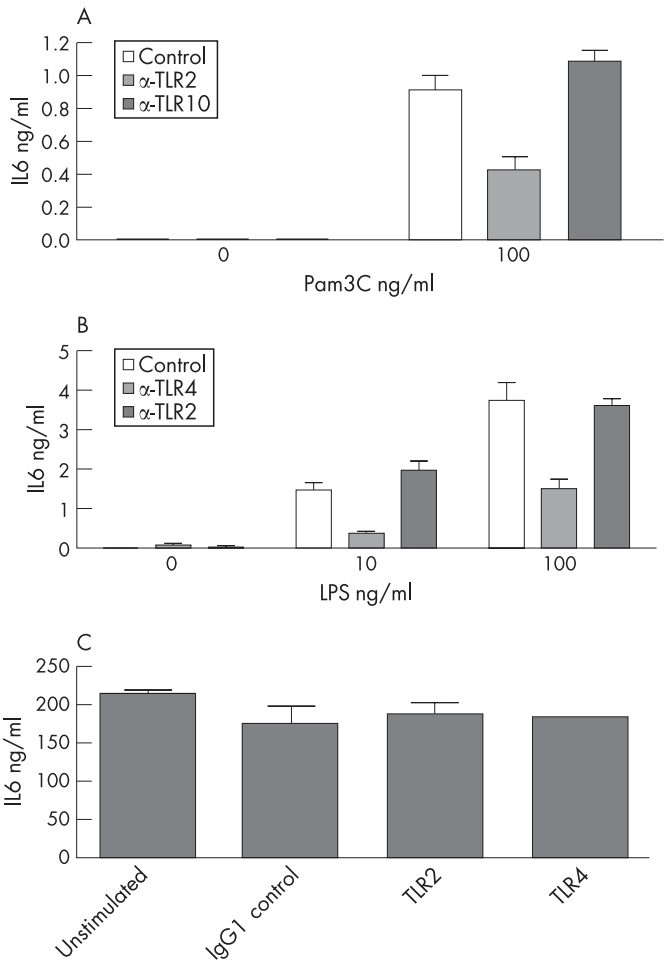


Figure 5 Spontaneous IL6 production from RA synovial membrane cultures is not inhibited by TLR2 or TLR4 blocking antibodies. Macrophages were generated from peripheral blood monocytes in culture with 100 ng/ml M-CSF for 3 days. (A) Macrophages were left untreated or incubated for 1 hr with 10 μg/ml isotype control antibody or 10 μg/ml α-TLR2 and then stimulated with 10 and 100 ng/ml Pam3Cys-Ser-Lys4 for 18 hrs. (B) Macrophages were left untreated or incubated for 1 hr with 10 μg/ml α-TLR2 or 10 μg/ml α-TLR4 and then stimulated with 0.5 or 1 ng/ml LPS for 18hrs. (C) RA synovial membrane cells were cultured with 10 μg/ml isotype control, α-TLR2 or α-TLR4 for 48 hrs, n = 5. Mean cytokine production (± SD) of triplicate cultures is shown and is representative of three independent experiments from unrelated donors in parts A and B, and five in part C.

in human macrophages.[29] However, as we have shown previously, TLR4 (LPS) signalling was unimpaired by either dnMyD88 or dnMal.[28] The reasons for this are unclear and infection with dominant negatives of Mal and TRAM either alone or in combinations with dnMy88 still failed to inhibit LPS activity in macrophages.[27]

Having established the activity of these inhibitory constructs in a simple TLR system, we applied the same reagents to studies on culture of primary human synovial tissue. As has been previously described, these spontaneously produce TNF, other cytokines, MMPs and other inflammatory mediators in the absence of any exogenous stimulus.[30] The factors driving the production of TNF are not yet defined. As shown in fig 4, infecting the cells with a control virus had no effect on the production of TNF or IL6 compared to uninfected controls. In contrast, the use of a viral vector encoding IκBα that would specifically inhibit the NF-κB pathway[4] reduced the level of TNF and other cytokines by 50–70% except for IL6 that was inhibited by over 90%. Dominant negative Myd88 reduced the production of both TNF and IL1 by approximately 30%, although the latter was not significant. IL6 and IL8 production was reduced by 60–70% and VEGF by approximately 50%. Studies with dnMal produced similar levels of inhibition of all these cytokines compared to dnMyD88. We also looked at the effect of inhibiting TLR signalling on the expression of several

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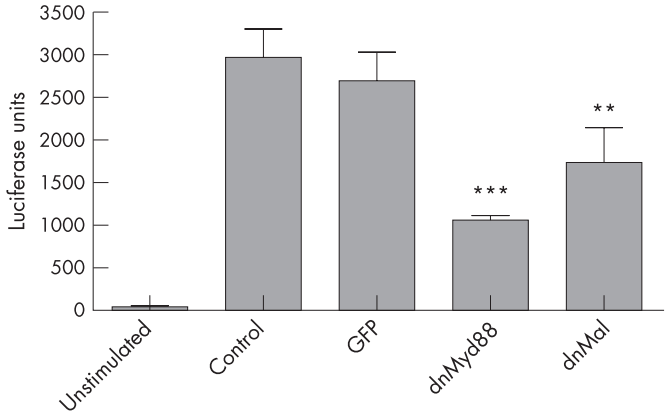


Figure 6 Overexpression of dnMyD88 or dnMal inhibits the activation of NFκB in human macrophages stimulated with the supernatant of rheumatoid membrane cell culture. Human M-CSF macrophages were infected with an adenovirus expressing a NF-κB luciferase reporter (MOI 50:1) in SF media for 1 hour. Four hours later cells were either untreated or additionally infected with a GFP control adenovirus or one expressing dnMyD88 or dnMal (MOI 100:1). After 24 hours cells were stimulated with conditioned supernatant generated from incubation with rheumatoid membrane cell cultures over 24 hours and then filtered to remove any cell debris. Absolute luciferase units ± SD of triplicate cultures are shown. For the statistical analysis a one-tailed Student t test was used to compare uninfected control cells with recombinant adenovirus-infected cells (**$p<0.01$, ***$p<0.001$).

MMPs. As shown in fig 4, dnMyd88 and dnMAL both reduced the production of all the MMPs by 50–70%. These data would indicate that there is a role for both the Myd88 and Mal signalling pathways in the production of TNF and, more so, IL6 in these RA membranes. While a role for Myd88 would suggest IL1 as well as the TLRs, the role of Mal would indicate an important role for TLR signalling, although a contribution from IL1 signalling might also be present. Given these results, we investigated whether TLR2 or TLR4 were involved in driving cytokine production in RA membranes. Antibodies to TLR2 and TLR4 are able to block the response of human macrophages to PAM3Cys or LPS, respectively (fig 5A, B). However, these antibodies were unable to block the spontaneous production of cytokines in the synovial membranes (fig 5C).

Given this result, we next investigated whether these TLR ligands might exist within the culture supernatants of these cultures. This is a relatively tricky experiment as the culture supernatant already contains cytokines. To investigate the presence of TLR ligands we utilised naive primary human macrophages that contained an NF-κB luciferase reporter gene construct. Luciferase activity will be induced in these cells by all the TLRs except 3 and 9 (data not shown). To ascertain if any induction of NF-κB by the synovial joint culture supernatants could be attributable to TLR ligands, the cells were co-infected with viruses for dnMyd88 or dnMal. As shown in fig 6, dnMyd88 and, to a lesser degree, dnMal inhibited the activation of the NF-κB luciferase reporter. As might be expected for cultures that would also contain IL1 and ligands for TLRs that do not use Mal, the effect of dnMal was less than that of dnMyd88. Also neither inhibitor produced a complete inhibition as also would be expected as these supernatants also contain other activators of NF-κB such as TNF that do not use the TLR/IL1 signalling pathway. Therefore, although the antibodies to TLR2 and TLR4 failed to block the spontaneous release of cytokines in RA membranes, there clearly are ligands for TLRs present in these cultures. At present our work is progressing to discover which TLRs are being activated by these potential ligands.

CONCLUSIONS

Our data would indicate that there is a potential role for the TLR system in driving the inflammatory and destructive processes in RA. It may not be necessary for TLRs to be involved in the inception of disease (although this could be via micro-organisms), but they could greatly contribute to chronicity through cycles of inflammation, disease destruction and further inflammation (fig 1). This activity may not be confined to a single TLR as several could be involved. Ongoing studies in this group are attempting to define which TLRs might be involved and the nature of the ligands being recognised. Are TLRs a therapeutic target? Assuming that a substantial proportion of the TNF-inducing activity is not spread among too many TLRs, then inhibition of one or two might produce a significant enough reduction in TNF to have a therapeutic benefit. In terms of safety that would potentially be a more selective suppression of TNF production than is obtained with TNF blockade, although this cannot really be assessed until actual human studies with an acceptable TLR inhibitor could be performed. Moreover, side effects through inhibiting one or several TLRs may be limited as other pattern recognition receptors such as nucleotide-binding oligomerisation domains, C-type lectin receptors and retinoic acid-induced gene I would still be left intact. A further indication for the feasibility of TLR inhibition as a therapy in terms of its side effects comes from humans with a mutation in the irak4 gene. IRAK4 is a common mediator of TLR signalling and, although individuals with mutated IRAK4 show severe infections in early childhood, this phenotype dissipates with age suggesting additional TLR-independent, protective mechanisms taking over.[31–33] TLRs, unlike many receptor systems, might be amenable to inhibition by small molecular weight compounds that would have an advantage over kinase inhibitors in that cell permeability is not a requirement or even desirable. Therefore the likelihood of off-target effects could be greatly reduced.

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Toll–like receptors: a new target in rheumatoid arthritis?

Stefan K Drexler, Sandra M Sacre and Brian M Foxwell†

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases. It is characterized by chronic inflammation of the joint leading to its destruction. Although the initiating cause remains elusive, environmental factors and genetic background are known to contribute to the etiology of RA. The role of Toll-like receptors (TLRs) in innate immunity and their ability to recognize microbial products has been well characterized. TLRs are able to recognize endogenous molecules released upon cell damage and necrosis, and are present in RA synovial fluid. Although it appears unlikely that a pathogen underlies the pathogenesis or progression of RA, the release of endogenous TLR ligands during inflammation may activate TLRs and perpetuate the disease. An increasing body of circumstantial evidence implicates TLR signaling in RA, although, at present, their involvement is not defined comprehensively. Targeting individual TLRs or their signaling transducers may provide a more specific therapy without global suppression of the immune system.

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Pathogenesis of rheumatoid arthritis

Rheumatoid arthritis (RA) affects approximately 0.5–1% of the adult population, with three-times more females than males affected. This condition is marked by a persistent inflammatory polyarticular synovitis, mainly affecting the small joints. During the early stages of RA, synovial lining cells proliferate and endothelial cells in the RA joint upregulate the expression of chemokines and adhesion molecules. This results in neovascularization and in the recruitment of large numbers of mononuclear cells [1]. As the disease progresses, the inflamed synovium (termed pannus) invades cartilage and bone. This process is mediated by the release of enzymes, including matrix metalloproteases (MMPs), aggrecanases and cathepsins, from activated osteoclasts and macrophages, which degrade the extracellular matrix (ECM). In the final stages of RA, bone and tendon damage eventually lead to deformation of the joints and functional disability.

Strong evidence suggests a role for activated inflammatory cells in the initiation and progression of RA. Macrophages are thought to be activated by infiltrating, activated $CD4^+$ T cells through cell-to-cell contact [2]. Once activated, macrophages release chemokines and cytokines that activate and recruit other inflammatory cells to the joint, creating a continuous cycle of inflammation (for a review see [2]). Autoreactive B cells are present in the lymphoid tissues of healthy individuals, but typically remain quiescent. Perturbation of inactive B cells leads to production of self-reactive antibodies that can have serious pathological consequences. B cells expressing an antigen receptor specific for self-immunoglobulin (Ig)G make rheumatoid factor (RF), an autoantibody used in the diagnosis and prognosis of RA.

Current therapies for rheumatoid arthritis

Until relatively recently, management of RA consisted of nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease-modifying antirheumatoid drugs (DMARDs) (including gold, penicillin and methotrexate). NSAIDs do not have disease-modifying properties, but help to reduce joint pain. Low-level glucocorticoids delay the progression of

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articular disease in RA [3], but have the disadvantage of many well-characterized adverse reactions, such as, osteoporosis and hypertension, so are often only used prior to the onset of DMARD treatment. For decades, DMARDs provided the most beneficial treatment of patient symptoms. Treatment often consists of combinations of DMARDs, for example, using methotrexate, sulphasalazine and hydroxychloroquine [4]. However, not all patients respond to DMARDs. The development of humanized antitumor necrosis factor (TNF)-α therapy has heralded the advent of the biologicals as novel therapies for RA. New treatments for RA have become available as our understanding of the role of cytokines in RA has improved. Targeting the key inflammatory cytokines TNF-α and, to a lesser degree, interleukin (IL)-1 has been the most beneficial therapeutic interventions to date. The treatments approved for use in the clinic are the TNF-α inhibitors, infliximab (Remicade®) [5,6], etanercept (Enbrel®) [7] and adalimumab (Humira®) [8], and the IL-1 inhibitor anakinra (Kineret®) [9,10]. Therapies targeting other inflammatory factors in the RA joint are under development, including anti-IL-6 receptor and anticytotoxic T-lymphocyte-associated antigen (CTLA)-4. Over production of IL-6 plays a pathological role in RA and treatment of RA patients with a humanized anti-IL-6 receptor antibody reduced disease activity in 78% of patients after 3 months [11]. T cells express CTLA-4 transiently, which binds to CD80 and CD86 on antigen-presenting cells (APCs) as well as CD28 on T cells. Inhibition of CTLA-4 engagement with CD28 was shown to impair activation of T cells [12]. Therefore, CTLA-4 may be a useful target in RA therapy [13]. A human CTLA-4–IgG_1 fusion protein is currently in Phase III trials in RA patients and has shown positive results in patients with an inadequate response to either methotrexate or anti-TNF-α therapy [14–16]. As with most immune system-modifying therapies, targeting inflammatory cytokines has a nonspecific effect on the whole of the immune system. As a result, immune function is compromised and infection may occur. Reported side effects of anti-TNF-α therapies are a lupus-like syndrome, demyelination syndrome and serious infections, including bacterial sepsis and reactivation of latent *Mycobacterium tuberculosis* [17]. However, these complications occur rarely. The aim of new therapies is to provide a less expensive, orally available treatment (anti-TNF-α is by injection) with a more specific mechanism of action.

Etiology of rheumatoid arthritis

Similar to other autoimmune diseases, environmental factors, such as mechanical stress and cigarette smoking, appear to play a role in RA susceptibility [18]. The initiating cause of RA has been suggested to be bacterial or viral infection [19]; however, no infectious pathogen has been identified conclusively in the inflamed joints of RA patients. With 15% concordance in identical twins, RA shows the lowest concordance of all autoimmune disease [20]. Nevertheless, there is a genetic influence in RA. An association between major histocompatibility complex (MHC) Class II antigens, which present antigenic peptides to $CD4^+$ T cells, and RA has been suggested [21]. The initial immune response of RA might be antigen presentation of a viral/bacterial pathogen or endogenous antigen through loss of peripheral tolerance [22]. Endogenous ligands such as human cartilage glycoprotein 39 [23] or self-DNA (IgG–chromatin) complexes [24], are equally capable of eliciting an immune response as exogenous infectious agents. These endogenous antigens might be stimuli in the early stages and maintenance of the pathogenesis of RA. However, the definitive cause of RA still remains elusive.

Signaling pathways of Toll-like receptors

Leukocyte recognize pathogen-associated molecular patterns (PAMPs) such as double-stranded (ds)RNA, unmethylated DNA, flagellin and lipopolysaccharide (LPS), through a family of receptors known as pattern recognition receptors (PRRs). TLRs are a major family of PRRs. The Toll receptor originally found in *Drosophila melanogaster* was first described as a protein involved in embryonic development and, only later, an additional role in innate immunity was defined [25,26]. Currently, ten human orthologs of Toll have been described. TLRs are expressed by APCs and synovial fibroblast-like cells in the RA synovial joint [27–29]. Some TLRs are found intracellularly in endosomes. These TLRs (TLR3, 7, 8 and 9) are the only members of the TLR family that recognize nucleotides (FIGURE 1) [30,31].

All of the TLRs have a cytoplasmic Toll/IL-1 receptor (IL-1R; TIR) domain shared with the IL-1R family, but show differences in their extracellular structure. While IL-1R possesses Ig domains, TLRs have leucine-rich repeats (LRRs) as their extracellular domains. With the exception of TLR3, TLRs and IL-1R family members signal using the adaptor myeloid differentiation primary response gene 88 (MyD88) [32–34], IL-1R-associated kinase (IRAK)-1, IRAK-4 [32,33,35] and TNF receptor-associated factor (TRAF)-6 [36]. The outcome of sequential activation of these signal components is the activation of inhibitors of nuclear factor (NF)-κB kinase (IKK) complex and the transcription factor NF-κB. Many NF-κB-dependent genes are important in the inflammation associated with RA, and increased NF-κB activation has been observed in the rheumatoid synovium [37].

MyD88-knockout mice still show activation of NF-κB after LPS stimulation [38]. This observation led to the discovery of the TLR adaptors MyD88 adaptor-like (Mal) [39], also termed TIR domain-containing adaptor protein (TIRAP) [40], TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF) [41] (also referred to as TICAM-1 [42]) and TRIF-related adaptor molecule (TRAM) [43], also known as TICAM-2 [44].

Stimulation of cells with dsRNA or LPS (TLR3 and 4 ligands, respectively) results in the recruitment of TRIF, and in case of TLR4, also TRAM, leading to the formation of a complex including IKK-ε, TANK-binding-kinase (TBK)-1 and TRAF-3 [45]. The dissociation of this complex leads to the activation of IFN regulatory factor (IRF)-3 and -7, consequently resulting in the expression of IFN-inducible genes, including IFN-β, IFN-inducible protein (IP)-10 and RANTES (FIGURE 1) [46–48]. Moreover, TRIF

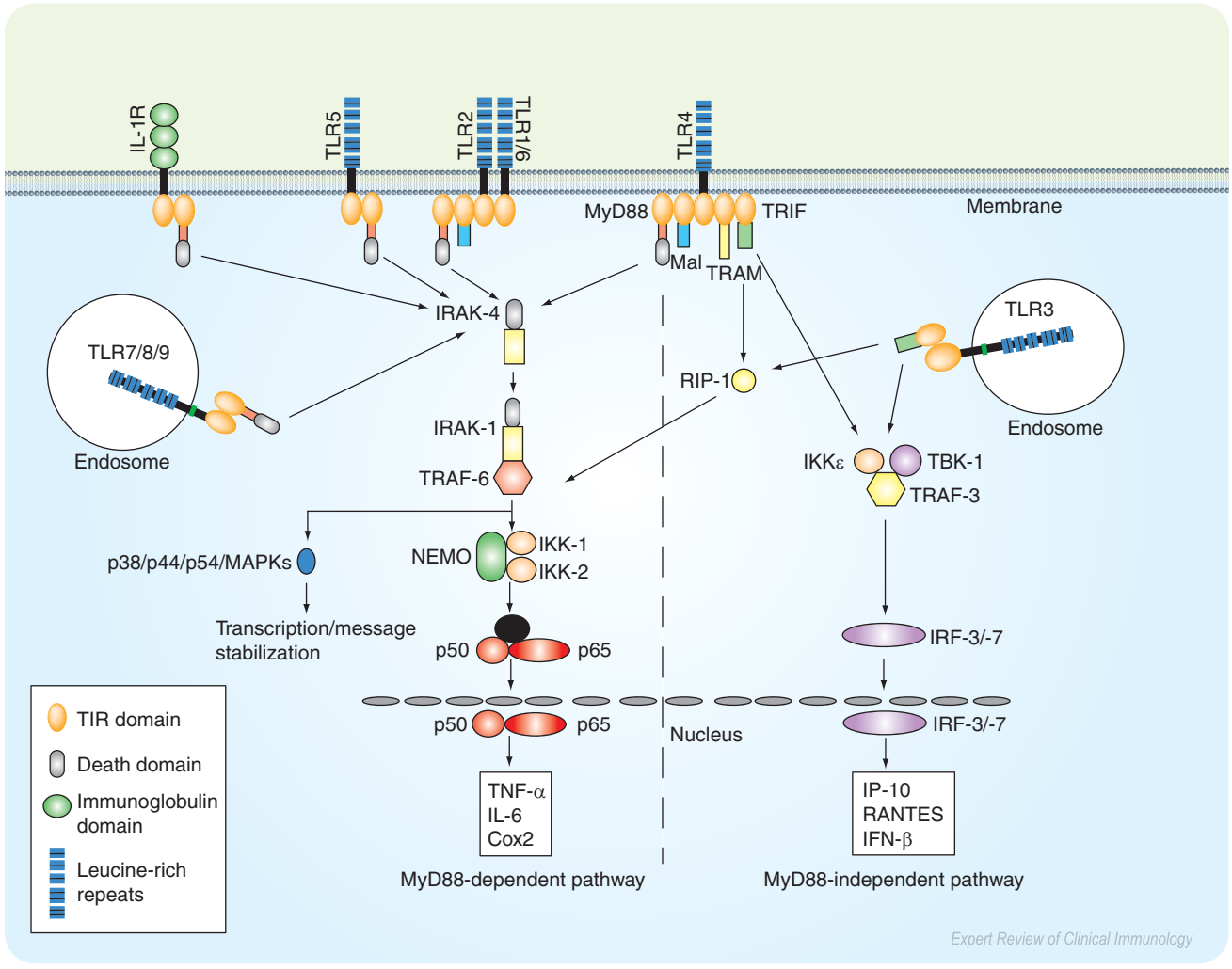


Figure 1. TLR signaling pathways. The IL-1R pathway is solely dependent on the adaptor molecule MyD88 and induces NF-κB through IRAK-4, IRAK-1 and TRAF-6. In general, TLR signaling begins following binding of the ligand and the recruitment of the adaptor molecules MyD88, Mal, TRIF and TRAM to the TIR domain of the TLR. **MyD88–dependent pathway**: IRAK-1 and -4 are recruited to the death domain (DD) of MyD88, where they become phosphorylated and recruit TRAF-6. IRAK-1 and TRAF-6 dissociate from the receptor and activate the IKK complex, resulting in the translocation of NF-κB to the nucleus and the upregulation of proinflammatory genes. Beside the induction of NF-κB, the MyD88-dependent pathway also activates the MAPKs p38 and JNK. This pathway is used by TLR1, 2, 4, 5, 6, 7 and 9. Mal, a second TIR domain-containing adaptor protein, is involved in the MyD88-dependent signaling pathway through TLR2 and TLR4, and acts as a bridging molecule for MyD88 to these receptors. **MyD88–independent pathway**: By contrast, TLR3- and TLR4-mediated activation of IRF-3 and the initiation of IFN-β are observed in a MyD88-independent manner. The adaptor molecule TRIF is essential in this pathway. TRIF recruits and activates RIP-1, which is responsible for a late induction of NF-κB as well as TBK-1 and IKK-ε, which form a complex with TRAF-3 activating IRF-3 and IRF-7 and the induction of type I IFNs. The fourth adaptor protein, TRAM, which is only involved in TLR4 MyD88-independent signaling, serves as a bridge between TLR4 and TRIF.
IKK: Inhibitors of NF-κB kinase; IFN: Interferon; IL: Interleukin; IRAK: IL-1 receptor associated kinase; IRF: IFN regulatory factor; JNK: c-Jun-N-terminal kinase; Mal: MyD88 adaptor-like protein; MyD88: Myeloid differentiation factor 88; NF: Nuclear factor; RIP: Receptor-interacting protein; TBK: TANK-binding kinase; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TRAM: TRIF-related adaptor molecule; TRAF: TNF receptor-associated factor; TRIF: TIR domain-containing adaptor inducing IFN-β.

recruitment also leads to a MyD88-independent activation of NF-κB. However, the exact mechanism of NF-κB activation by TRIF is still unclear. Studies show that binding of receptor-interacting protein (RIP)-1 to the RIP interacting homology motif (RIHM) domain of TRIF leads to the induction of NF-κB, while Covert and colleagues suggest that an autocrine effect of TNF-α, initially induced through IRF-3, is responsible for NF-κB activation [49,50]. Besides the activation of NF-κB and IRFs, stimulation of TLRs also leads to the activation of the mitogen-activated protein kinase (MAPK) cascades, including p38 MAPK, extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK).

Each TLR recognizes a specific group of ligands, resulting in the induction of the signaling pathways described above. The most well-characterized ligands are of exogenous origin (reviewed in [46]). However, there is increasing evidence that TLRs also recognize endogenous ligands released during inflammation, injury or necrotic cell death.

Toll-like receptors are also stimulated by endogenous ligands

The main role of TLRs is considered to be the recognition and response to microbial pathogens, but they have also been reported to recognize endogenous ligands. The evolutionary conserved Toll pathway in *D. melanogaster* is induced by binding of the endogenous protein Spaetzle. This suggests a role for Toll in recognizing endogenous as well as exogenous ligands. The recognition of endogenous ligands by mammalian TLRs has recently gained momentum with increasing numbers of publications about endogenous TLR ligands (reviewed in [51–54]).

Endogenous ligands are thought to be released during necrotic cell death induced by tissue damage, stress factors or infection, and result in the release of cell components that initiate an inflammatory response [55]. In contrast to apoptosis, necrotic cell death is typically associated with inflammation. This is probably owing to the release of the cell contents into the surrounding environment, leading to the activation of adjacent cells [56]. Apoptotic cells retain their membrane integrity and are cleared rapidly by macrophages before lysis [57]. This has been demonstrated with dendritic cells (DCs) that are activated by exposure to necrotic cells, but not apoptotic cells [58,59]. However, apoptotic particles are often present in inflammatory conditions and contain single-stranded (ss)RNA that is capable of stimulating either TLR7 or TLR8, and by forming hairpin loops, also TLR3 [60]. The contents released from necrotic cells may activate TLRs, generating further inflammation and thus more necrosis. This cycle of inflammation may explain the chronic inflammatory state found in autoimmune diseases, such as RA (FIGURE 2). TLRs known to respond to endogenous ligands are TLR2, 3, 4, 7, 8 and 9. TLR2 and TLR4 respond to structural proteins released from damaged cells, whereas TLR3, 7, 8 and 9 respond to nucleic acids.

Endogenous ligands for TLR2 & TLR4

One of the first reported groups of endogenous TLR ligands were the heat-shock proteins (HSPs), HSP60 [61], HSP70 [62] and glycoprotein (gp)96 [63]. Recent studies also suggest HSP22 as a TLR4 ligand that is able to activate monocyte-derived DCs [64]. HSP60, HSP70 and gp96 were reported to signal through TLR2 and TLR4 [61–63]. Murine DCs and macrophages signal in a MyD88- and TRAF6-dependent manner when stimulated with HSPs [61–63]. Signaling of HSP60, HSP70 and gp96 through TLR2 was dependent on endocytosis, whereas signaling via TLR4 was dependent on the co-receptor MD-2, similar to LPS signaling [61–63].

However, recent studies have raised doubts about the ability of HSPs to act as endogenous TLR ligands. Highly purified murine liver HSP70 did not induce TNF-α release from murine macrophages, even at high concentrations [65], leading to suggestions that the TNF-α-inducing activity previously attributed to HSP70 [62] was due to endotoxin contamination [66]. Similarly, other studies using LPS-free preparations of HSP70, HSP60 and gp96 have all suggested that the TLR activity reported previously may have been due to LPS contamination [66–68]. Contamination in commercially sourced preparations may occur owing to the isolation process, since all of these products are recombinant proteins often from *Escherichia coli* and therefore may be contaminated with lipoproteins as well as LPS. Other studies using expression of gp96 or addition of gp96-secreting fibroblasts into mice have avoided potential contamination. Activation of APCs was observed in these studies, but it was unclear if this was due to a direct effect gp96 [69,70]. High-mobility group box (HMGB)-1 is a molecule released during cell necrosis [71]. HMGB-1 has been suggested to signal through TLR4 as it shares a similar signaling pathway to LPS when stimulating human endothelial cells and neutrophils [72,73]. However, neutrophils lacking TLR4 still respond to HMGB-1 [74]. A recent study demonstrated a role for TLR2 and TLR4 in recognizing HMGB-1 and the subsequent activation of NF-κB [75].

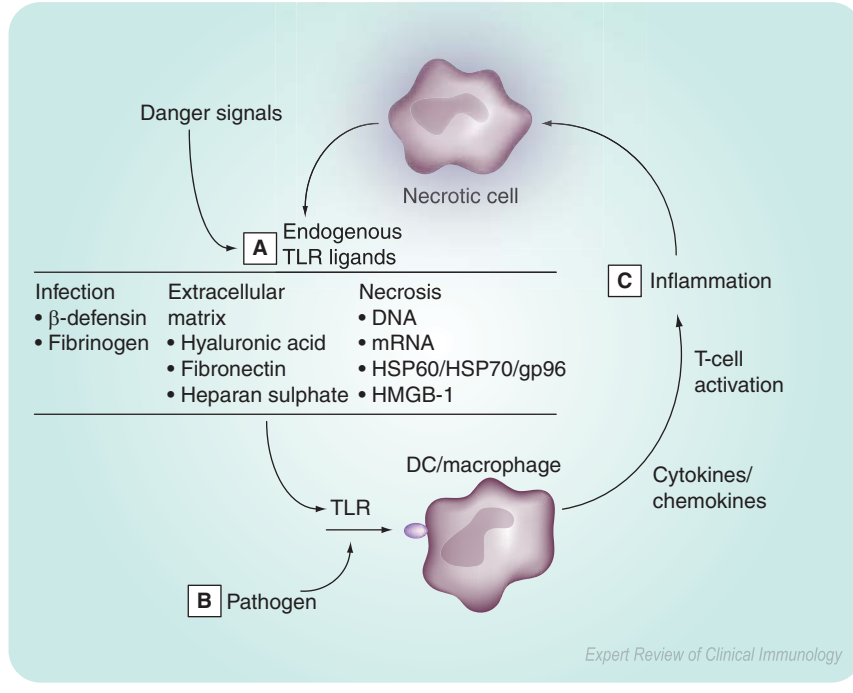


Figure 2. Hypothesis for TLR participation in rheumatoid arthritis. (A) The release of endogenous TLR ligands (e.g., HMGB-1, RNA) due to cell necrosis, breakdown of extracellular matrix or infections subsequently leads to the activation of TLRs on the synovial membrane (which mainly consists of synovial fibroblasts, macrophages). As a result, proinflammatory cytokines and chemokines that induce inflammation are produced. **(B)** Exogenous TLR ligands from bacterial or viral infection may activate TLRs resulting in inflammation. **(C)** The inflammation generated by endogenous and/or exogenous ligands may cause cells to undergo necrotic cell death and release endogenous TLR ligands.
DC: Dendritic cell; HMGB: High-mobility group box; HSP: Heat shock protein; TLR: Toll-like receptor.

Another source of endogenous TLR2 and TLR4 ligands is the remodeling and/or destruction of the ECM due to tissue damage. This process results in the release of fragments from ECM components that can induce inflammation. Heparan sulphate is associated with the cell membrane as well as part of the ECM [76] and is only found at low levels in healthy tissue. Increased levels of heparan sulphate are detected in the extracellular fluid around injuries [77], in RA synovium [78] and in urine from patients with infections [79]. Hyaluronic acid (HA), a major component of the ECM, is degraded rapidly at sites of inflammation [80,81]. Both heparan sulphate and HA have been shown to signal through TLR4. DCs from the C3H/HeJ mouse, which expresses a nonfunctional TLR4, do not respond to either stimulus [81,82]. Fragments of hyaluronan released during injury can also stimulate TLR4 [83] and biglycan, a ubiquitously expressed proteoglycan in the ECM, has been reported to signal through TLR2 and TLR4 in macrophages [84]. Fibronectin is released from cells in response to tissue damage [85–88] and is able to stimulate synovial cells to release proinflammatory cytokines and MMPs [87]. Studies using transfected TLR4 human embryonic kidney (HEK)293 cells have shown fibronectin to be a ligand for TLR4 [89].

While HSPs, HA, heparan sulphate and fibronectin are released as a result of cell necrosis or ECM remodeling, other endogenous TLR ligands are a sign of inflammation or infection. These include fibrinogen and β-defensins. Fibrinogen is an early hallmark of inflammation. It migrates from the vasculature into the extravascular space during inflammation owing to increased permeability of endothelial cells [90]. Data from macrophage cell lines have shown that fibrinogen-induced secretion of macrophage inflammatory protein (MIP)-1α, MIP-1β and MIP-2 requires functional TLR4 [91].

Murine β-defensin2, an antimicrobial peptide of the innate immune response, has been shown to stimulate TLR4 on immature DCs, inducing maturation and upregulation of costimulatory molecules [92]. β-defensin can also act as a chemoattractant of immature DCs [93,94].

Endogenous ligands for TLR3, 7 & 8

The exogenous ligand for TLR3 is dsRNA and not ssRNA [95], which is instead recognized by TLR7 and TLR8 [96]. Although cellular RNAs are mainly regarded as ssRNA, they can form hairpin loop formations that act as sources of endogenous dsRNA [97]. Endogenous dsRNA released from necrotic cells stimulates DCs to produce IFN-α, whereas pretreatment of necrotic cells with RNase prior to incubation with DCs prevented this response [95]. ssRNA-associated autoantigens have been reported to activate B cells by combined B-cell antigen receptor–TLR7 engagement in mice [98].

Endogenous ligands for TLR9

Genomic DNA also triggers the maturation of DCs and macrophages. Nonmethylated CpG motifs in bacterial and viral DNA are recognized by TLR9, but endogenous DNA on its own is normally inert [99]. The defining difference between bacterial and endogenous DNA is that bacterial DNA is unmethylated, while endogenous DNA is 70–80% methylated [100]. However, cells from autoimmune humans and mice show decreased CpG methylation [101], yet demethylation of murine DNA does not enable it to stimulate B cells [102]. Thus, the mechanism of activation by bacterial and endogenous DNA appears to be more complex than simply methylation. Given that TLR9 is capable of recognizing self-DNA (IgG–chromatin) complexes in B cells [24], in situations where necrotic cells are abundant, the DNA released may form autoantibody immune complexes that can stimulate TLR9. The discovery of endogenous TLR ligands described above suggests a possible role for TLRs in sensing tissue damage and in the generation of inflammation in autoimmune diseases.

Role of Toll-like receptors in inflammatory diseases

The potential of TLRs to recognize endogenous ligands may have an effect on the consequent development of autoimmune disease and chronic sterile inflammatory disorders. Several studies suggest a role for TLRs in the promotion of systemic lupus erythematosus (SLE), Crohn's disease, multiple sclerosis, Type 1 diabetes, in the repair of lung injuries and in RA (TABLE 1). Although most studies show a role in disease progression or development, some TLRs can also facilitate tissue protection from injury; for example, TLR2 and TLR4 recognize microbes in the gut or hyaluronan degradation products in the lungs [103,104]. The outcome of TLR activation, be it inflammation or tissue protection, may be dependent on the type of stimulus encountered.

Evidence for a role for Toll-like receptors in rheumatoid arthritis

TLRs were first suggested to have a role in RA in response to a pathogen initiating disease [105]. Bacterial TLR ligands have been detected in human RA synovium [105], but they have also been found in synovial tissue of healthy donors without any inflammatory response [106]. B cells, activated by chromatin–IgG complexes binding to the antigen receptor and TLR9, produce RF independently of T cells. However, reports of bacterial DNA in the synovium of RA patients [105,107,108] were called into question by a more specific study detecting the bacterial marker muramic acid that did not identify any bacterial components in RA patients [109]. The search for a pathogenic stimulus initiating RA has proven unsuccessful and the focus now has turned to the involvement of endogenous TLR ligands. Possible sources of endogenous TLR ligands include cell necrosis due to tissue damage or inflammation. Hyaluronan oligosaccharides, fibronectin fragments, HSPs, necrotic cells, antibody–DNA complexes and HMGB-1 have all been identified in the RA joint [110–113].

Several animal models used bacterial cell wall components to induce experimental arthritis. The injection of streptococcal cell wall (SCW), containing a peptidoglycan–polysaccharide fragment, into rats led to a chronic arthritis comparable to human RA [114]. Similar experiments in mice showed that the induction of joint inflammation by SCW was dependent on

Table 1. Toll-like receptors were shown to be involved in inflammatory diseases and injury.

Disease/injury	Toll-like receptor	Potential ligands	Ref.
Systemic lupus erythematosis	TLR7, TLR8, TLR9	RNA, immune complexes, double-stranded DNA	[60,98,172–174]
Atherosclerosis	TLR2, TLR4	Fatty acids	[175–188]
Diabetes (Type 1)	TLR3, TLR7	RNA	[189–191]
Multiple sclerosis	TLR4, TLR9	?	[192–200]
Crohn's disease	TLR2, TLR5, TLR9	Microbial antigens, bacterial DNA	[201–209]
Rheumatoid arthritis	TLR2, TLR3, TLR4, TLR9	HMGB-1, RNA, HSP22, bacterial DNA	[27–29,64,71–75,105–126,210–218]
Tissue damage	TLR2, TLR4	Hyaluronan, microbial antigens	[103,104]

HMGB: High-mobility group box; HSP: Heat shock protein; TLR: Toll-like receptor.

TLR2 and MyD88, since TLR2- or MyD88-knockout mice showed a reduced severity of SCW-induced joint inflammation [115,116]. In addition, the intra-articular administration of the TLR2 and NOD2 ligand peptidoglycan also led to the development of destructive arthritis in mice [117]. As well as TLR2, there is also evidence for a possible role for TLR4 in RA [118]. The administration of LPS together with arthritogenic serum in IL-1R-deficient mice, but not in MyD88-deficient mice, resulted in acute paw swelling. Moreover, serum-transferred arthritis was not sustained in TLR4 mutant mice compared with controls [118]. Thus, animal disease models show a possible role for TLR2 and TLR4 in RA.

Expression of TLR2, 3, 4, 7 and 9 has been observed in human synovial tissue from RA patients [27–29,119,120]. TLR2 expression was identified in synovial fibroblasts, $CD16^+$ monocytes and $CD16^+$ macrophages in the synovium [28,29,121]. Interestingly, expression of TLR2, 3 and 4 is increased in the synovial tissue of RA patients compared with healthy donors or osteoarthritis (OA) samples used as controls [27,119]. Stimulation of TLR2- and TLR9-expressing RA synovial fibroblasts with peptidoglycan, a TLR2 ligand, leads to the expression of MMPs and secretion of IL-6 and IL-8, while there is no activation in response to the TLR9 ligand CpG oligodeoxynucleotides [121]. More recently, RNA released from synovial fluid cells, necrotized by freeze/thawing, was shown to activate synovial fibroblasts via TLR3 [119]. Evidence for a TLR4 ligand in the serum and synovial fluid of RA patients has been observed. Serum and synovial fluid from RA patients stimulated TLR4-expressing Chinese hamster ovary (CHO) cells to upregulate CD25, but had no effect on untransfected CHO cells [120].

The DNA-binding protein HMGB-1 is released passively during cell necrosis as well as actively upon LPS, TNF-α or IL-1 stimulation of macrophages [71,122]. Therefore, it can function as a mediator of inflammatory responses through binding to TLR2 and TLR4, the activation of NF-κB and the induction of proinflammatory cytokines [72,73,75]. A role for HMGB-1 in RA has been suggested as it was detected in the synovial fluid of RA patients and Lewis rats with *M. tuberculosis*-induced adjuvant arthritis [123]. Moreover, synovial fibroblasts show higher expression levels of HMGB-1 in RA patients compared with OA patients, which was suggested to result in the release of proinflammatory cytokines by RA synovial fibroblasts [113].

Naturally occurring TLR polymorphisms may indicate the relevance of TLR function in human RA. The amino acid substitution Asp299Gly in the *TLR4* gene results in the disruption of TLR4 signaling and two studies investigating this polymorphism found no correlation with susceptibility to RA [124,125]. Similarly, polymorphisms Arg677Trp and Arg753Gln in the *TLR2* gene and Asp299Gly and Thr399Ile in the *TLR4* gene also showed no association with RA [126]. However, a study by Radstake and colleagues found a lower susceptibility to RA while the severity seemed unaffected [127]. The presence of TLRs and their ligands in RA may indicate a role for these receptors in the onset and progression of the disease.

Potential ways to target Toll-like receptor signaling pathways in rheumatoid arthritis

The data from murine models and human studies could suggest that TLRs have a potential role in RA. Ultimately, if TLRs prove to be functionally linked to inflammation in RA, inhibitors of TLR-associated signaling pathways will become attractive candidates for future therapies, offering the potential of a more target-specific inhibition of inflammation in RA. Two of the DMARDs used in the clinic may attribute some of their beneficial action to modulation of TLR signaling. Hydroxychloroquine inhibits endosomal acidification, thus inhibiting the TLRs that are localized intracellularly, but would have no effect on those expressed at the cell surface. Hydroxychloroquine has also been reported to decrease mRNA expression of TLR4 and TLR9 [128]. However, it also has many other modes of action that may produce the beneficial effects observed, including inhibition of processing of pro-TNF-α and thus secretion of the mature form of TNF-α, as well as inhibition of IL-6 and IL-1β mRNA stability [129]. Glucocorticoids, another treatment used in RA, have recently been shown to disrupt MyD88-dependent formation of IRF/p65 complexes [130].

New strategies to inhibit some aspects of the TLR signaling pathway are already under investigation. For an overview of TLR signaling, see FIGURE 1. Numerous endogenous negative regulators of TLR signaling, including single-Ig-IL-1R-related (SIGIRR) and IRAK-M, have been described (reviewed in [131,132]), but these proteins would be difficult to administer as a therapy. NF-κB is a transcription factor with an important role in regulating inflammatory cytokines and chemokines. TLR stimulation leads to the nuclear translocation of NF-κB and the induction of inflammatory genes, including TNF-α and IL-6, as well as costimulatory molecules and MHC Class I and II in APCs [133]. NF-κB activation is also involved in bone resorbtion due to osteoclast maturation [134] and in the inhibition of chondrocyte differentiation and the repair of damaged cartilage [135,136]. In addition, increased nuclear localization of NF-κB has been shown in macrophages and fibroblast-like synovial cells from RA patients [137–139]. Our own *in vitro* studies show that overexpression of inhibitor of NF-κBα (IκBα) in synovial tissues result in suppression of proinflammatory cytokines, such as TNF-α [140,141], while anti-inflammatory mediators, including IL-10, were unaffected [142]. Similar results were obtained in murine models of RA where NF-κB inhibition resulted in a downregulation of MMP1 and MMP3 [37,143–145].

A NF-κB inhibitor already approved by the US FDA for the treatment of multiple myeloma is the proteasome inhibitor bortezomib (Velcade®; Millenium). By inhibiting the 26S subunit of the proteasome, it prevents the degradation of IκBα and therefore the translocation of NF-κB to the nucleus. However, because the proteasome is involved in a great variety of regulatory processes, it is currently unclear if the effects observed are due specifically to inhibition of IκBα degradation. Moreover, the treatment of chronic inflammatory disease, such as RA, makes it necessary to administer bortezomib over a long period of time and thus may lead to unacceptable side effects.

Instead of targeting the proteasome, which is involved in a number of cellular functions, the inhibition of IKK2, which phosphorylates IκBα and results in its degradation, is a more specific approach to block NF-κB. Although major efforts were undertaken in the development of IKK2 inhibitors, no compound is in further clinical trials, so far [146]. However, initial experiments showed that the inhibition of IKK2 can reduce the severity of collagen-induced arthritis, indicating a potential use in the treatment of inflammatory diseases (for review see [146]).

MAPKs are a family of kinases involved in cellular stress and TLR signaling pathways. These include p38, p42/44 (extracellular signal-regulated protein kinases [ERK]1/2) and p46/54 (JNK). Signaling via MAPKs leads to activation of the transcription factor activating protein (AP)-1 and the transcription of proinflammatory cytokines. Although all three MAPKs appear to be activated in the synovial tissue of RA patients [147,148], only the inhibition of p38 MAPKs leads to a suppression of TNF-α production in LPS-stimulated macrophages [149–151]. Different methods of TNF-α regulation by p38 have been suggested. In human rheumatoid synovial cells, mRNA stabilization mediated through p38 was suggested to control TNF-α production, while, in human macrophages, p38 was also shown to regulate NF-κB activation and thereby TNF-α expression [152]. *In vivo* studies in murine and rat models of arthritis using the p38 MAPK inhibitors, SB 203580 [153], SB 220025 [154], SB 242235 [155], RWJ 67657 [156] or PRP200765A [157], showed a reduction in the severity of arthritis. The major problem of p38 MAPK inhibitors is their toxicity. However, this seems to be related to unspecific effects of the inhibitors rather than to p38 inhibition.

Upstream of MAPKs and IKK2 are the family of IRAKs, consisting of four distinct members, two of which, IRAK-1 and -4, are involved in signaling from the IL-1R and TLRs. IRAK-1 and -4 are recruited to the receptor by MyD88 where they become phosphorylated and complex with TRAF-6 (FIGURE 1). This complex then dissociates from the receptor, activating the IKK complex, ultimately allowing translocation of NF-κB to the nucleus. Other IRAK family members include the inactive kinases IRAK-2 and IRAK-M. IRAK-M is an endogenous inhibitor of TLR signaling that inhibits the dissociation of IRAK-1 and -4 from the TLR complex [36]. Patients with mutated, inactive IRAK-4 suffer from repeated, severe bacterial infections, are highly susceptible to Gram-positive infections and exhibit hyporesponsiveness to LPS, IL-1 and IL-18 [158–160]. Although there are endogenous inhibitors of TLR signaling, such as IRAK-M, these suppressors would be difficult to administer successfully during treatment. Therefore, development of inhibitors of IRAK1 and IRAK4 could lead to IL-1R- and TLR-specific inhibition of NF-κB. This approach would provide a strategy to inhibit the MyD88-dependent pathway selectively, leaving the MyD88-independent pathway intact (FIGURE 1).

A novel inhibitor of TLR signaling pathways is the phytoalexin resveratrol found in the skin of red grapes and plums. Resveratrol is a polyphenol that protects the plant from fungal infection and a variety of plant diseases [161,162]. In mammalian models, resveratrol demonstrates anti-inflammatory effects such as the inhibition of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) [163], and the suppression of macrophage activation [164]. Initially, these effects were attributed to inhibition of NF-κB activation and the suppression of target gene expression, but more recently the direct molecular targets of resveratrol were shown to be TBK-1 and RIP-1, which are part of the MyD88-independent pathway (FIGURE 1) [165]. Inhibition of NF-κB and IRF-3 activation by resveratrol seems to be selective for the MyD88-independent pathway, while the MyD88-dependent pathway is unaffected [165].

Another plant extract that was shown to inhibit TLR signaling is ginsan, a polysaccharide found in *Panax ginseng*. Mice pretreated with ginsan and consequently challenged with *Staphylococcus aureus* showed a lower incidence of septic shock. This protection against lethal doses of *S. aureus* seems to be due to the downregulation of TLR2 and MyD88 and consequently the inhibition of MAPKs and NF-κB and attenuated cytokine production [166].

An immune modulatory role has been suggested for vitamin D3 [167,168]. Recently, a study found that vitamin D3 downregulated the expression of TLR2 and TLR4 in human monocytes in response to lipoteichoic acid and LPS, respectively, which led to an inhibition of MAPK and NF-κB and consequently to the inhibition of TNF-α [169].

The most specific way to target TLR signaling in RA would be to identify which TLRs are important in inflammatory signaling and to target these individually at the receptor level. One approach to inhibit TLR activation is to use TLR neutralizing antibodies that are currently available for some of the TLRs. However, since different ligands using the same TLR may bind to different sites on the receptor, a disadvantage of this approach is that individual blocking antibodies may not work to inhibit signaling of different ligands sharing the same TLR. Yet another way to inhibit TLR activation has been demonstrated for TLR9 by use of immunoregulatory DNA sequences (IRS). Although the mechanism of these IRS is still unknown, it has been suggested that the cellular uptake of CpG motifs is unaffected and that the IRS do not compete for binding of the receptor [170]. This technique has been used to inhibit collagen-induced arthritis, initiated by bacterial CpG-containing DNA. In this case, mice treated with IRS before the onset of arthritis showed decreased production of inflammatory cytokines and chemokines [171]. These studies suggest that IRS might be a promising therapeutic agent in the treatment of RA.

Most of the current inhibitors of the TLR signaling pathway, including the transcription factor NF-κB and p38 MAPK, also target several other signaling pathways and therefore, their use as therapeutics may cause major side effects, including hyporesponsiveness to endotoxin and severe bacterial infections. In chronic inflammatory disease, such as RA, it is necessary to administer a drug over a long period of time, making unwanted side effects particularly unacceptable. Therefore, it is necessary to develop a more specific inhibition of TLR signaling. An ideal therapy would be to target individual TLRs, although, at present, their involvement in RA still needs to be defined. Identification of the function of TLRs in RA and those that are actually involved is required before specific therapies can be developed. It is likely that, in such a complex multicellular environment, there will be a cocktail of ligands and several receptors influencing the pathogenesis of RA and thus it is likely that more than one TLR is involved. The generation of a specific TLR-targeted therapy could offer benefits of improved specificity with a reduced impact on the immune system. It is also likely that different sets of TLRs will influence different inflammatory diseases.

Expert commentary & five-year view

TLRs have an important role in the innate immune response recognizing microbial products as well as having a protective effect in tissue damage. However, the recent recognition that endogenous ligands for TLRs exist has potentially implicated these receptors in the pathogenesis of a number of autoimmune diseases, including RA, and may have a common role in the initiation of chronic inflammatory conditions. Evidence for a role of TLRs in RA has come from *in vitro* studies and murine models where pathogens are used to induce arthritis. While the initiating cause of RA still remains elusive, it seems unlikely that, during chronic inflammation, the TLRs in the joint are stimulated by a pathogen. A more probable source of TLR ligands is endogenous molecules released from damaged tissue and necrotic cells. Necrotic cells are known to induce inflammation and this may be a source of more endogenous TLR ligands whose release produces a further cycle of inflammation. In recent years, expression and upregulation of TLRs in the synovium of RA patients have been observed and evidence for TLR4 ligands in the synovial fluid of RA patients has been published. However, a functional link between TLRs and RA remains still elusive.

Key issues

- Rheumatoid arthritis (RA) affects approximately 0.5–1% of the adult population, making it one of the most prevalent autoimmune diseases.
- Toll-like receptors (TLRs) are suggested to have a role in the inflammatory and destructive processes of RA through the recognition of endogenous TLR ligands.
- Increasing evidence suggests an involvement of TLRs in other autoimmune disease, such as atherosclerosis, multiple sclerosis and Type 1 diabetes.
- Emerging evidence implicates a role for pattern recognition receptors in the pathogenesis of gout, another inflammatory disease of the joints.
- Animal models of RA suggest an involvement of TLRs in the disease.
- Endogenous TLR ligands may be released owing to cell necrosis and tissue damage, initiating a cycle of inflammation.
- TLR receptors are expressed in the RA synovium and endogenous TLR3 and TLR4 ligands have been identified in RA synovial fluid.
- Inhibition of TLR signaling is an attractive future therapy for RA. It could provide a more specific effect without globally impairing the immune system, making it more suitable for long-term treatment.

TLRs make attractive candidates for a future therapeutic target for autoimmune diseases, including RA. Although inhibitors targeting some components of the TLR signaling pathway are available, their actions are often on molecules shared by other signaling pathways impeding their use in long-term therapy. In such a complex multicellular environment, it is plausible that more than one TLR will be involved in the inflammatory response to endogenous ligands. Inhibition of combinations of TLRs to suppress the chronic inflammation observed in RA without affecting other TLRs or immune signaling pathways would be advantageous in dampening down inflammation in RA, while permitting continued surveillance of pathogens and tissue damage by other receptors to mount an immune response.

Future work will expand our knowledge of TLR expression in RA and will determine the cell types expressing each TLR and how expression levels change with disease progression. Further characterization of the TLRs known to be expressed in the rheumatoid synovium, for example, TLR2, 3, 4 and 7 will be intensified through the use of specific blocking antibodies as well as emerging technologies, such as short interfering RNA. Furthermore, it will be necessary to consider a dual function of TLRs in tissue protection as well as inflammation. Studies in mice models of gut and lung injury support this emerging role for TLRs. Consequently, impairment of TLR signaling may reduce inflammation in the joint, but may also enable accelerated tissue destruction. Therefore, further research will be necessary to establish the role of TLR signaling in the rheumatoid synovium.

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Clinical prospects of NF-κB inhibitors to further targeted therapies in rheumatology

Stefan K Drexler, Jeremy JO Turner, and Brian M Foxwell

Introduction • The NF-κB transcription factor family • Involvement of NF-κB in rheumatological diseases • Non-specific NF-κB inhibitors • Targeted therapies • Potential future targets • Conclusion • References

INTRODUCTION

Many anti-inflammatory drugs currently used in clinical rheumatology are limited by lack of target specificity and a broad range of side effects consequent upon this. Thus, there is much interest in the possibility of developing improved targeted therapies that will have greater clinical efficacy combined with a narrower range of side effects.

The most significant recent advance in the clinical management of auto-inflammatory conditions including rheumatoid arthritis (RA) has been the advent of the biopharmaceuticals that target cytokines. Chief amongst these are the blockers of tumor necrosis factor (TNF)-α, infliximab (Centocor), a chimeric murine-human anti-TNF monoclonal antibody (mAb);[1] etanercept (Amgen), a TNF receptor-Fc fusion protein;[2] and adalimumab (Abbott Laboratories), a fully human anti-TNF mAb.[3] In addition, there has been success with blockade of interleukin (IL)-1 in RA using recombinant IL-1 receptor antagonist (IL-1Ra; anakinra, Amgen),[4,5] and with blockade of IL-6 by the humanized anti-IL-6 receptor mAb, tocilizumab (Hoffman-La-Roche).[6] However, the biopharmaceuticals have problems associated with their use including, cost, route of administration, and side effects.[7] They are also not universally efficacious in all patients. Thus, there is still a great unmet medical need for the development of targeted anti-inflammatory drugs that will overcome some of these problems.

In order to build on the clinical success of the biopharmaceuticals while circumventing some of the associated problems, attention has focused on alternative strategies for inhibiting cytokine signaling. The transcription factor nuclear factor-κB (NF-κB) is activated by TNF-α, IL-6, and IL-1, and these genes are also under regulation by NF-κB.[8] Thus, inhibition of components of the NF-κB signaling pathway has come to be regarded as a promising alternative strategy for development of novel drugs to block the effects of excessive cytokines in auto-inflammatory diseases.

As the understanding of pathological cellular signaling in disease states improves, it is becoming feasible to apply this knowledge to identify novel drug targets and then seek compounds that act on these as a route to develop novel drugs. The efficacy of this approach has already been demonstrated by the development of the tyrosine kinase inhibitor imatinib (Novartis)[9] that targets constitutive tyrosine kinase signaling in chronic myeloid leukemia. In the future it is likely that an increasing number of drugs will be developed in this way.

This chapter discusses evidence that several anti-inflammatory agents currently in routine clinical use, in part, owe their anti-inflammatory

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effects to NF-κB inhibition. Current work to develop more targeted inhibitors will then be reviewed before discussing potential routes for the development of novel NF-κB inhibitors in the future.

THE NF-κB TRANSCRIPTION FACTOR FAMILY

The transcription factor NF-κB is involved in numerous cellular processes including development, inflammation, and immunity.[10] It consists of homo- or hetero-dimers of the subunits, c-Rel (also known as Rel); RelA (also known as p65 and NF-κB3); RelB, NF-κB1 (also known as p50), and NF-κB2 (also known as p52). All five subunits contain the characteristic Rel homology domain (RHD), which enables the subunits to dimerize and to bind to the promoter of target genes.[8]

NF-κB signaling is divided into two distinct signaling pathways, known as the canonical and the alternative (non-canonical) NF-κB pathways. Central to the canonical pathway is the formation of the NEMO (NF-κB essential modulator)/IKK (inhibitor κB kinase)1/IKK2 complex, the degradation of inhibitor-κB (IκB)α, following its phosphorylation, and the translocation of p50/RelA heterodimers to the nucleus (Figure 47.1). In contrast, the alternative pathway is activated by IKK1 phosphorylating NF-κB2 and marking it for processing by the proteasome. The processed p52, dimerized with RelB, subsequently translocates to the nucleus (Figure 47.1).

The most common dimer in the canonical NF-κB pathway, consisting of p50/RelA, is bound to the inhibitor, IκBα, in the cytosol in the quiescent state (Figure 47.1). IκBα is the archetypical NF-κB inhibitor in an evolutionary conserved inhibitor family consisting of IκBβ, IκBe, B cell CLL/lymphoma (Bcl)-3, IκBζ, and IκBNS as well as IκBγ, which is a degradation product of NF-κB1 and of NF-κB2.[8,11,12] With the exception of Bcl-3 and IκBζ, these proteins can inhibit the translocation of NF-κB dimers to the nucleus by interacting with the RHD and thereby inactivate the nuclear localization signal (NLS). IκBβ resembles IκBα in its primary as well as tertiary structure. However, while IκBα degradation leads to a rapid oscillatory NF-κB activation, IκBβ degradation results in prolonged NF-κB activation.[8,13,14] This prolonged activation appears to be required for numerous pathological conditions including asthma and cystic fibrosis.[13,15–21] The oscillatory propensity in the NF-κB signaling system is counteracted by a negative feedback mechanism mediated by IκBε, which is delayed and functions in anti-phase to IκBα.[22] This indicates that cells have the capacity to modulate NF-κB activity depending on cell type and stimuli. Beside the canonical inhibitors IκBα, IκBβ, and IκBε, the two IκB family members Bcl-3 and IκBζ seem to have a more multifaceted role. Bcl-3 specifically interacts with the intrinsically inactive NF-κB1 and NF-κB2 homodimers in the nucleus and is not degraded after cellular stimulation.[23–26] Depending on its phosphorylation status it can either act as an inhibitor, dissociating p50 or p52 homodimers from DNA, or as a co-activator that is recruited to the gene promoter by NF-κB1 or NF-κB2 homodimers.[23–28] The recently described IκBζ is highly homologous to Bcl-3. [29–34] Its transcription is rapidly induced upon stimulation with Toll-like receptor (TLR) ligands and IL-1. Cells from IκBζ-deficient mice show a severe impairment of IL-6 production in response to a variety of TLR ligands and IL-1 but not in response to TNF-α.[35] Similar results were obtained using NF-κB1 knockout mice, which might indicate that IκBζ specifically interacts with the NF-κB1 subunit.[35] On the other hand, IκBNS seems to have the opposite role to IκBζ, as it was shown to selectively inhibit IL-6 production in response to lipopolysaccharide (LPS).[36] Furthermore, mice deficient in IκBNS are highly susceptible to LPS-induced endotoxic shock and intestinal inflammation, most likely due to prolonged activation of NF-κB.[37]

In order to allow the release of NF-κB dimers IκBs need to become phosphorylated by IKKs.[38] The IKK complex, inducing the canonical NF-κB pathway, consists of the regulatory subunit NEMO and the catalytic subunits IKK1 and IKK2.[39] While NEMO and IKK2 appear to be essential for NF-κB activation, cells lacking IKK1 still show normal levels of NF-κB DNA binding activity.[40–45] Subsequently, IKK1 kinase activity was found to be involved in the resolution of inflammation, induced by NF-κB activation, by accelerating the turnover of NF-κB and its

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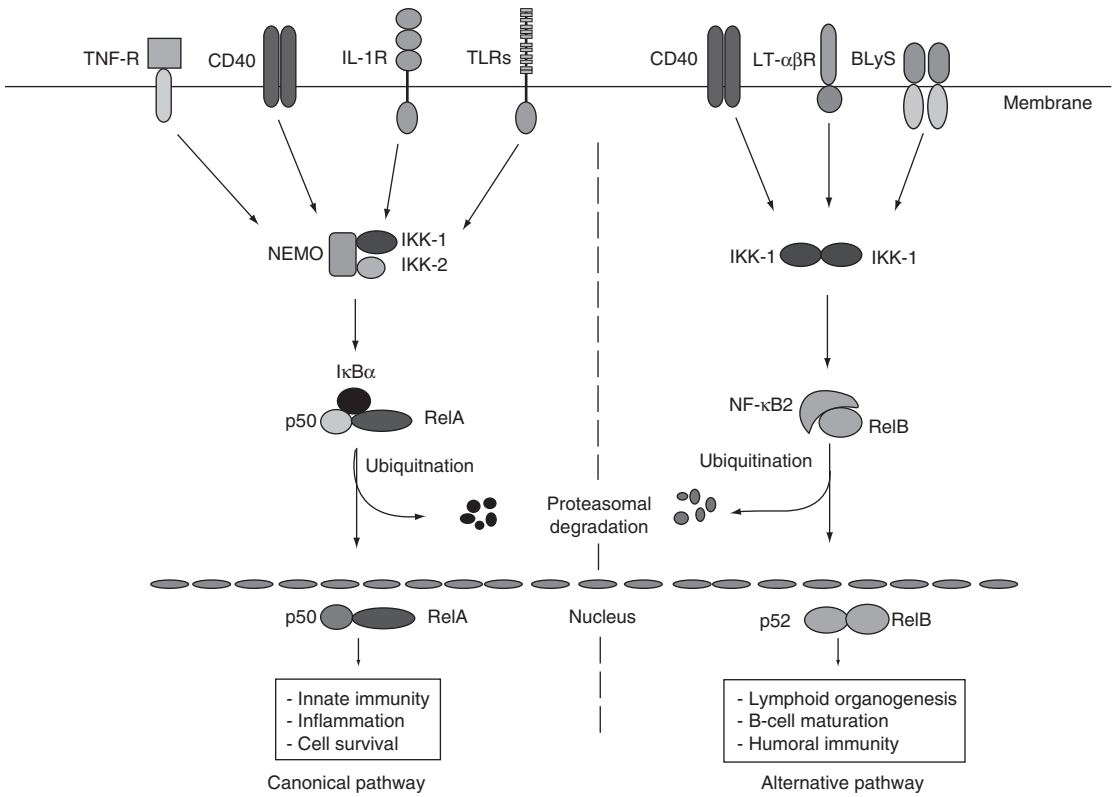


Figure 47.1 Illustration of the canonical and alternative NF-κB signaling pathways. The canonical NF-κB is depicted on the left side of the diagram. It is activated by proinflammatory mediators including TNF-α, IL-1, TLR ligands, and CD40L. Ligand binding ultimately leads to the activation of the IKK complex consisting of the regulatory subunit NEMO and the catalytic subunits IKK1 and IKK2. The endogenous inhibitor of NF-κB, IκBα, is phosphorylated by the IKK complex, which leads to its ubiquitination and as a result to its degradation by the proteasome. The released NF-κB dimer translocates to the nucleus where it induces transcription of genes involved in immunity, inflammation, and cell survival. The alternative NF-κB signaling pathway is depicted on the right. It is induced by lymphotoxin αβ, CD40L, and BLyS. Activation of these receptors leads to the activation of IKK1. IKK1 phosphorylates p100, which becomes degraded to p52 by the proteasome and as a result translocates to the nucleus as a dimer with RelB. The alternative pathway is involved in the organogenesis of lymphoid organs and in humoral immunity. NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IL, interleukin; TLR, Toll-like receptor; IKK, inhibitor of kappa kinase; NEMO, NF-κB essential modulator; IκBα, inhibitor-κBα; BLyS, B-lymphocyte stimulator.

release from the gene promoter.[46] In addition, IKK1 was shown to be an activator of the alternative NF-κB pathway[47–49] (Figure 47.1). IKK1 homo-dimer complexes induce the processing of NF-κB1 to p50 and consequently the nuclear translocation of p50/RelB hetero-dimers. While the NEMO/IKK1/IKK2 complex is required for NF-κB activation in response to most NF-κB stimuli, the role of the two non-canonical IKKs IKKε (also known as IKKi) and TBK-1 (TNF receptor associated factor family member-associated NF-κB activator binding kinase), is unclear. Both were shown to be involved in the induction of interferon (IFN) regulatory factor (IRF)-3 in response to the TLR4 ligand LPS and the TLR3 ligand dsRNA.[50] Their role in the activation of NF-κB, however, is still not well understood.

Phosphorylation of IκBα by the IKKs and its subsequent proteasomal degradation releases NF-κB dimers to translocate to the nucleus and activate target genes.[47] However, NF-κB dimers need to undergo additional post-translational modifications, including site-specific phosphorylation and acetylation to produce a maximal

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transcriptional response.[51,52] The most well studied NF-κB subunit in terms of post-translational modifications is RelA. Several serine phosphorylation sites for RelA have been described including serines 276, 311, 529, and 536.[51] Kinases involved in the phosphorylation of these serine residues include protein kinase A (PKA), mitogen- and stress-activated kinase (MSK)-1, protein kinase C (PKC)ζ, ribosomal subunit kinase (RSK)-1, glycogen-synthase kinase (GSK)-3β, and phosphatidylinositol 3-kinase (PI3K), as well as the canonical IKKs and TBK1.[51] The kinases involved in serine phosphorylation appear to be stimuli-specific, e.g. PKA phosphorylates serine 276 in response to LPS while TNF-α triggers the phosphorylation of serine 276 by MSK1.[51] Ultimately serine phosphorylation leads to an enhancement in the overall transcriptional response. Similarly, acetylation is important for regulating NF-κB activity. Also, three main acetylation sites have been identified within RelA (lysines 218, 221, and 310).[53] The acetyltransferases p300 and cAMP response element binding protein (CBP) seem to be involved in RelA acetylation *in vivo*[53,54] and acetylation of RelA was reported to be regulated by phosphorylation of its serine residues 276 and 536, as phosphorylation increases the assembly of phosphoRelA-p300 complexes.[55] Acetylation of RelA results in different outcomes, as acetylation of lysine 221 enhances DNA binding and impairs assembly with IκBα, the acetylation of lysine 310 is required for full transcriptional activity of RelA.[51]

Inhibiting the amplification of NF-κB activity due to post-translational modification may provide a way to dampen the response generated by proinflammatory stimuli without affecting the basal activity of NF-κB, which is suggested to be beneficial by preventing undesired apoptosis.[56]

Nuclear translocation of NF-κB dimers allows their binding to κB sites in the promoter of the target gene. Several studies have attempted to identify DNA sequence specificity of different NF-κB dimers.[8,57–63] However, κB sites display a remarkably variable consensus sequence.[8] The classical κB nucleotide sequence is G-G-G-R-N-N-Y-Y-C-C[8] (where N = any base, R = purine, and Y = pyrimidine). This may suggest the possibility that the κB site, rather than determining the ability of a particular dimer to bind effectively, affects which coactivator forms productive interactions with the bound NF-κB dimer. Unpublished results from our group suggest that even greater complexity of regulation of gene expression by κB sites exists. The κB sites of the TNF-α gene may be both inhibitory and stimulatory under specific conditions. One possible explanation is that the binding sites may affect the tertiary structure of the activation complex.

NF-κB activators and its outputs

The NF-κB pathway described above integrates signals from a wide range of stimuli and in turn activates numerous cellular responses.[64] This section will discuss NF-κB activation in the context of inflammation.

The canonical pathway is mainly stimulated by inflammatory signals including TNF-α, IL-1, IL-17, and CD40 ligand (CD40L), as well as pathogen-associated molecular patterns (PAMPs) recognized by TLRs and nucleotide oligomerization domain (NOD) receptors.[65–68] The activation of NF-κB by these stimuli leads to the expression of inflammatory mediators such as cytokines, chemokines, cell adhesion molecules, inducible nitric oxide synthase (iNOS), anti-apoptotic proteins, costimulatory molecules, matrix metalloproteinases (MMPs), and cyclooxygenase (COX).[69] The canonical NF-κB pathway leads to the maturation and activation of macrophages and dendritic cells (DCs) and consequently to the induction of the innate and adaptive immune response and inflammation. In the case of TNF-α-induced signaling, NF-κB has been shown to be an activator of anti-apoptotic gene expression.[70–73] This outcome of NF-κB signaling has been shown to depend on the termination of c-Jun N-terminal kinase (JNK) activation.[74,75] Therefore, TNF-α signaling may result in apoptosis, if JNK is activated, or proliferation, if NF-κB activation prevails.[76] This indicates that these signaling pathways are not strictly linear but that there is cross-talk between different signaling pathways taking place which influences the outcome of receptor stimulation. Therefore, inhibition of one signaling pathway may also affect the outcome of other

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signaling pathways. In addition, NF-κB exhibits different activation dynamics depending on the stimulus. While showing an oscillatory dynamic when stimulated with TNF-α, this is not observed when stimulated with the TLR4 ligand LPS.[77] These differences are associated with distinct signaling pathways induced by those stimuli.

Strong inducers of the alternative NF-κB pathway are lymphotoxin (LT), CD40L, and B-lymphocyte stimulator (BLyS). Unlike the canonical pathway, activation of the alternative NF-κB pathway plays a major role in lymphoid organogenesis and humoral immunity through inducing B-cell maturation.[49]

The strong induction of immunity and inflammatory mediators requires a robust regulation of NF-κB activation. Evidence suggests that a defect or breakdown of regulatory mechanisms could lead to an exaggerated immune response and inflammatory diseases.[78] Therefore, NF-κB is a logical target for the treatment of inflammatory diseases.

INVOLVEMENT OF NF-κB IN RHEUMATOLOGICAL DISEASES

Initial evidence for an involvement of NF-κB in RA originated from the detection of NF-κB dimers in the nucleus of macrophages and fibroblast-like synoviocytes of patients with early and later stage RA.[79–81] This observation has also been made in other inflammatory diseases including sarcoidosis.[82] Overexpression of the endogenous NF-κB inhibitor IκBα in dissociated synovial membrane cultures from RA patients resulted in attenuation of proinflammatory mediators such as TNF-α, IL-1β, IL-6, and IL-8.[83,84] In contrast, expression of the anti-inflammatory IL-1Ra and IL-10 were unaffected, further confirming a specific functional role of NF-κB in driving inflammation in conditions such as RA[84] (Figure 47.2). Similarly, the overexpression of IκBα potently inhibited the expression of MMP-1, -3, and -13, while the expression of their endogenous inhibitor, tissue inhibitor of

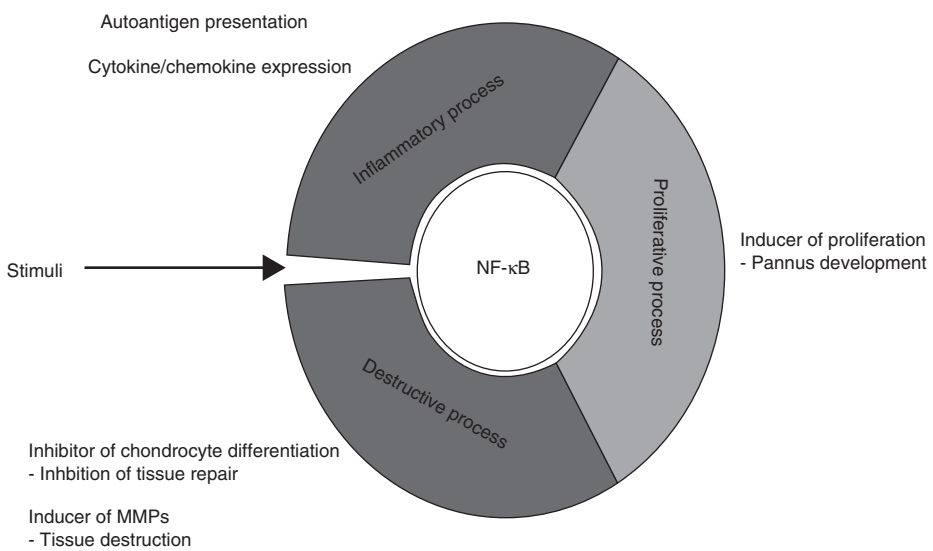


Figure 47.2 NF-κB activation affects numerous aspects of the disease process of RA. NF-κB activation leads to the expression of proinflammatory cytokines and chemokines as well as an enhancement of autoantigen presentation by DCs. This leads to inflammation through further recruitment of T cells and macrophages. The joint destruction observed in RA is also driven by NF-κB activation, which induces the expression of MMPs as well as inhibiting the differentiation of chondrocytes and therefore inhibiting repair of damaged cartilage. A third aspect of RA etiology is the formation of the pannus, which is driven by the induction of anti-apoptotic genes following NF-κB activation. NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; DC, dendritic cell; MMP, matrix metalloproteinase.

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metalloproteinase (TIMP)-1, was unaffected.[84] Comparable results were obtained in synovial cells obtained from osteoarthritis (OA) patients.[85] Further support for an involvement of NF-κB in the production of proinflammatory cytokines and destructive enzymes, such as MMPs, resulted from experiments using a dominant negative version of the upstream activator of NF-κB, IKK2 (IKK2dn). Overexpression of IKK2dn resulted in a significant inhibition of IL-6 and IL-8, as well as MMP-1, -2, -3, and -13. However, the expression of TNF-α was only marginally affected.[86] These results would suggest different mechanisms of NF-κB-dependent gene expression. Besides its function as an inducer of proinflammatory cytokine expression, NF-κB activation promotes another aspect of inflammation by up-regulating cell surface markers important for antigen presentation by DCs that lead to T-cell activation. This was shown to depend on the activation of the canonical NF-κB pathway, as the expression of IKK2dn inhibited this process.[87] Therefore, in the context of RA the activation of NF-κB could lead to enhanced autoantigen presentation by DCs (Figure 47.2).

Activation of NF-κB has an important role in the generation and maturation of osteoclasts, which are involved in bone resorption while at the same time inhibiting differentiation of chondrocytes that are essential for repair of damaged cartilage.[88–91] This contributes to the bone destruction observed in RA. Chondrogenesis requires activation of sex determining region Y-box (Sox) 9, which is down-regulated by TNF-α and IL-1 produced in response to NF-κB activation, thus inhibiting cartilage formation.[90] On the other hand, NF-κB drives synovial proliferation which leads to the formation of the pannus, characteristic of RA (Figure 47.2). Synovial tissue from patients with RA shows a significantly higher expression of NF-κB1 in cells at the cartilage–pannus junction compared with other areas.[92]

Animal models support this central role of NF-κB. Mice deficient in c-Rel or NF-κB1 are protected from the development of collagen-induced arthritis (CIA).[93] Similarly, transgenic expression of the constitutively active form of the inhibitor IκBα (N-terminally truncated from amino acids 37 to 317) in the T-cell lineage resulted in a decreased incidence and severity of CIA.[94] Furthermore, the use of decoy oligonucleotides, which obstruct the binding of NF-κB to the promoter of the target gene, prevented the recurrence of streptococcal cell wall (SCW)-induced arthritis as well as CIA, most probably due to inhibition of TNF-α and IL-1. Inhibition of IκBα degradation by the proteasome inhibitor PS-341 after the onset of polyarthritis in rats also showed beneficial effects as measured by the Total Arthritis Index.[95]

The evidence presented here indicates an involvement of NF-κB in numerous processes leading to inflammatory diseases and driving their progression, making NF-κB an attractive target for the treatment of rheumatological diseases.

NON-SPECIFIC NF-κB INHIBITORS

A number of anti-inflammatory drugs already in clinical use may produce some of their anti-inflammatory action by inhibiting components of the NF-κB signaling pathway. These include non-steroidal anti-inflammatory drugs (NSAIDs), sulfasalazine, glucocorticoids, thiazolidinediones (TZDs), triptolide, and thalidomide. However, in the case of all these drugs it must be emphasized that there is a multiplicity of targets in addition to NF-κB.

Non-steroidal anti-inflammatory drugs and derivatives

The prototypical NSAID is aspirin (aminosalicylic acid), the molecular target of which is COX, which leads to the formation of prostaglandins that cause inflammation, swelling, pain, and fever.[96] At high doses salicylic acid can also inhibit IKK2.[97] However, this effect is only seen at very high doses of salicylic acid, higher than would routinely be used in the treatment of RA.

Sulfasalazine

Sulfasalazine was originally developed in the 1940s as a combined antimicrobial/anti-inflammatory agent containing sulfapyridine and aminosalicylic acid. It is an established disease-modifying anti-rheumatoid drug (DMARD) and is also extensively used in the

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management of ankylosing spondylitis.[98] Its anti-inflammatory effects appear to be mediated through a number of mechanisms including induction of autocrine adenosine signaling,[99] COX inhibition, and inhibition of NF-κB signaling.[100] Although the endpoint of inhibited NF-κB signaling is reduced DNA binding (e.g. to the IL-12 subunit gene, p40-κB site),[100] it is not as yet clear at which exact point(s) along the NF-κB pathway sulfasalazine mediates its effects.

Glucocorticoids

Glucocorticoids remain among the most commonly used anti-inflammatory drugs. Their numerous anti-inflammatory actions are mediated by the glucocorticoid receptor (GR), a classical nuclear hormone receptor that dimerizes upon ligand binding and acts as a ligand-inducible transcription factor. The anti-inflammatory effects of glucocorticoids are mediated by numerous mechanisms including inhibition of the transcription factor activating protein-1 (AP-1), induction of lipocortin-1, and inhibition of NF-κB signaling.[101] Inhibition of NF-κB occurs by at least two distinct mechanisms. Firstly, the GR reportedly induces expression of the endogenous NF-κB inhibitor IκBα,[102,103] which forms a negative feedback loop inhibiting nuclear translocation of NF-κB. Secondly, the GR inhibits expression of NF-κB target genes,[104–106] which is probably mediated by direct protein–protein interaction between the GR and NF-κB.[107] However, glucocorticoids can have significant side effects including osteoporosis, type 2 diabetes, hypertension, upper gastrointestinal tract ulceration, and increased susceptibility to infection.[108] Therefore, the challenge is to design compounds that show comparable anti-inflammatory efficacy to glucocorticoids without major side effects. Other glucocorticoid derivatives with potent NF-κB-inhibiting effects but fewer side effects than traditional glucocorticoids are under development, such as the inhaled glucocorticoid derivative ciclesonide, for the treatment of asthma.[109]

Thiazoledinediones

The TZDs are synthetic agonists at the peroxisome proliferator-activated receptor (PPAR)γ, a nuclear receptor involved in the regulation of metabolic function in tissues including liver, muscle, and adipose.[110] This class of drugs was developed for the treatment of type 2 diabetes mellitus and includes troglitazone (now withdrawn due to several cases of liver failure),[111] pioglitazone (Takeda),[112] and rosiglitazone (Glaxo Smith Kline).[113] In addition to their anti-diabetic actions, several lines of evidence also suggest that these drugs have clinically useful anti-inflammatory properties and these have now been ascribed, at least in part, to inhibition of NF-κB signaling. In animal models including the carrageenin paw edema model of inflammation, the CIA model of arthritis,[114] and the dextran sodium sulfate (DSS) model of colitis, rosiglitazone reduces inflammation. While there are as yet no clinical trials of TZDs in arthritis, in an open-label trial of rosiglitazone in patients with ulcerative colitis, 4 mg twice daily led to a reduction of severity of the colitis.[115] Although congestive cardiac failure may be exacerbated in patients taking TZDs[116] and a small number also develop abnormal liver function tests,[117] these drugs are generally well tolerated and safe for the majority of patients. Several mechanisms of inhibition of NF-κB by TZDs have been hypothesized,[118–120] including reduction in RelA expression.[118,119]

Thalidomide

Thalidomide was originally developed in the 1950s as a treatment for hyperemesis gravidarum but was withdrawn in 1961 as its teratogenic effects became apparent. However, it has become recognized as an immunomodulatory agent and has been successfully used in the treatment of inflammatory conditions including human immunodeficiency virus (HIV)-associated aphthous ulceration and inflammatory bowel disease.[121] NF-κB inhibition has been suggested as a mechanism of action of thalidomide in inflammation.[122,123] In particular, it appears that thalidomide may inhibit the phosphorylation of IκBα.[124] There are as yet no reported trials of thalidomide in RA; however, clinical trials of thalidomide in inflammatory bowel disease indicate that not only is it a clinically efficacious anti-inflammatory agent but it also has an acceptable safety and side effect profile.[125]

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Triptolide

The anti-rheumatic properties of extracts of the Chinese herbal remedy *Tripterygium wilfordii* Hook F (thunder god vine, lei gong teng)[126] have been recognized for many years. The active ingredient has been identified as a diterpene triepoxide, triptolide (Table 47.1).[127] *In vitro* triptolide inhibits TNF-α- and IL-1β-induced transcription of inflammatory cytokines. Inhibition occurs after NF-κB DNA-binding, by either interfering with post-transcriptional modifications of RelA or interfering with the recruitment of transcription cofactors.[128] Beside the inhibition of NF-κB, triptolide was also shown to inhibit the transcription factors, nuclear factor activating T cells (NF-AT) and AP-1 at the level of nuclear translocation.[128–130] A recent double-blind placebo-controlled trial of an ethanol/ethyl acetate extract of *Tripterygium wilfordii* Hook F in patients with long-standing RA that was resistant to conventional therapy showed that most patients achieved an American College of Rheumatologists score (ACR) 20% response and significant improvements in objective markers of inflammation. No serious side effects were observed.[131]

TARGETED THERAPIES

Although a number of anti-inflammatory agents currently in clinical use may act in part by inhibition of NF-κB, they also have other targets and are all limited by significant side effects. It is therefore desirable that drugs with more specific mechanisms of action are developed providing a decreased risk of undesirable side effects. In this section we will therefore review some of the compounds currently under development that are designed to inhibit specific components of the NF-κB signaling pathways.

IKK2 inhibitors

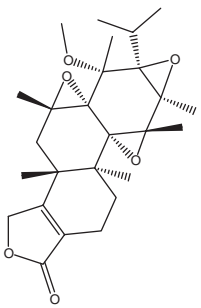
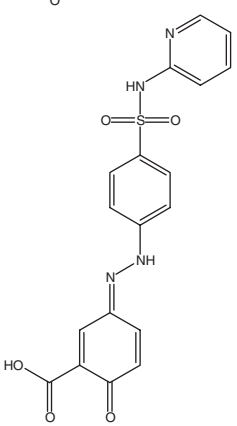
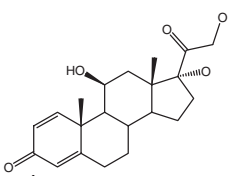
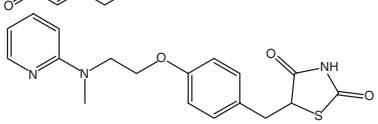
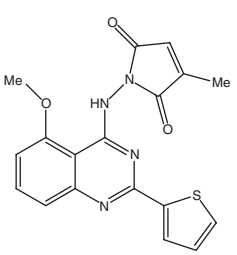
Numerous elements in the activation pathway of NF-κB are currently under investigation for their feasibility as therapeutic targets. Inhibitors for the kinases IKK1 and IKK2 would provide a specific inhibition of NF-κB activation while leaving other signaling pathways intact. Therefore, intensive effort has been made in the development of these inhibitors (reviewed by Karin et al.).[132] Although IKK1 would be an attractive target for the treatment of autoimmune diseases, no potent and specific inhibitor has been described so far. However, several IKK2 inhibitors are in their preclinical developmental stages and showed an effect in models of arthritis.

SPC-839 is a quinazoline analog and an ATP-competitive inhibitor of IKK2.[132] It inhibits IKK2 with an IC_{50} of 62 nM compared with inhibition of IKK1 with an IC_{50} of 13 μM.[132] Treatment of rat adjuvant arthritis with SPC-839 led to a reduction in paw swelling and radiographic damage.[133] An inhibition of NF-κB activation in LPS-challenged rats was already detectable at a dose of 10 mg/kg.[132] Another well-studied IKK2 inhibitor is BMS-345541. Tested in the human monocytic cell line THP-1, this compound blocked the release of LPS-induced TNF-α, IL-1β, IL-8, and IL-6 with an IC_{50} in a range between 1 and 5 μM.[134] More recently, it was shown that BMS-345541 successfully blocks the release of MMP-9 in RAW 264.7 cells in response to the TLR9 ligand CpG DNA.[135] Furthermore, CIA mice treated with BMS-345541 showed significantly reduced joint inflammation and destruction.[136] However, this effect was only observed if BMS-345541 was administered prophylactically before the induction of disease.

A more recently described IKK2 inhibitor is ML120B, with an IC_{50} of 60 nM, as evaluated in an *in vitro* kinase assay.[137] ML120B inhibited the induction of RANTES (regulated on activation, normal T-cell expressed and secreted) in TNF-α- or IL-1β-stimulated human fibroblast-like synoviocytes.[137] Moreover, the expression of TNF-α, IL-1β, and IL-6 was also inhibited in LPS- or peptidoglycan (PGN)-stimulated human mast cells as well as the expression of MMP-1 and MMP-13 in human chondrocytes stimulated with IL-1.[137] These results suggest that ML120B is a potent inhibitor of NF-κB in RA-relevant cell systems. Furthermore, ML120B was shown to block the differentiation and maturation of T cells and B cells and to inhibit the expression of numerous proinflammatory mediators such as IL-6, IL-8, and RANTES in human airway smooth muscle cells.[138] The inhibition of these cytokines and chemokines using ML120B was

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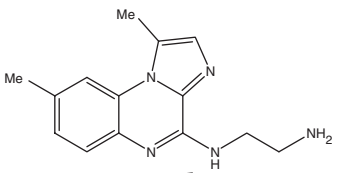
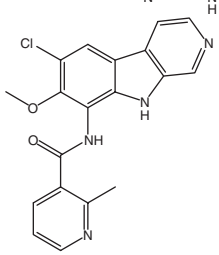
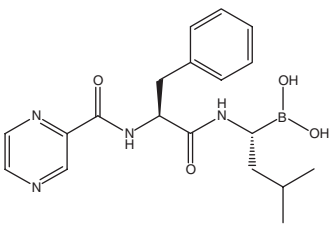
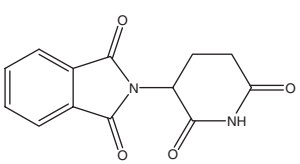
Table 47.1 NF-κB targeted anti-inflammatory drugs in current clinical use and undergoing development

Drug	*Structure*	*Target/mode of action*	*Trials*	*Refs*
Triptolide		p65 post-transcriptional modifications NF-κB co-activator recruitment	RA, phase II	127–130,163
Sulfasalazine		NF-κB DNA binding	RA, RCTs	100,164
NFkappaB decoy oligo	5′-CCTTGAAGGGATTT CCCTCC-3′ (phosphorothioate)	NF-κB DNA binding	Phase I in HIV skin disease, phase II in atopic dermatitis	143,165, 166,167
Steroids, e.g. prednisolone		Trans-repression of NF-κB	RA, RCTs	106,107,168
PPARγ agonists, e.g. rosiglitazone		Trans-repression of NF-κB	DM, RCTs	114
SPC-839		IKK2 inhibitor	No clinical trial data available	132,133

Continued

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Table 47.1 NF-κB targeted anti-inflammatory drugs in current clinical use and undergoing development—cont'd

Drug	*Structure*	*Target/mode of action*	*Trials*	*Refs*
BMS-345541		IKK2 inhibitor	No clinical trial data available	134–136,140
ML120B		IKK2 inhibitor	No clinical trial data available	138,137
Bortezomib		Proteasome inhibitor	Approved by FDA for multiple myeloma	142,141
NBD	C-terminus of IKK1 and IKK2	NF-κB dimerization	No clinical trial data available	89,152–154
SN50	NLD of NF-kappaB1	Nuclear localization of NF-kappaB	No clinical trial data available	144–149
PTP-p65-P1	Amino acids 271–282 of RelA	Transactivation of RelA	No clinical trial data available	150
Thalidomide		IKK	IBD, juvenile RA, and ankylosing spondylitis	124,169–171

RCT, randomized controlled trials; IBD, inflammatory bowel disease; CIA, collagen-induced arthritis; IKK2, inhibitor of kappa kinase 2; FDA, Federal Drug Authority; NBD, NEMO binding domain; T2DM, type 2 diabetes mellitus; NLD, nuclear localization domain.

either as effective as or more effective than dexamethasone.[138]

Proteasome inhibitors

Following the phosphorylation and ubiquitination of IκBα, it is degraded by the proteasome and releases the NF-κB dimer to translocate to the nucleus.[139] Therefore, targeting the proteasome could prevent the nuclear translocation of NF-κB. A proteasome inhibitor already approved by the US Food and Drug Administration (FDA) for the treatment of multiple myeloma is bortezomib (Millenium).[140] Another proteasome

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inhibitor currently in clinical trials for multiple myeloma is PR171 (Proteolix). However, because the proteasome is involved in a great variety of regulatory processes, the effects generated are non-specific. Adverse effects observed after bortezomib treatment include infections of the upper respiratory tract, lymphopenia, and thrombocytopenia.[141,142] Because the treatment of chronic inflammatory diseases such as RA makes it necessary to administer therapies over a long period of time, such side effects are likely to be unacceptable.

NF-κB decoy oligos

Since NF-κB functions by binding specific DNA sequences in the promoters of target genes, novel NF-κB targeted anti-inflammatory drugs utilizing the DNA-binding properties of the NF-κB heterodimers may provide future therapies. One approach to achieve this is the therapeutic use of phosphothiorate deoxyribonucleic acid oligomers. Several *in vitro* experimental models of this approach have been reported, such as the use of folate-linked lipid-based nanoparticles to transfect macrophages with NF-κB decoy oligos.[143] In the RAW264.7 murine macrophage cell line, these were found to potently inhibit the translocation of NF-κB from cytoplasm to the nucleus, following LPS stimulation.[143] Such agents are already in clinical trials for atopic dermatitis (NIH Clinical Trials Identifier NCT 00125333). These trials are ongoing and no results are available yet. If it were possible to safely and effectively deliver such decoy oligos to the inflamed joint then these would also form attractive treatments for RA.

NF-κB inhibitory peptides

An alternative approach to inhibit NF-κB activation is to block the interaction of molecules involved in NF-κB signaling using short peptide sequences. The first member of this class of inhibitors described was SN50, which contained the nuclear localization domain sequence of NF-κB1.[144] SN50 was shown to block NF-κB in numerous cell lines in response to the HIV envelope protein glycoprotein (gp) 120 and staphylococcal enterotoxin B.[145,146] Furthermore, SN50 was reported to protect rats from acute pancreatitis as well as showing efficacy in the treatment of corneal alkali burns in mice.[147,148] However, SN50 also affects the nuclear translocation of STATs (signal transducer and activator of transcription), AP-1, and NF-AT.[149] This proof of principle of inhibiting NF-κB through blocking specific interaction of signaling components led to the development of several other inhibitory peptides. In contrast to SN50, the PTD-p65-P1 peptide was reported to specifically inhibit TNF-α-induced NF-κB activation without affecting other transcription factors.[150] PTD-p65-P1 corresponds to the amino acid residues 271–282 of RelA. It was reported to block the phosphorylation of serines 276, 529, and 536 of RelA, thereby inhibiting transactivation of RelA without affecting IκBα degradation.[150] However, no *in vivo* studies confirming the inhibitory effect of PTD-p65-P1 are available so far. The cell-permeable peptide NBD consists of six amino acids (Leu, Asp, Trp, Ser, Trp, Leu), which correspond to the C-terminus of IKK1 as well as IKK2 that is responsible for the association with NEMO.[151] Numerous studies have investigated the effect of NBD administration on NF-κB activation in models of inflammatory arthritis. The administration of NBD to mice before the onset of inflammatory arthritis inhibited cytokine-induced osteoclasts formation and thereby inhibited bone erosion in the joints of those mice.[152] Similarly, the administration of NBD into mice at the onset of CIA reduced the severity of the inflammatory arthritis by impairing the production of TNF-α and IL-1 and thereby reducing joint swelling and the destruction of bone and cartilage.[89] Also, NBD given at the onset of carrageenin-induced paw edema led to a reduction in edema formation and cellular infiltration into the paws of the mice.[153] In all of the animal studies described above NBD was administered before or at the onset of inflammation, therefore, it is unclear if NBD exhibits an effect on an already established inflammatory disease. However, initial results obtained from *in vitro* studies using fibroblast-like synoviocytes and macrophages from RA patients also suggest a positive effect of NBD on RA treatment in humans.[154] When given to *ex vivo* cultured RA synovial tissue or RA fibroblast-like synoviocytes, it inhibited the spontaneous

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release of IL-1β, TNF-α, and IL-6.[154] Moreover, NBD inhibited IL-1β-induced expression of TNF-α in human macrophages.[154] These results indicate that NBD might be a promising future therapeutic in rheumatological diseases, which is able to inhibit NF-κB specifically. However, the use of peptide drugs is limited by the requirement for a parenteral route of administration. The ultimate goal would therefore be to identify peptidometic, orally bioavailable, small molecular weight compounds to replace such peptides.

POTENTIAL FUTURE TARGETS

In addition to the targets describe above, there remain a number of targets in the NF-κB signaling pathway that have so far not been exploited for development of targeted anti-inflammatory drugs. In this section, the potential for developing targeted therapies to inhibit some of these will be discussed.

Ubiquitin ligase inhibition

Following IκBα phosphorylation by IKKs, E3 ubiquitin ligase targets IκBα for proteasomal degradation by addition of poly-ubiquitin side chains. Inhibition of the E3 ligase is therefore a potential mechanism for modulating NF-κB signaling. Such an approach is already being utilized in the oncology field[155] by means of high throughput screening to identify inhibitors of anaphase promoting complex (APC) E3 ligase. However, E3 ubiquitin ligases are involved in numerous cellular processes, therefore, their inhibition might lead to a broad range of adverse effects as is observed with proteasome inhibitors.

Phosphorylation of RelA

Post-translational covalent modification of Rel family members appears to be a key regulatory step in NF-κB signaling.[46,156] Thus phosphorylation of serine residues in RelA represents potential targets. Since kinase inhibition is a well established route of drug development, such an approach should be readily amenable to a high throughput small molecule screening approach similar to that used in the identification of IKK inhibitors.[157] Kinases reported to be involved in serine phosphorylation of RelA include PKA, MSK-1, PKCζ, RSK-1, GSK-3β, PI3K, IKK1, IKK2, TBK1,[51] and Bruton's tyrosine kinase (Btk).[156,158] As some of these kinases appear to be restricted to particular stimuli, their inhibition raises the prospect of highly specific future therapies. Moreover, this approach would potentially have the advantage of inhibiting the amplification of the stimulated NF-κB signal while leaving its basal activity unaffected.

Inhibition of translocation

One of the key steps in NF-κB activation is nuclear translocation. Thus, nuclear–cytoplasmic shuttling presents an attractive potential target for inhibition of NF-κB-induced inflammation and it can be envisaged that drugs to inhibit transport through the nuclear pore complex (NPC)[159] could be developed. Some interest has already been shown in this target for drug development by researchers in other areas such as HIV.[160,161]

Gene therapy

An approach to circumvent potential systemic toxicity of NF-κB targeted therapies for arthritis is to administer therapy selectively to the affected joints by means of gene therapy. A study of the use of recombinant adeno-associated virus (rAAV) in the rat adjuvant arthritis model[162] showed that a single intra-articular injection of rAAV encoding IKK2dn produced significant reductions in paw swelling and production of IL-6 and TNF-α in the treated joint. Such work raises the possibility of NF-κB targeted gene therapy becoming a viable option for the treatment of arthritis in humans. Indeed, a phase II gene therapy trial sponsored by the Targeted Genetics Coporation, USA is already recruiting (clinical trials identifier, NCT00126724) in which patients with inflammatory arthritis (including patients who are already on anti-TNF therapy or on classical DMARDS) receive repeat intra-articular injections of an adeno-associated vector designed to express human TNF receptor (TNFR)-immunoglobulin (IgG1) Fc fusion protein.

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CONCLUSION

The central role of NF-κB in inflammation and immunity makes it a legitimate target for the treatment of inflammatory diseases. *In vitro* as well as *in vivo* studies confirm the central involvement of NF-κB in rheumatological diseases including RA and OA. NF-κB inhibition causes significant reduction in the disease activity of CIA and SCW models of RA and the improvement observed in these models of RA appears to be due to inhibition of NF-κB-dependent expression of proinflammatory mediators as well as enzymes including MMPs that are responsible for joint destruction.

Considerable effort has been made to develop therapeutically useful inhibitors of NF-κB. However, the treatment of chronic inflammation requires a therapy to be administered over a long period of time, making significant side effects – as is observed, for example, with proteasome inhibitors – unacceptable.

The NF-κB pathway shows a high degree of convergence, whereby signals from a wide range of receptors are processed through a central signalling pathway comprising the IKK/IκB/NF-κB cascade (Figure 47.3). Subsequently, NF-κB signaling diverges and results in numerous cellular responses due to the induction of a great variety of genes (Figure 47.3). However, more recent work is beginning to unveil the complexity of the NF-κB system and its regulation. For example, the discovery of the role of NF-κB transactivation in 'fine tuning' gene induction adds another layer of complexity to this signaling system.[51]

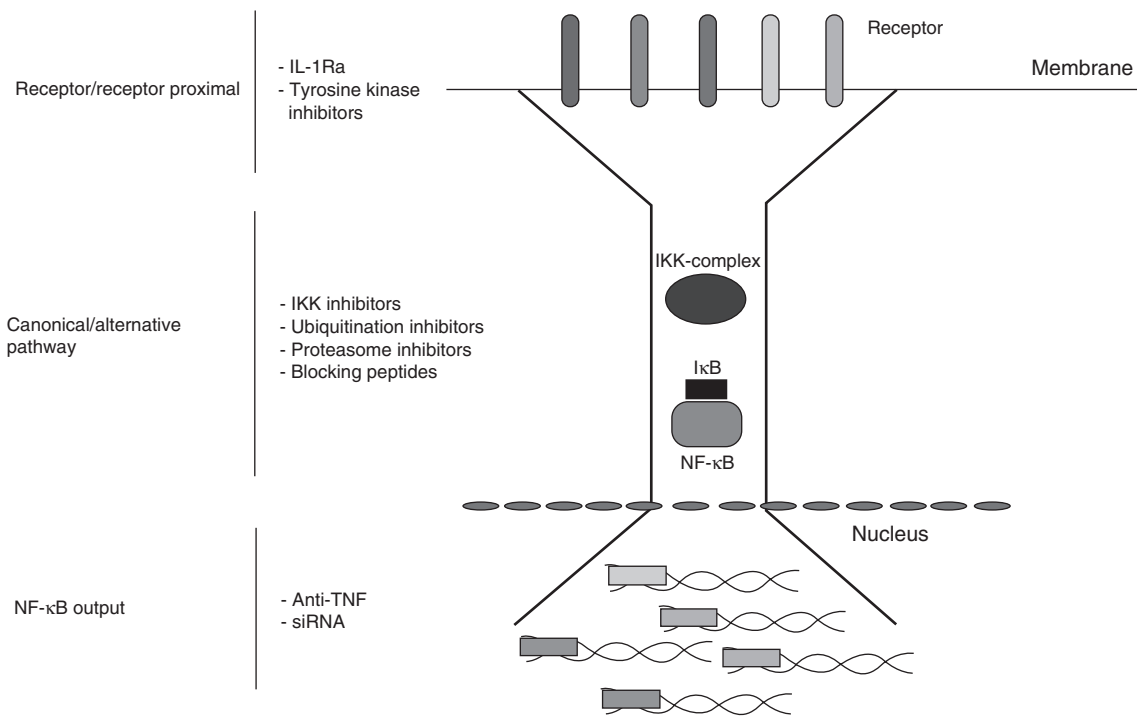


Figure 47.3 Possible approaches to inhibit NF-κB signaling. Numerous receptors initiate signaling via the NF-κB pathway. Signaling then converges through either the canonical or non-canonical pathway before diverging through induction of a wide range of genes. Examples of classes of compounds that inhibit signaling upstream of the convergence of this pathway (Receptor/receptor proximal in the diagram) include receptor antagonists (e.g. IL1-Ra). Examples of inhibitors that act on the canonical and alternative pathways include IKK inhibitors, ubiquitination inhibitors, proteasome inhibitors, and blocking peptides. Examples of inhibitors acting after divergence of the pathway (NF-κB output in the diagram) include anti-TNF agents and siRNA-based therapies. NF-κB, nuclear factor-κB; IL-1Ra, interleukin-1 receptor antagonist; TNF, tumor necrosis factor; siRNA, small interfering RNA.

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Future therapies based on inhibiting NF-κB signaling could be divided into three possible categories (Figure 47.3). Inhibition of the receptor or receptor proximal signal transducers would be specific for one stimulus; however, it might affect, several signaling pathways induced by this receptor. This would be a promising therapy if the stimulus, responsible for the condition which is treated, is already identified. On the other hand, while inhibiting central components of the NF-κB pathway (e.g. IKK complex, IκBα degradation) would exert only limited effects on other signaling pathways, it may be expected to produce a more global inhibition of NF-κB and therefore result in significant adverse effects. A third possibility would be the targeting of specific NF-κB outputs. Anti-TNF therapy is one such example; in the future siRNA-based therapies may provide another approach.

However, in addition to mediating pathological inflammation, NF-κB is also necessary for host defense and thus global inhibition of NF-κB would lead to an unacceptable range of side effects including serious infections. In order to utilize the therapeutic potential of NF-κB inhibition, it will be necessary to further study the regulation of NF-κB and its involvement in specific conditions including RA and OA. This knowledge will facilitate future development of compounds that are more precisely targeted, thereby giving greater therapeutic efficacy with fewer side effects.

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