MAPPIT analysis of the early events in Toll-Like receptor signalling

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Thesis submitted to fulfil the requirements for achievement of the degree of Ph.D. in Biomedical sciences

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Abbreviation list

aa	amino acid
ABIN	A20-binding inhibitor of NF-κB
AP-1	Activator protein 1
APCs	Antigen presenting cells
ASC	Apoptosis-associated Speck-Like protein containing a CARD
ASK	Apoptosis Signal-regulating Kinase 1
ATF3	Activating transcription factor 3
BiFC	Bimolecular Fluorescence Complementation
BIR	Baculoviral Inhibitor of apoptosis Repeats
CARD	Caspase Recruitment Domain
CD	Clusters of Differentiation
CRP	C-reactive protein
DAG	Diacylglycerol
DAI	DNA-dependent activator of IFN-regulatory factors
DAMPs	Danger-Associated Molecular Patterns
DAP	meso-diaminopimelic acid
DC	Dendritic Cell
DD	Death Domain
DNA	Deoxyribonucleic Acid
ds	double-stranded
ECM	Extracellular Matrix
EMCV	Encephalomyocarditis Virus
ERK	Extracellular signal-regulated Protein Kinase
FADD	Fas receptor associated death domain
Fliih	Flightless I Homolog
FRET	Fluorescence Resonance Energy Transfer
GPCR	G-protein coupled receptor
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HEK	Human Embryonic Kidney

HEV	High Endothelial Venule
HIV	Human Immunodeficiency Virus
HMGB1	High-Mobility Group Box 1
HSP	Heat Shock Protein
HSV	Herpes Simplex Virus
ICE	IL-1β converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IL-1RAP	Interleukin-1 Receptor Accessory Protein
IPAF	ICE protease activating factor
IPS-I	Interferon Promoter Stimulator I
IRAK	IL-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISRE	Interferon-stimulated Response Element
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JAK	Janus Kinase
LBP	LPS-binding protein
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
LTA	Lipoteichoic Acid
Mal	MyD88 Adaptor-Like
MALP-2	Macrophage-Activating Lipopeptide-2kDa
MAPK(KK)	Mitogen Activated Protein Kinase (Kinase Kinase)
MAPPIT	Mammalian Protein-Protein Interaction Trap
MASP	MBL-associated serine proteases
MBL	Mannan-binding lectin
MCMV	Murine Cytomegalovirus
Mda5	Melanoma differentiation associated gene 5
MDP	Muramyl Dipeptide
MEF	Mouse Embryonic Fibroblast

MEK(K)	MAPK-ERK Kinase (Kinase)
MHC	Major Histocompatibility Complex
MKK	MAPK Kinase
MSR	Macrophage scavenger receptor
MyD88	Myeloid Differentiation primary response gene 88
MyD88$_s$	MyD88 short
NALP	NACHT-, LRR-, and pyrin-domain-containing proteins
NAP1	NF-κB-activating kinase-Associated Protein 1
NDV	Newcastle Disease Virus
NK	Natural Killer
NLR	NOD-Like Receptor
NOD	nucleotide-binding oligomerisation domain
PAMP	Pathogen-Associated Molecular Pattern
pDC	plasmacytoid DC
PG	Peptidoglycan
Pin1	Peptidyl-prolyl-isomerase
PKC	Protein kinase C
PRR	Pathogen Recognition Receptor
RHIM	Rip Homotypic Interacting Protein
RIG-I	Retinoic-acid-Inducible protein I
RIP1	Receptor-Interacting Protein 1
RLR	RIG-I-Like Receptor
RNA	Ribonucleic Acid
RP105	Radioprotective 105
RSV	Respiratory syncytial virus
SAP	serum ameyloid protein
Sarm	Sterile alpha and HEAT-Armadillo motifs containing protein
SH2	Src Homology 2
SHP-2	SH-2 containing protein tyrosine phosphatase-2
SIGIRR	Single immunoglobulin IL-1R-related protein
SOCS	Suppressor of cytokine signalling
SR-A	Scavenger receptors type A
ss	single-stranded
STAT	Signal Transducer and Activator of Transcription

SYK	Spleen Tyrosine Kinase
TAB	TAK1-binding Protein
TAK1	Transforming growth factor-β-activated Kinase 1
TANK	TRAF-family-member-associated NF-κB activator
TAP	Tandem Affinity Purification
TBK1	TANK-binding Kinase 1
TGF-β	Transforming Growth Factor β
Th	T-helper
TIR	Toll IL-1 Receptor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
Tollip	Toll-interacting protein
TPL2	Tumor progression locus 2
TRAF	Tumor Necrosis Factor-associated factor
TRAIL(-R)	TNF-related apoptosis-inducing ligand (receptor)
Tram	Trif-related adapter molecule
Trif	TIR-domain-containing adaptor inducing interferon
Ubc 13	Ubiquitin-conjugating enzyme 13
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus

Samenvatting

Vertebraten bezitten een ingenieus verdedigingssysteem waarmee ze zich wapenen tegen het constante gevaar van invasieve pathogenen. Het immuunsysteem bij zoogdieren kan onderverdeeld worden in een adaptieve en een aangeboren respons. De adaptieve immuniteit is gebaseerd op clonale expansie van T and B lymphocyten die antigen-specifieke receptoren expresseren. Door deze clonale selectie en expansie is de adaptieve respons pas volledig operationeel drie tot vijf dagen na infectie. De aangeboren respons daarentegen vormt de eerstelijnsdefensie tegen pathogenen en is daarom essentieel om de infectie onder controle te houden tot de adaptieve respons geactiveerd wordt. De aangeboren immuniteit maakt gebruik van een gelimiteerde set aan pathogeen-herkennende receptoren waarvan de familie van de Toll-Like receptoren (TLRs) de belangrijkste zijn. TLRs herkennen een brede waaier aan bacteriële en virale structuren zoals lipopolysaccharide, dsRNA of bacterieel DNA. Deze structuren worden vaak pathogeen-geassocieerde moleculaire patronen of PAMPs genoemd. PAMPs zijn essentieel voor het overleven en de replicatie van deze pathogenen en zijn daarom onveranderd gebleven doorheen de evolutie. Herkenning van een pathogen door TLRs leidt tot de productie van pro-inflammatoire cytokines. Deze cytokines activeren de aangeboren immuuncellen zoals macrofagen en neutrofielen. De TLRs zijn bovendien ook betrokken bij de activatie en modulatie van de daaropvolgende adaptieve immuunrespons. Echter, ongepaste en overmatige activatie van de TLR signaalweg leidt tot excessieve inflammatie wat vaak enorm schadelijk is. Daarom is de TLR signaalweg strikt gereguleerd. Verscheidene modulatoren zijn beschreven die ofwel interfereren in ligand binding, ofwel de receptorexpressie beïnvloeden ofwel de intracellulaire signaalweg moduleren.

Dit werk richt zich voornamelijk op de initiële stappen van de TLR signaalweg. Daarbij zijn vier adaptor molecules betrokken: MyD88, Mal, Trif and Tram. Verschillende TLRs gebruiken verschillende combinaties van adaptoren, wat gedeeltelijk de ligand specifieke respons kan verklaren. Door gebruik te maken van MAPPIT (Mammalian Protein-Protein Interaction Trap), een twee-hybride methode die in ons laboratorium werd ontwikkeld, creëerden we een TLR-adaptor interactiemap. Bovendien toonden we aan dat Mal een brugfunctie vervult in TLR2 en TLR4 signalisatie, door MyD88 naar de geactiveerde receptor te derigeren. We identificeerden CIS, een lid van de SOCS proteïne familie, als een mogelijke regulator van de MyD88-afhankelijke signaalweg en toonden aan dat de bindingsmodaliteiten van CIS verschilden tussen receptor en MyD88 interactie.

Summary

Vertebrates developed an ingenious defence system, by which they counteract the continuous peril of invading pathogens. This mammalian immune system can be divided in an innate and an adaptive branch. Adaptive immunity relies on clonally expanded T and B lymphocytes expressing antigen-specific receptors. However, due to this clonal selection and expansion, the adaptive response is only fully functional three to five days after infection. The innate immune response, on the other hand, provides a first line of defence against pathogens and therefore is essential in containing the infection until the adaptive response is initiated. The innate immune system makes use of a distinct set of germ-line encoded pathogen-recognition receptors (PRRs) of which the family of Toll-Like receptors (TLRs) form the major constituent. TLRs recognize a wide spectrum of bacterial and viral structures including bacterial lipopolysaccharide, dsRNA or bacterial DNA. These structures are referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are essential for the survival and replication of these pathogens and therefore are conserved throughout evolution. Pathogen recognition by TLRs ultimately leads to the production of pro-inflammatory cytokines, essential for the activation of innate immune cells, like macrophages and neutrophils. Moreover, TLRs are involved in the activation and modulation of the subsequent adaptive response. However, aberrant activation of TLR signalling leads to excessive inflammation which is extremely harmful for the host. Therefore this signalling pathway is tightly controlled by numerous regulators, which interfere in TLR signalling either by inhibiting ligand binding, by downregulation of receptor expression or by interference of downstream signalling.

This work focuses on the initial steps of TLR signalling, mediated by four adaptor molecules: MyD88, Mal, Trif and Tram. Selective usage of these adaptors by different TLRs in part accounts for ligand specific responses. Using MAPPIT (Mammalian Protein-Protein Interaction Trap), a mammalian two-hybrid method developed in our laboratory, we generated a TLR-adaptor interaction map. Moreover, we provided evidence that Mal acts as a bridging adaptor, linking MyD88 to TLR2 or TLR4. In addition, we identified CIS, a member of the SOCS protein family, as a possible regulator of MyD88-dependent signalling, and showed that the binding properties of CIS differ between cytokine receptor and MyD88 binding.

Résumé

Les vertébrés ont développé un système de défense ingénieux, par lequel ils combattent les menaces continues des pathogènes envahissants. Ce système immunitaire mammifère peut être divisé en une branche innée et adaptative. L'immunité adaptative est basée sur l'expansion clonale des lymphocytes T et B qui expriment des récepteurs d'antigène spécifique. Mais en raison de cette sélection et expansion clonale, la réponse adaptative n'est fonctionnelle que trois à cinq jours après l'infection. D'autre part, l'immunité innée fournit une première ligne de défense contre les microbes pathogènes et est donc essentielle pour contrecarrer l'infection jusqu'à ce que la réponse adaptative soit induite. Le système immunitaire inné se sert d'un ensemble de récepteurs, appelés les Pattern-Recognition Receptors (PRRs) dont la famille de récepteurs Toll-Like (TLRs) forment le composant principal. Les TLRs peuvent identifier une gamme étendue de structures bactériennes et virales comme le lipopolysaccharide bactérien, l'ARN double brin ou l'ADN bactérien. Ces structures sont appelées Pathogen-Associated Molecular Patterns (PAMPs) et elles sont cruciales pour la survie et la réplication de ces microbes pathogènes, et pour cela leurs structures ont été fort conservées à travers l'évolution. Finalement, l'identification de microbes pathogènes par les TLRs mène à la production de cytokines pro-inflammatoires qui sont essentielles dans l'activation des cellules immunitaires innées, comme les macrophages et les neutrophiles.   De plus, les TLRs sont aussi impliqués dans l'activation et la modulation de la réponse adaptative qui en suit. Cependant, une activation aberrante de la signalisation émanant des TLR mène à une inflammation excessive qui peut être extrêmement nocive pour l'hôte. Par conséquent, cette voie de signalisation est strictement contrôlé par de nombreux régulateurs interférant avec la cascade de signalisation des TLRs en empêchant l'interaction du ligand, en diminuant l'expression des récepteurs ou en interférant avec la signalisation en aval.

Ce projet se focalise sur les premières étapes de la cascade de signalisation des TLRs, menés par quatre molécules adaptatrices: MyD88, Mal, Trif et Tram. L'emploie sélective de ces molécules adaptatrices par les différents TLRs contribue entre autre aux réponses spécifiques induites par les différents ligands. En utilisant MAPPIT (Mammalian Protein-Protein Interaction Trap), une méthode deux-hybride dans des cellules mammifères qui a été développée dans notre laboratoire, nous avons généré une carte d'interactions des TLRs et leurs molécules adaptatrices. Par ailleurs, nous avons fourni l'évidence que Mal agit en tant que molécule adaptatrice reliant MyD88 à TLR2 ou à TLR4. Finalement, nous avons identifié CIS, un membre de la famille de protéine des SOCS, comme régulateur potentiel de la cascade de signalisation dépendant de MyD88, et nous avons démontré que les caractéristiques des interactions de CIS diffèrent pour l'association avec les récepteurs des cytokines d'une part et l'interaction avec MyD88 d'une autre part.

PART 1: Pathogen recognition receptors in innate immunity

CHAPTER 1: General introduction

I.   Introduction

Since invading pathogenic micro-organisms are a continuous threat, vertebrates developed an ingenious defence system. This vertebrate immune system commonly is divided in an innate and an adaptive branch (Table 1). The adaptive or acquired immune system is characterised by specificity and memory and is mediated by T and B lymphocytes. Each single lymphocyte expresses a structurally unique antigen receptor, resulting in a large and diverse population. This diversity is achieved by a process of gene rearrangement and should enable recognition of almost any antigen. Binding of a specific antigen to an individual lymphocyte, results in the activation and proliferation of the cell. This clonal selection and expansion is fundamental for a successful immune response. However, it is a time-consuming process, which generally takes three to five days, giving pathogens enough time to overwhelm the host.

The innate immune response, on the other hand, provides an immediate response against pathogens and is essential in restraining the infection in the first couple of days. Next to containing infection, innate immunity activates and defines the nature of the subsequent adaptive responses by promoting antigen presentation and the production of co-stimulatory molecules and pro-inflammatory cytokines. Innate-induced pathogen clearance relies on efficient detection mediated by pattern-recognition receptors (PRRs) (Janeway, Jr., 1989). These restricted sets of germ-line encoded receptors recognize highly conserved microbial structures like bacterial lipopolysaccharide (LPS), dsRNA or bacterial DNA, commonly referred to as Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs are essential for the replication and viability of a broad range of pathogens. Since these PAMPs are restricted to micro-organisms and invariant throughout evolution, detection of these structures by PRRs provides an elegant way of discriminating between self and non-self antigens and circumvents the need for a highly variant set of antigen-specific receptors. PRRs can be secreted in the bloodstream or expressed intracellularly or on the cell surface of innate immune cells like macrophages, neutrophils and dendritic cells. Their main functions include opsonisation, activation of the

complement system, mediating phagocytosis and induction of pro-inflammatory signalling pathways.

	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns	Details of molecular structure
Self vs non-self	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Type I IFN, IL-6, IL-12	Clonal expansion or anergy IL-2, IL-4, IFN-γ

Table 1: Characteristics of innate and adaptive immunity (adapted from Janeway, Jr. and Medzhitov, 2002)

II.   *Secreted and endocytic PRRs*

Some secreted PRRs, like Mannan-binding lectin (MBL), C-reactive protein (CRP), and the serum amyloid protein (SAP) are produced by the liver during the acute phase response following infection. MBL is a member of the collectin family, which is characterised by a collagenous domain linked to a lectin-domain. MBL recognizes carbohydrates of gram-positive and gram-negative bacteria as well as viral envelope proteins via its lectin domain (Fraser et al., 1998). MBL activates the

complement system by interaction with two serine proteases, MBL-associated serine proteases (MASP) 1 and 2, resulting in the activation of the lectin pathway of complement (Fig.1) (Thielens et al., 2002; Rossi et al., 2001). CRP and SAP are members of the pentraxin family and act as opsonins by interacting with phosphorylcholine and LPS (Garlanda et al., 2005). Moreover, these pentraxins can interact with C1q, leading to the activation of the classical complement pathway, normally activated by immune complexes (antibody-antigen complexes) (Fig.1) (Agrawal et al., 2001). Activation of these complement pathways leads to direct killing of pathogens, recruitment of inflammatory cells and phagocytosis of opsonised pathogens mediated by complement receptors expressed on myeloid cells (Gasque, 2004).

Next to these secreted PRRs, several cell-surface PRRs expressed on macrophages also mediate pathogen phagocytosis. One such endocytic PRR, macrophage mannose receptor, is a C-lectin family member and senses mannose, fucose and N-acetyl glucosamine residues in the cell-wall of various pathogens, thereby inducing opsonin-independent phagocytosis. The scavenger receptor type A (SR-A) family forms another group of endocytic PRRs. SR-A member Macrophage scavenger receptor (MSR) recognizes a variety of polyanionic ligands, like LPS, dsRNA and Lipoteichoic Acid (LTA). Analysis of MSR-deficient mice revealed a key function for MSR in host defence as these mice were highly susceptible to *Listeria monocytogenes*, *Staphylococcus aureus*, herpes simplex virus (HSV) and malaria infections (Suzuki et al., 1997; Thomas et al., 2000). Another family member, MARCO, recognizes LPS and also mediates phagocytosis (Elomaa et al., 1995).
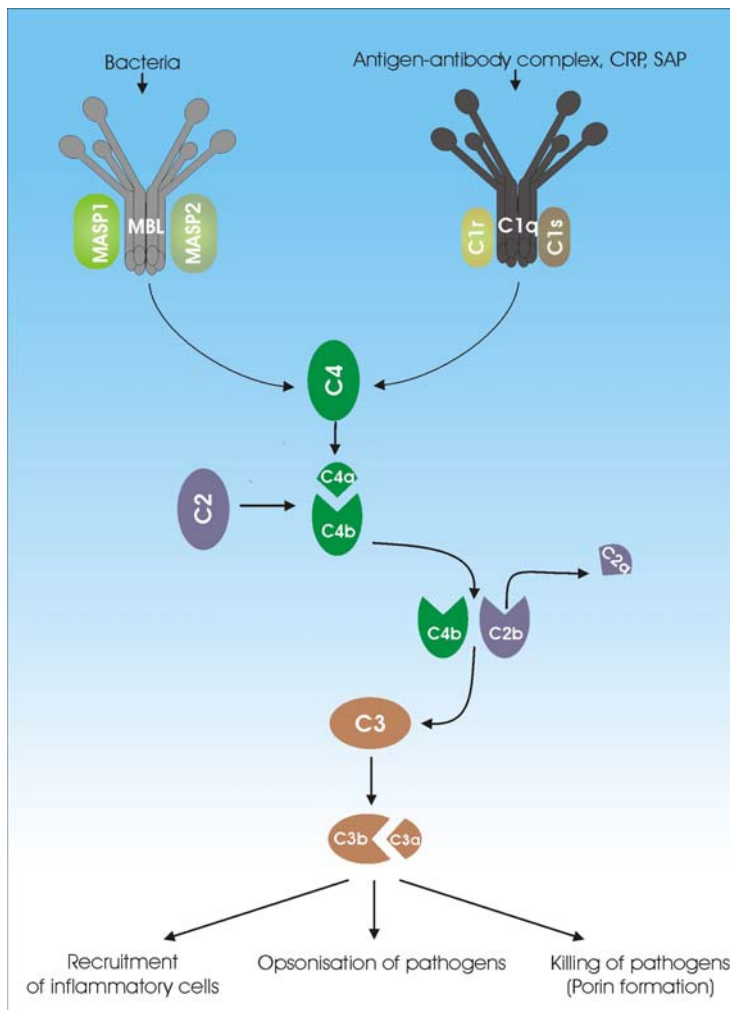
Fig.1: The lectin and classical complement pathway adapted from Medzhitov and Janeway, Jr., 2000. Mannose-binding lectin recognizes carbohydrates on the bacterial cell surface. Ligand recognition leads to the activation of two associated serine proteases, MASP1 and MASP2, which cleave complement component C4 and C2. Next, cleavage products C4b and C2a form a C3 convertase, further activating the complement system by cleavage of C3. The classical complement pathway relies on C1q binding with antigen-antibody complexes, CRP or SAP, resulting in the activation of serine proteases C1r and C1s, which in analogy with MASP1 and MASP2 cleave complement component C4 and C2. Complement activation ultimately leads to the opsonisation of pathogens, the direct killing of pathogens and the recruitment of inflammatory cells.

III. Signalling PRRs

For a long time it was known that some bacterial and viral structures, like bacterial LPS, peptidoglycan or viral double-stranded RNA, can activate the immune system. However, it took until the late nineties before their receptors could be identified. First, Lemaitre and Hoffman demonstrated that the Drosophila melanogaster protein Toll, known for its role in embryonic dorso-ventral patterning, is also an essential component of the immune response against the fungus *Aspergillus fumigatus*. The following year, the first human analogue of *Drosophila* Toll (human Toll), now known as Toll-Like receptor 4 (TLR4), was identified (Medzhitov et al., 1997). Finally, the observation that a single P→H mutation in the TLR4 gene rendered the mouse strain

C3H/HeJ unresponsive to LPS triggering, highlighted the major role of this TLR in PAMP-based vertebrate innate immunity (Poltorak et al., 1998). At present 10 human and 13 murine TLRs are identified, detecting a wide spectrum of bacterial and viral PAMPs at the cell-surface or within phagocytic endosomes.

Next to TLRs, an emerging group of other signalling PRR families were discovered, for example NOD-Like and RIG-I-Like receptors, that broadcast the presence of pathogens intracellularly, or dectin-1, a transmembranary receptor important in fungal defence (Fig.2). The importance and function of all these signalling PRRs in innate immunity, as well as the synergy between different PRR families, will be discussed throughout this thesis.

PRR signalling ultimately leads to the activation of transcription factors like NF-κB, AP-1 and interferon regulatory factors (IRFs) and concomitant gene induction of pro-inflammatory cytokines, chemokines and co-stimulatory molecules (Hirotani et al., 2005). As such, PRRs are crucial components of the innate immune system but are also involved in the activation and shaping of subsequent adaptive responses, which will be discussed in the following section.

Triggering of RIG-Like receptors and a subset of TLRs leads to the production of type I interferons (IFNs), thereby inducing an antiviral state. Upon viral infection most cells produce type I IFNs but recently a subset of dendritic cells (DCs), plasmacytoid DCs, was shown to produce enormous amounts of these cytokines upon PRR stimulation (Cao and Liu, 2007). Therefore these 'professional' type I IFN-producing cells are crucial mediators of antiviral innate immune responses. IFN stimulation of target cells leads to the induction of a set of antiviral genes of which dsRNA-dependent protein kinase PKR, 2'5' oligoadenylate synthetase and MX proteins are the best-characterised examples (Levy and Garcia-Sastre, 2001). These proteins interfere in viral replication and induce apoptosis of the infected cells. Moreover, type I IFNs activate specific subsets of innate immune cells, like DCs (see below) and natural killer (NK) cells (Gidlund et al., 1978). These NK cells express receptors for major histocompatibility complex (MHC) class I, and are programmed to lyse cells lacking this MHC type I. Normal host cells are MHC class I positive, but its expression is often downregulated by viral infection. That way, a CD8+ cytotoxic T cell response is inhibited (see below), but makes the cell vulnerable to NK cell-mediated lysis, a process often referred to as missing self recognition. In addition, type I IFNs enhance the expression of MHC type I, thereby promoting the afore

mentioned adaptive CD8+ cytotoxic T cell response (Ehrlich et al., 1989). Next to their crucial role in innate immunity, these type I IFNs regulate adaptive T-cell responses, for instance biasing naive Th cells towards a Th1 response (see below) (Smith et al., 2005; Belardelli and Ferrantini, 2002).
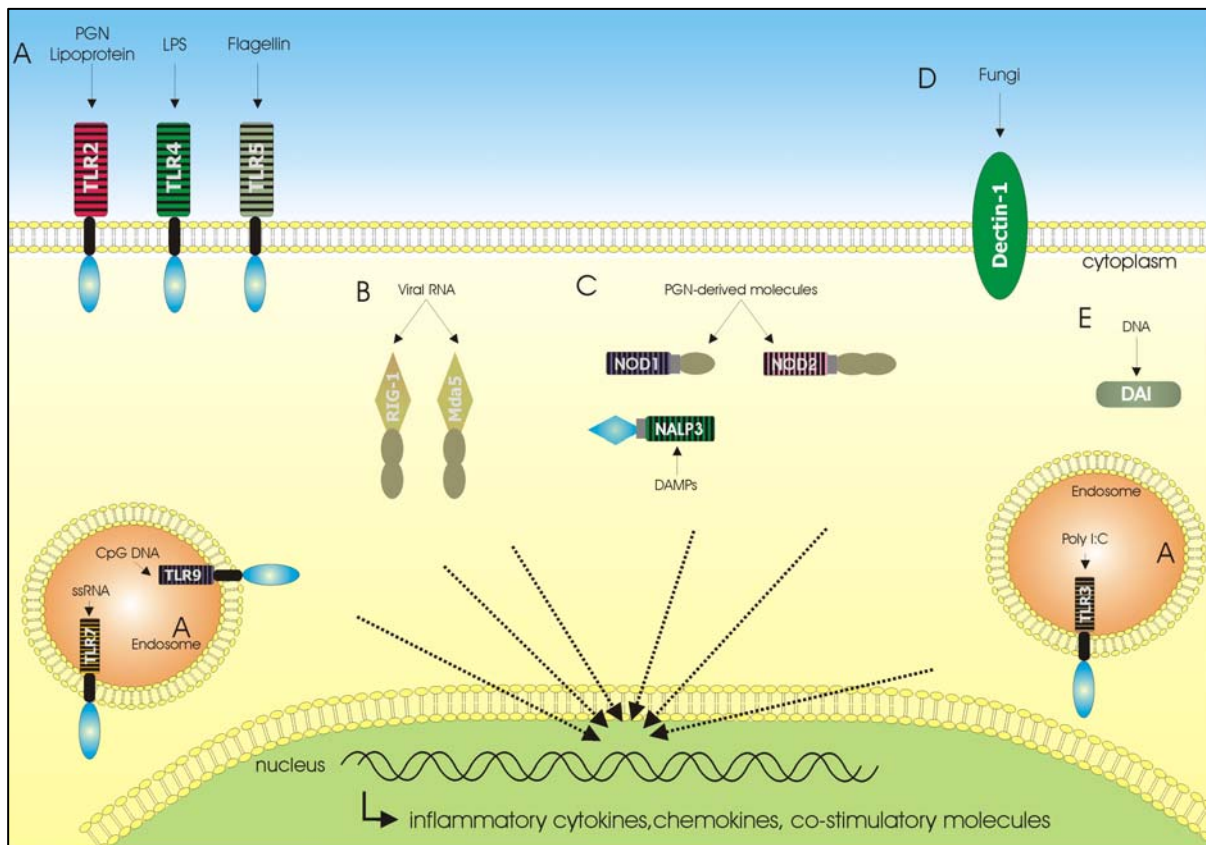
Fig.2: Pattern Recognition Receptors in innate immunity; different receptors for different needs. Innate immunity relies on the detection of evolutionary conserved pathogen structures (Pathogen-Associated Molecular Patterns or PAMPs) by so called Pathogen-Recognition Receptors (PRRs). Over the past years, a growing number of PRRs have been described: (A) Toll-Like receptors (TLRs) recognize diverse viral or bacterial products (LPS, flagellin, viral RNA, bacterial DNA) either in the extracellular lumen or in endosomal compartments. (B) RIG-I-Like receptors (RLRs), on the other hand, sense intracellular viral RNA, while (C) NOD-Like receptors (NLRs) respond to a multitude of PAMPs like peptidoglycan (PGN)–derived molecules and danger-associated molecular patterns or DAMPs. (D) Another transmembrane receptor, Dectin-1, ensures a potent immune reaction in response to fungal threat. (E) Finally, DNA-dependent activator of IFN-regulatory factors (DAI) senses cytosolic DNA. Cellular responses induced by these receptors include the production of chemokines, co-stimulatory molecules and inflammatory cytokines, not only necessary for the activation of innate immune cells but also involved in the activation and shaping of the subsequent adaptive response.

PRR activation induces the production of a multitude of pro-inflammatory cytokines including interleukin-6 (IL-6), IL-12 and tumor necrosis factor (TNF)-α.

IL-6 is produced by both innate and adaptive immune cells like T-lymphocytes, macrophages and monocytes. It exerts pleiotropic effects on different target cells like induction of acute phase response genes in hepatocytes, neutrophils activation, stimulation of B-cell proliferation and inhibition of regulatory T-cells (Treg) (see below) (Pasare and Medzhitov, 2003; Kopf et al., 1995; Borish et al., 1989).

IL-12 is a heterodimeric cytokine generally produced by macrophages and DCs. It predominantly functions as IFN-γ-inducing factor on NK cells and T-cells. IFN-γ is a potent activator of macrophages, NK cells and DCs and biases naive T-cells towards a Th1 response. IL-12 also induces the differentiation of naive T-cells into Th1 cells and it potentiates the cytotoxic activity of cytotoxic T lymphocytes (CTLs) and NK cells (Trinchieri, 2003).

One of the best-characterised cytokines induced by PRRs is TNF-α. It is produced by DCs, NK cells, macrophages and endothelial cells among others and is a key cytokine in inflammation. TNF-α was originally identified as a cytotoxic agent for certain tumor cells but over the years a broader role in immunity became apparent (Locksley et al., 2001; Aggarwal, 2003; Pfeffer, 2003). It induces pleiotropic effects including neutrophils and macrophage activation, promotion of vascular endothelial permeability, chemokines production and activation of the acute phase response. The functions of TNF-α are not restricted to modulation of immune responses as it can induce insulin resistance, fever and a hypermetabolic state.

One of the major innate functions of PRR-induced chemokines, like RANTES, IP-10 and MIP-1β, is attracting innate immune cells, like neutrophils, macrophages and monocytes to the site of infection, ensuring a potent innate response. However, chemokine function is not restricted to this chemotactic activity since chemokines and their receptors are also involved in multiple other immune processes like DC and T-cell maturation and regulation of DCs, B-cell and T-cell migration (see below) (Rot and von Andrian, 2004).

IV. Innate control of adaptive immunity through modulation of dendritic cell function

Recent data reveal an essential link between innate and adaptive immune responses. PAMP recognition by PRRs is proven to be crucial for the initiation and orientation of adaptive immune responses. A key role in this process is played by DCs. Activated peripheral DCs migrate to the lymph nodes, where they present antigens on MHC molecules to the relevant T lymphocytes, thereby activating the latter cells in an antigen-specific manner. Depending on the type of MHC-antigen complex presented and on the subset of secreted cytokines and chemokines, DCs activate and induce differentiation of CD4+ Th1 lymphocytes (MHCII, IFN-γ/IL-12/IFN-α/IFN-β), CD4+ Th2 lymphocytes (MHC class II, IL-4/IL-5) and CD8+ Cytotoxic T lymphocytes (MHC class I). This process requires phagocytosis of pathogens, upregulation of MHC molecules for efficient antigen presentation, induction of co-stimulatory molecules and cytokines and migration to lymphoid tissues. All of these processes were shown to be mediated by innate PRRs expressed on DCs. Of all the PRRs expressed by DCs, including scavenger receptors, mannose receptors, NOD-Like receptors, RIG-I-Like receptors and TLRs, only the latter have been shown to be involved in this regulation, although other PRRs are expected to exhibit similar functions.

The initial phagocytosis and subsequent antigen processing and presentation by DCs were shown to be TLR-dependent. Blander and Medzithov found that TLR activation by bacteria, but not by apoptotic cells, regulated phagocytosis at multiple steps including internalization and phagosome maturation (Blander and Medzhitov, 2004). These authors also showed that the efficiency of presenting antigens from phagocytosed cargo is dependent on the presence and detection of TLR ligands within the cargo (Blander and Medzhitov, 2006).

Next, DCs migrate to the lymph nodes, a process initiated by a switch of chemokine receptor expression. LPS-stimulation of immature DCs leads to the downregulation of chemokine receptors which respond to ligands found in inflammatory sites including CCR1, CCR5 and CCR6 whereas expression of CCR7, which responds to chemokines in lymphoic organs and thus induces DC migration, is upregulated (Sallusto et al., 1998; Dieu et al., 1998; Forster et al., 1999).

Once in the lymph nodes, DCs provide several signals to activate naive T-cells. First, the specific T-cell receptor interacts with the peptide-MHC complex expressed on DCs. The second signal is based on TLR-induced expression of co-stimulatory molecules like B7-1 (CD80) and B7-2 (CD86) on the DCs, which trigger CD28 on the T-cell. The third signal involves the abrogation of suppressive effects of Tregs. Additionally to the expression of co-stimulatory molecules, this subset of T lymphocytes maintains tolerance to self-antigens. However, DCs activated by pathogen recognition produce high amounts of IL-6, which acts on the antigen specific T cells thereby inhibiting the inhibitory effects of Tregs (Pasare and Medzhitov, 2003; Yang et al., 2004).

Next, activated CD4+ T cells differentiate into Th1 or Th2 effector cells. This choice is in part based on cytokines expressed by DCs. TLR-activated DCs seem to bias naive T-cells towards a Th1 response, induced by the secretion of Th1 cytokines like IL-12 and IL-6 (Schnare et al., 2001). Accordingly, mice deficient in MyD88, a central adaptor in TLR signalling, exhibit severely impaired Th1 responses and instead support Th2 differentiation (Schnare et al., 2001; Kaisho et al., 2002). This Th1 response is consistent with the viral, fungal, prokaryotic or protozoan nature of the PAMPs recognized by TLRs. Th2 responses, on the other hand, are mounted upon infection with multicellular eukaryotes, which are recognized by an unknown sensor.

Antigen presentation by dendritic cells can only lead to efficient adaptive responses when the antigen-specific T-cells are quickly transported from the blood stream into the lymph nodes. This lymphocyte entry is mediated by a structure called high endothelial venule (HEV). Maturation of the lymph nodes following infection is accompanied with the growth of these HEVs, which results in higher lymph node entry. This process is in part dependent on activated DCs as lymph node maturation was observed upon DC injection (MartIn-Fontecha et al., 2003). Moreover, depletion of these cells was shown to result in diminished numbers of lymphocytes in lymphoid tissues (Zammit et al., 2005). This lymph node maturation is induced by vascular endothelial growth factor (VEGF), produced upon DC-dependent recruitment of unknown blood-borne cells (Webster et al., 2006).

Together, these findings reveal a tight link between innate and adaptive responses, mediated by PRRs expressed on dendritic cells. Therefore, PRR signalling components are valuable therapeutic targets and TLR agonist as well as

TLR antagonists are already applied in antitumor and antiviral therapy, in treatment of allergic and autoimmune diseases and as vaccine adjuvants (see Chapter 4).

V. References

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CHAPTER 2: Toll-Like Receptors

II.   Structure

All thirteen mammalian Toll-Like receptors (TLR1-13) are type I transmembrane glycoproteins, comprised of extracellular Leucine-Rich Repeat (LRR) motifs, essential in ligand binding, a single α–helical transmembrane domain and a cytosolic Toll-IL-1R ('TIR') interaction domain, mediating receptor dimerisation and downstream signalling. Toll-Like receptors recognize extremely diverse PAMPs (see below; Table 1), varying from dsRNA (TLR3), to lipopeptides (TLR2), flagellin (TLR5), and even small synthetic molecules (TLR7/8). Therefore, the TLR ectodomain, composed of 19-25 LRRs, is designed for highly variable molecule recognition. The LRR motif consists of 24 amino acids (aa), characterised by a $xL_2xxL_5xL_7xxN_{10}x\varphi_{12}xx\varphi_{15}xxxxF_{20}xxL_{23}x$ consensus motif (φ represents any hydrophobic aa, and x any aa) (Bell et al., 2003). Each LRR is composed of a short parallel β-sheet, an α-helix and a variable region. Initially, structural knowledge was based on the extracellular domain of TLR3, since it was the only resolved TLR-ectodomain crystal structure (Choe et al., 2005; Bell et al., 2005). The extracellular domain of TLR3 forms a highly glycosylated horseshoe-shaped solenoid composed of 23 LRRs (Fig. 2A-B). The β-sheets of the LRRs form the inner or concave surface of the solenoid and the conserved hydrophobic residues form the core. These hydrophobic residues are N- and C-terminally shielded by cysteine-rich capping domains. LRR12, -14, -18 and -20 all exhibit insertions after residue 10 of the consensus motif, which could account for specific ligand recognition. Based upon the two independently solved crystal structures, models were setup identifying the dsRNA binding site of TLR3. Additional data should give us more insight in the exact nature of ligand binding. Considering the highly glycosylated nature and overall negative charge of the concave surface, Chloe et al. proposed that binding of dsRNA occurs at the non-glycosylated convex side of TLR3. Basic residues herein facilitate binding. Based on the position of bound sulphate ions from the crystallisation

medium, which could mimic phosphates from the RNA nucleotide backbone, Bell and colleagues proposed three possible binding sites. Two binding sites map to the glycosylated concave surface, whereas the third is located in a shallow non-glycosylated groove between the two insertions on LRRs 12 and 20 (Fig.2C). The latter binding site was further confirmed by mutational analysis (Bell et al., 2006). Additionally, two positively charged, non-glycosylated patches were described, greatly adding to ligand binding. Moreover, a recent mutagenesis study revealed that only 3 of the 23 LRRs are dispensable for TLR3 function and that 3 C-terminal LRRs (LRR20-22) are essential for ligand-induced receptor dimerisation and intracellular signal transmission (Takada et al., 2007).
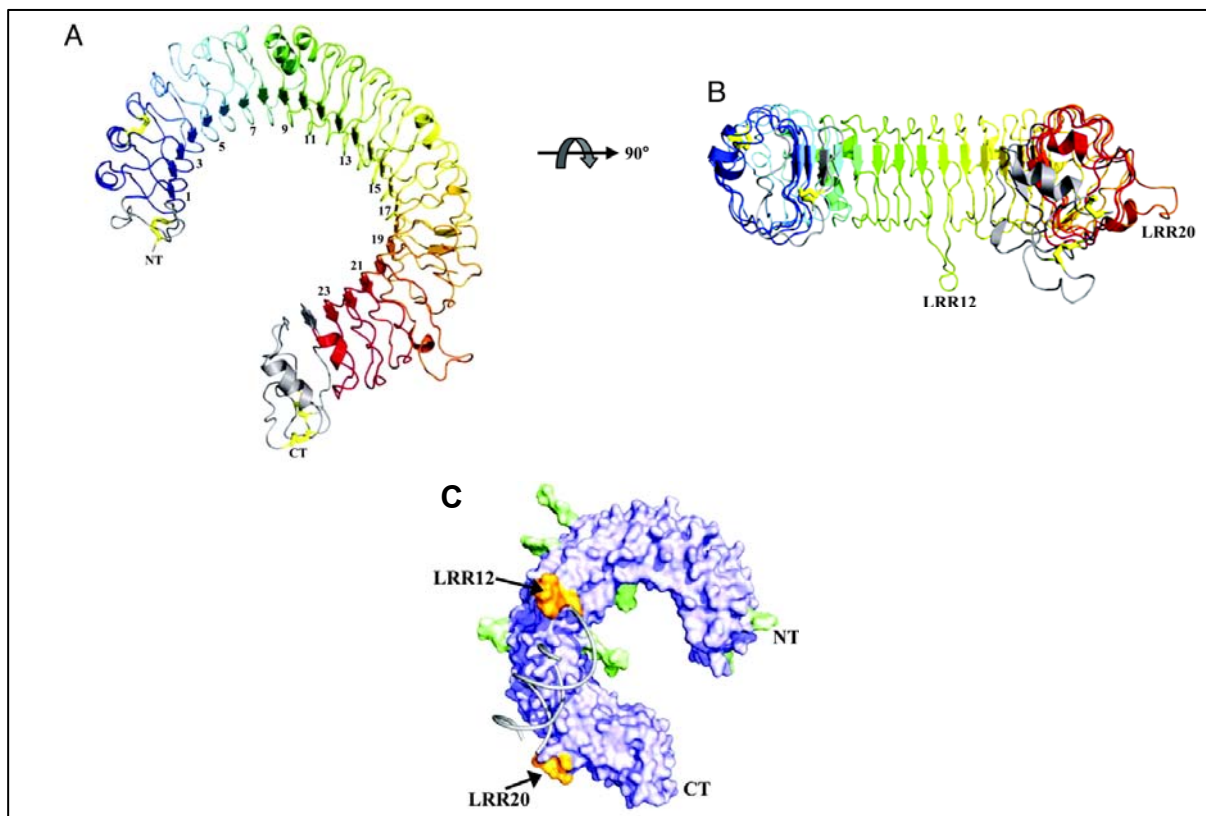
Fig.2 : TLR3 ectodomain (adapted from Bell et al., 2005). (A)The extracellular domain of TLR3 forms a horseshoe-shaped solenoid composed of 23 LRR, stabilized by N- and C-terminal capping structures. (B) The concave surface of the TLR3 ectodomain is composed of parallel β-sheets originating from each LRR. (C) The unglycosylated groove between LRR12 and LRR20 likely contributes to dsRNA binding.

Two recent studies by Lee and colleagues greatly contributed to unravel the mode of TLR ligand binding. Using hybrid proteins consisting of portions of TLR-LRR and LRRs from variable lymphocyte receptor (VLR) found in hagfish, the crystal structure of both the TLR4/MD-2 and the TLR1/TLR2 complex respectively bound to eritoran, an antagonistic LPS analogue, and Pam_3CSK_4, a synthetic lipopeptide TLR2 agonist, was resolved (Kim et al., 2007; Jin et al., 2007). Surprisingly, the nature of ligand binding completely differs between both complexes. In contrast with the abovementioned predictions of TLR3-ligand binding and with the structure of the TLR1/TLR2/Pam_3CSK_4 complex, TLR4 associates with MD-2-eirtoran at its concave surface. Another striking feature of this structure is that no direct interaction between eritoran and TLR4 was observed. Much in contrast, a lipid-binding pocket in the convex side of TLR2 ectodomain was shown to directly associate with two of the acyl chains of Pam_3CSK_4 whereas a similar binding pocket in TLR1 accommodates the third chain. This feature provides a structural basis for the requirement of TLR2-TLR1 heterodimer formation in the recognition of tri-acetylated lipopeptides. These studies suggest a bivalent model of ligand recognition by TLRs; Protein ligands are likely to bind the concave surface of the TLR ectodomain, whereas non-protein ligands could associate with the convex side. Additional studies should provide us clearer insight in the exact nature of ligand binding and specificity of TLRs.

Toll-Like receptors share their intracellular domain with the IL-1 receptor superfamily (IL-1R, IL-1RAPL, IL-18R and IL-33R). The TIR domain of TLRs and downstream adapters encompasses approximately 150 aa. Alignment of different TIR sequences revealed three highly conserved boxes of which box2 and box3 are involved in signalling (Fig.3A). TIR domain structures are available for TLR1, TLR2 and IL-1RAPL (Xu et al., 2000; Khan et al., 2004). The structures contain a central five-stranded parallel β-sheet, surrounded by five helices on both sides (Fig.3B). The BB loop, linking the second β-strand with the second α-helix, on the surface of the TIR domain, contains a proline critical for signalling and therefore is thought to be involved in homotypic TIR domain interactions.
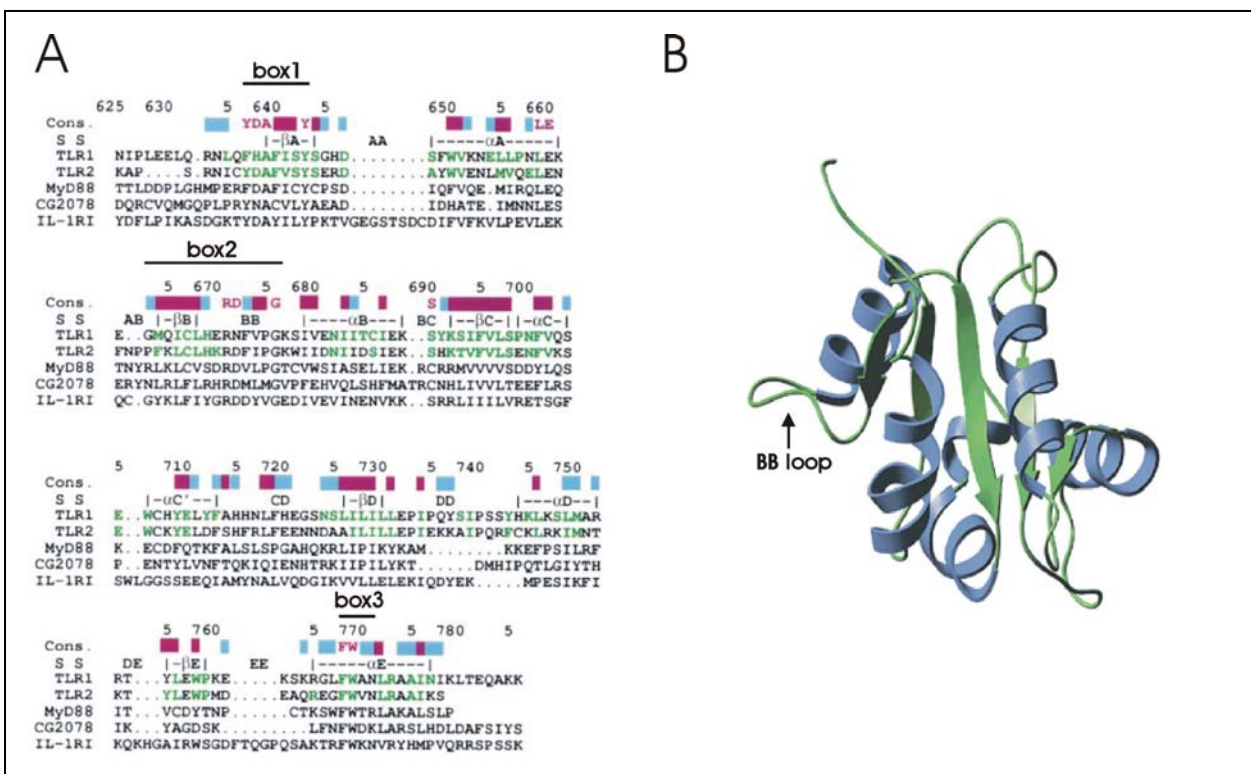
Fig.3: TIR domain sequence and structure (adapted from Xu et al., 2000). (A) TIR domain sequence alignment. Three regions, box1, box2 and box3 are conserved throughout all TIR domains. (B) TIR domain is comprised of 5 parallel β-sheets enclosed by 5 α–helices. The BB loop harbours a conserved proline or arginine residue, critical for signal transduction.

III. Expression pattern and cellular localisation of the different TLRs

Toll-Like receptors are expressed on a diverse spectrum of haematopoietic and non-haematopoietic cells. However, expression of the different TLRs is not equally distributed among these cell types: macrophages, neutrophils and dendritic cells express most of the TLRs, whereas only a subset of TLRs can be found on fibroblasts, epithelial and endothelial cells. Based on their subcellular expression pattern, TLRs can be split up in two groups: TLRs expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6), involved in recognition of bacterial and fungal structures and endosomal/lysosomal expressed TLRs (TLR3, TLR7, TLR8, TLR9), capable of sensing viral and bacterial nucleic acids. Endosomal targeting of TLR3 is achieved by a short linker sequence between its transmembrane and TIR domain (Nishiya et al., 2005). In contrast, intracellular localisation of TLR7 and TLR9 was only dependent on their transmembrane domains (Barton et al., 2006; Nishiya et al., 2005). The lysosomal and endosomal environment facilitates degradation of viruses and bacteria, resulting in the release of nucleic acids which are sensed by these intracellular TLRs. An additional reason for endosomal expression of some TLRs was provided through the analysis of a chimeric TLR9 protein. TLR9 recognizes unmethylated CpG DNA and suppression of this motif in vertebrate DNA is thought to be the discriminating factor between self and non-self DNA (Hemmi et al., 2000; Krieg et al., 1995). However, the TLR9N4C chimera, composed of the TLR9 ectodomain and the TLR4 transmembrane and intracellular domain, is still responsive to self DNA (Barton et al., 2006). Contrary to wild type TLR9, this TLR9C4N chimera is expressed at the cell surface. Thus, next to the nature of the recognized DNA, intracellular localisation of TLR9 is involved in the discrimination of self and non-self DNA.

IV. Ligand specificity of Toll-Like receptors

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Toll-Like receptors recognize a diverse pattern of viral, fungal and bacterial motifs, commonly designated as PAMPs. However, TLRs also sense chemical components and even endogenous or self ligands are detected. The following section summarizes the diversity of TLR ligand recognition.

TLR1/2/6

LPS and peptidoglycan (PG), both bacterial cell wall components, were first identified as TLR2 ligands (Yang et al., 1998; Kirschning et al., 1998; Yoshimura et al., 1999), but more recent studies showed that purified LPS and PG do not provoke a TLR2-dependent response (Hirschfeld et al., 2000; Travassos et al., 2004). The originally observed effects of LPS and PG on TLR2 are caused by lipopeptide and lipoteichoic acid (LTA) contamination of the samples (Takeda et al., 2002; Schwandner et al., 1999). In response to certain ligands, TLR2 forms heterodimers with either TLR1 or TLR6. Analysis of TLR1-deficient macrophages revealed a role for TLR1/TLR2 heterodimers in response to bacterial triacetylated lipoproteins and the synthetic triacetylated lipopeptide Pam_3CSK_4 (Takeuchi et al., 2002). Moreover, TLR1/TLR2 heterodimers are also involved in the recognition of the outer-surface lipoprotein of *Borrelia burgdorferi* and some soluble factors secreted by *Neisseria meningitis* (Alexopoulou et al., 2002; Wyllie et al., 2000). TLR2/TLR6 dimers on the other hand detect diacetylated lipopeptides since both $TLR2^{-/-}$ and $TLR6^{-/-}$ mouse embryonic fibroblasts (MEFs) failed to detect the diacetylated mycoplasmal macrophage-activating lipopeptide-2kDa (MALP-2) (Takeuchi et al., 2001; Takeuchi et al., 2002).

Additionally, TLR2 recognizes protozoal glycosylphosphatidylinositol (GPI) anchors (Bafica et al., 2006; Campos et al., 2001), fungal phospholipomannan and zymosan (Underhill et al., 1999b; Kataoka et al., 2002; Jouault et al., 2003), Atypical LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* (Hirschfeld et al., 2001; Werts et al., 2001), Modulin, a factor secreted by *Staphylococcus epidermidis* (Hajjar et al., 2001), Neisserial porins (Massari et al., 2002), hemagglutinin protein of wild-type measles virus (Bieback et al., 2002) and envelope proteins from Human Cytomegalovirus (HCMV) (Compton et al., 2003).

TLR	Cellular compartment	Exogenous Ligands	Ligand origin	Reference
TLR1*	Cell surface	Triacyl lipopeptides	Bacteria/Mycobacteria	(Takeuchi et al., 2002)
		Soluble factors	Neisseria meningitis	(Wyllie et al., 2000)
		Outer-surface lipoprotein OspA	Borrelia burgdorferi	(Alexopoulou et al., 2002)
TLR2	Cell surface	Peptidoglycan	Gram-positive bacteria	(Yoshimura et al., 1999; Schwandner et al., 1999)
		Lipotecichoic acid and related glycolipids	Gram-positive bacteria	(Schwandner et al., 1999; Opitz et al., 2001)
		Phospholipomannan	Fungi	(Jouault et al., 2003)
		Lipoarabinomannan	Mycobacteria	(Underhill et al., 1999a)
		Atypical LPS	Porphyromonas gingivalis	(Hirschfeld et al., 2001)
		Atypical LPS	Leptospira interrogans	(Werts et al., 2001)
		Phenol-soluble modulin	Staphylococcus epidermidis	(Hajjar et al., 2001)
		Lipopeptides	Bacteria	(Takeda et al., 2002)
		GPI anchor	Protozoa	(Campos et al., 2001; Bafica et al., 2006)
		Envelope protein	Virus	(Compton et al., 2003)
		Hemagglutinin	Virus	(Bieback et al., 2002)
		Porins	Bacteria	(Massari et al., 2002)
		Zymosan	Fungi	(Underhill et al., 1999b)
TLR3	Endosomal/ Cell surface**	dsRNA	Virus	(Alexopoulou et al., 2001; Wang et al., 2004)
TLR4	Cell surface	Lipopolysaccharide	Gram-negative bacteria	(Poltorak et al., 1998)
		HSP60	Chlamydia pneumoniae	(Bulut et al., 2002)
		Flavolipin	Flavobacterium meningosepticum	(Gomi et al., 2002)
		Mannan	Fungi	(Tada et al., 2002)
		Glycoinositolphospholipids	Protozoa	(Oliveira et al., 2004)
		Taxol	Plant	(Perera et al., 2001)
		Envelope protein	Virus	(Rassa et al., 2002)
		RSV fusion protein	Virus	(Kurt-Jones et al., 2000)
TLR5	Cell surface	Flagellin	Bacteria	(Hayashi et al., 2001)
TLR6*	Cell surface	Diacyl lipopeptides	Bacteria/Mycobacteria	(Takeuchi et al., 2001)
TLR7/ TLR8	Endosomal	Imidazoquinoline-like molecules	Synthetic molecule	(Hemmi et al., 2002)
		ssRNA	Virus	(Heil et al., 2004)
		Guanosine analogs	Synthetic molecule	(Lee et al., 2003)
TLR9	Endosomal	CpG DNA	Bacteria/Protozoa/Virus	(Hemmi et al., 2000)
		Hemozoin	Protozoa	(Coban et al., 2005)
TLR11	Cell surface	Components of uropathogenic bacteria	Bacteria	(Zhang et al., 2004)
		Profilin like molecule	Protozoa	(Yarovinsky et al., 2005)

Table 1: Recognition of pathogen-associated molecular patterns by mammalian TLRs.
*   Ligand recognition by TLR1 and TLR6 requires heteromeric dimers with TLR2.
**   TLR3 is mostly expressed in endosomal compartments, but can also be found on the cell surface of human fibroblasts (Matsumoto et al., 2003).

TLR3

Double-stranded RNA (dsRNA) is produced during the course of some viral infections and is the natural ligand of TLR3. Overexpression of TLR3 in Hek293 cells resulted in responsiveness to synthetic dsRNA (poly(I:C)) and TLR3-deficient mice were less susceptible to these ligands (Alexopoulou et al., 2001). Recent findings suggest that TLR3, in addition to dsRNA, also recognizes single strand polyinosinic acid (Marshall-Clarke et al., 2007).

Gene-targeting studies questioned the importance of TLR3 as a dsRNA sensor. Absence of TLR3 did neither alter the viral pathogenesis nor the production of type I IFNs following lymphocyticchoriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), reovirus, Sendai Virus (SeV) and Newcastle disease virus (NDV) infection in fibroblasts and conventional DCs (Edelmann et al., 2004; Honda et al., 2003; Lopez et al., 2004), although some data could not be confirmed by others (Hoebe et al., 2003). Moreover, several other viral RNA sensors, like the RIG-I-Like receptor family, have been described over the past years (see later), further questioning the precise role of TLR3 in viral defence.

TLR4

TLR4 was the first human homologue to Drosophila Toll to be described (Medzhitov et al., 1997). Identification of a P$\rightarrow$ H mutation in the TLR4 gene of the LPS-hyporesponsive mouse strain C3H/HeJ together with the observation that TLR4-deficient macrophages did not respond to LPS stimulation, established LPS as a natural ligand for TLR4 (Poltorak et al., 1998; Hoshino et al., 1999). LPS is a major glycolipid component of the outer cell wall of Gram-negative bacteria with strong immunostimulatory capacities, emphasized by its role in endotoxic shock. Susceptibility of the mouse strain C3H/HeJ to Salmonella typhimurium, Escherichia coli and Neisseria meningitis infection underscores the importance of TLR4 in vivo (Woods et al., 1988; Hagberg et al., 1985; O'Brien et al., 1982).

TLR4 requires co-receptors, like CD14, MD-2 and the LPS-binding protein (LBP), to provoke a potent LPS-response (Jerala, 2007). First, the serum protein LBP binds LPS, resulting in disruption of LPS aggregation. Next, LBP delivers monomeric

LPS to CD14, which in turn transfers LPS to MD-2. Finally, the MD-2:LPS complex associates with the ectodomain of TLR4, which triggers intracellular signal transduction.

Next to LPS, other bacterial components are recognized by TLR4 such as Heat shock protein 60 (HSP60) of *Chlamydia pneumoniae* (Bulut et al., 2002) and flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum* (Gomi et al., 2002).

TLR4 is also involved in fungal, protozoal and viral defence, as it respectively responds to mannans from *Saccharomyces cerevisiae* and *Candida albicans (Tada et al., 2002)*, glycoinositolphospholipids from *Trypanosoma cruzi* (Oliveira et al., 2004) and the fusion protein from Respiratory Syncytial Virus (RSV) (Tada et al., 2002; Oliveira et al., 2004; Kurt-Jones et al., 2000). In addition, it was shown that TLR4 interacts with Taxol, an antimitotic drug used in cancer therapy (Perera et al., 2001)

TLR5

TLR5 is mostly expressed on epithelial cells, monocytes and immature cells and is capable of sensing flagellin, an essential protein of the bacterial flagella (Hayashi et al., 2001). It seems that only monomeric flagellin is recognised, as the TLR5 recognition site is embedded in the intact filamentous flagellar structure (Smith et al., 2003). TLR5 is expressed exclusively on the basolateral surface of intestinal epithelia, where it detects flagellin of invading bacteria (Gewirtz et al., 2001). Knock-out studies revealed that the contribution of TLR5 to antibacterial host response is highly redundant with other TLRs (Feuillet et al., 2006). Moreover, TLR5-independent recognition of cytosolic flagellin by members of the NOD family is also observed (Franchi et al., 2006; Miao et al., 2006) and will be discussed later.

Surprisingly, TLR5$^{-/-}$ mice are more susceptible to infection of the urinary tract induced by Escherichia coli, a feature shared with the highly related TLR11 (see below). No functional TLR11 is expressed in humans, suggesting that human TLR5 is the predominant receptor for uropathogenic E.coli detection (Andersen-Nissen et al., 2007) .

TLR7/8

For a long time, the natural ligand for murine TLR7 and human TLR8 remained unidentified. However, it was known that small antiviral components, like imiquimod (R-837), resiquimod (R-848) and loxoribine can elicit a strong TLR7/8 response (Hemmi et al., 2002; Jurk et al., 2002). Together with the endosomal expression pattern analogous to TLR3, these observations suggested a role for TLR7/8 in antiviral defence. As these antiviral components are structurally related to nucleic acids, it came as no surprise that TLR7 and TLR8 recognize guanosine- and uridine-rich single stranded RNA from HIV-1 (Heil et al., 2004). Moreover, DCs and B-cells derived from TLR7$^{-/-}$ mice display a reduced response against ssRNA viruses like influenza and VSV (Lund et al., 2004; Diebold et al., 2004). Recently it was shown that nucleoside modification and the origin of RNA has a drastic effect on RNA recognition by TLR3/7/8 (Kariko et al., 2005). In this report, the authors show that modified nucleosides, like N6-methyldenosine, pseudouridine, 5-methylcytidine, 2-thiouridine and 5-methyluridine generally suppresses TLR-induced responses and that this suppression is proportionally to the number of modified nucleosides. This observation suggests that RNA modification rather than endosomal expression of TLRs is the mechanism to discriminate between host and pathogen RNA since much more nucleoside modifications are observed in mammalian RNA compared to viral and bacterial DNA. The observed immunogenic effects of host RNA could be attributed to the poorly-modified mitochondrial RNA. Of note, nucleoside modification (i.e. N6-methyldenosine) is observed in various viruses, like RSV, HSV and SV40, and this modification could allow viruses to evade TLR-mediated immune responses. These findings have implications on the therapeutic applications of synthetic RNA. Avoiding nucleotide modifications results in an increased immunogenicity and therefore could improve RNA-based vaccines or the application of RNA as adjuvant. Conversely, introducing RNA modifications may enhance RNAi efficiency.

TLR9

Unmethylated bacterial CpG DNA is recognized by TLR9, as was shown by the study of TLR9-deficient mice who failed to produce inflammatory cytokines in response to CpG challenge (Hemmi et al., 2000). In analogy with RNA recognition by TLR3/7/8, discrimination between self and non-self DNA is based on the nature of the recognized ligand and the endosomal expression of TLR9. CpG motifs in mammalian DNA are very rare and readily methylated and host DNA does not frequently enter the endosomal compartments (Barton et al., 2006). Another source of CpG motifs are viruses, and these sequences are also recognized by TLR9 (Krug et al., 2004; Lund et al., 2003). Although haemozoin, a hydrophilic heme polymer produced by malaria parasites, was reported as a non-DNA ligand of TLR9 (Coban et al., 2005), recent data revealed that DNA contamination of haemozoin could be the real activator (Parroche et al., 2007). In addition, TLR9-independent mechanisms have evolved to detect foreign DNA intracellularly, and these will be discussed later.

TLR11

In contrast to its human analogue which contains a stop codon, mouse TLR11 is fully functional. Recently, mouse TLR11 was shown to detect uropathogenic bacteria (Zhang et al., 2004) and profilin-like molecules from Toxoplasma gondii (Yarovinsky et al., 2005).

TLR responses to self ligands.

Next to pathogen-derived ligands, TLRs recognize some endogenous products, such as heat shock proteins (HSPs) and extracellular matrix proteins (Table 2). These self ligands are mostly released after tissue damage or inflammation and are called therefore danger-associated molecular patterns or DAMPs.

HSPs, chaperone proteins involved in protein folding and trafficking, form a substantial subset of self ligands. The mitochondrial HSPs, HSP60 and HSP70 respectively induce TNF-α/NO and IL-12 production in macrophages in a TLR2- (HSP60) or TLR2/TLR4(HSP70)-dependent manner (Ohashi et al., 2000; Vabulas et al., 2001; Vabulas et al., 2002b; Asea et al., 2002). Moreover, an endoplasmic reticulum HSP, gp96, was shown to induce pro-inflammatory cytokines production and upregulation of co-stimulatory molecules in bone-marrow derived DCs. This immunostimulatory effect was shown to be dependent on TLR2 and TLR4 signalling since DCs derived from TLR4 mutant and TLR2/TLR4 double mutant mice lost responsiveness to gp96 (Vabulas et al., 2002a). Of note, this gp96 recently was identified as the key chaperone involved in cell surface and intracellular expression of TLRs (Yang et al., 2007). Moreover, the small HSPs αA crystalline and HSPB8 were identified as a TLR4 ligands (Roelofs et al., 2006). Synovial tissue from patients with rheumatoid arthritis abundantly expresses the small nuclear HSPB8 or HSP22 protein which activates DCs through interaction with TLR4 (Roelofs et al., 2006). These data point to a possible role for HSPB8 in the inflammatory state of rheumatoid arthritis.

Cleavage of extracellular matrix (ECM) proteins is a common phenomenon observed in tissue injury. Some of these degradation products are detected by TLRs, leading to the activation of DCs and macrophages. These ligands include hyaluronan (TLR2/TLR4), heparan sulphate (TLR4), fibrinogen (TLR4), fibronectin (TLR4), biglycan (TLR2/TLR4) and surfactant protein-A (TLR4) (Termeer et al., 2002; Johnson et al., 2002; Smiley et al., 2001; Okamura et al., 2001; Schaefer et al., 2005; Guillot et al., 2002). These findings suggest a role for Toll-Like receptors as "tissue-state sensors".

Endogenous TLR Ligand	TLR	Reference
Heat Shock Proteins		
HSP60	TLR4	(Ohashi et al., 2000; Vabulas et al., 2001)
HSP70	TLR2/TLR4	(Asea et al., 2002)
gp96	TLR2/TLR4	(Vabulas et al., 2002a)
αA Crystallin	TLR4	(Roelofs et al., 2006)
HSPB8/HSP22	TLR4	(Roelofs et al., 2006)
Extracellular matrix proteins		
Hyaluronan	TLR4	(Termeer et al., 2002)
Heparan sulphate	TLR4	(Johnson et al., 2002)
Fibrinogen	TLR4	(Smiley et al., 2001)
Fibronectin extradomain A	TLR4	(Okamura et al., 2001)
Biglycan	TLR2/TLR4	(Schaefer et al., 2005)
Surfactant protein-A	TLR4	(Guillot et al., 2002)
Others		
β-defensin	TLR4	(Biragyn et al., 2002)
Minimally modified LDL	TLR4	(Miller et al., 2003)
HMGB1	TLR2/TLR4	(Park et al., 2004)
DNA containing immune complexes	TLR9	(Tian et al., 2007)

Table 2: Endogenous TLR ligands

Next to HSPs and ECM proteins, other endogenous TLR ligands were described. Beta-defensins, epithelial antibacterial peptides produced upon mucosal infection, activate DCs in a TLR4-dependent way, thereby inducing upregulation of co-stimulatory molecules and cell maturation (Biragyn et al., 2002). High-mobility group box 1 (HMGB1) protein, a chromatin-binding protein, was also identified as endogenous TLR2/TLR4 ligand (Park et al., 2004). HMGB1 is released in the extracellular medium either in a passive (released by necrotic cells) or an active manner (secreted by a variety of both immune and non-immune cells following inflammatory cytokine stimulation). Next to binding TLR2/TLR4, HMGB1 was also shown to interact with the receptor for advanced glycation end products (RAGE) (Hori et al., 1995). Recognition of HMGB1 by TLR2/TLR4 leads to the production of pro-

inflammatory cytokine and DCs maturation (Park et al., 2004; Dumitriu et al., 2005). This DC maturation following HMGB1 stimulation was recently shown to be crucial for the outcome of anticancer therapy (Apetoh et al., 2007). In this report it was shown that the release of the high mobility group box 1 protein (HMGB1) by dying tumor cells is essential for instructing DCs to process and present tumor antigens. This DCs maturation was dependent on TLR4, since breast cancer patients carrying a loss-of-function TLR4 polymorphism (see below) display a faster relapse after anti-tumor therapy than patients with the normal allele. It remains unclear whether HMGB1 directly induces its pro-inflammatory effects since studies using highly purified protein revealed that HMGB1 itself has little immunostimulatory properties (Rouhiainen et al., 2007). Interaction of HMGB1 with nucleic acids or LPS was shown to drastically improve the pro-inflammatory activity of HMGB1 and therefore it was proposed that these complexes account for the immunostimulatory effects (Rouhiainen et al., 2007). In line with these findings, Tian and colleagues describe a role for HMGB1 in mediating TLR9 activation in response to DNA-containing immune complexes (Tian et al., 2007).

In addition, minimally modified low density lipoprotein (mmLDL) was shown to induce actin polymerisation and macrophage spreading in a TLR2/TLR4-dependent manner (Miller et al., 2003). This endogenous TLR ligand is thought to be essential in the progression of atherosclerosis (see below).

Taken together, these findings provide conclusive evidence for a broader function of TLRs, not only detecting pathogen invasion but also controlling tissue state.

V.   TLR signalling.

1. Early steps in TLR signalling

Following ligand binding, Toll-Like receptor signalling is intiated by the recruitment of adaptor molecules through homotypic TIR-TIR domain interactions. At present four adaptor molecules are described: MyD88, Mal, Trif and Tram. Different TLRs can recruit different TLRs (Table 3) which, in part, explains TLR-specific responses. The following section overviews the structural and fuctionnal characteristics of these adaptor molecules.

TLR adaptor	Recruited by	References
MyD88	All TLRs (TLR1-TLR11) except TLR3	(Hacker et al., 2000; Hemmi et al., 2002; Kawai et al., 1999)
Mal/TIRAP	TLR2/TLR4	(Horng et al., 2002; Yamamoto et al., 2002a)
Trif/Ticam-1	TLR3/TLR4	(Yamamoto et al., 2003a)
Tram/Ticam-2	TLR4	(Yamamoto et al., 2003b)

Table 3: Adaptor usage by different mammalian Toll-Like receptors.

MAPPIT analysis of early Toll-Like receptor signalling events.

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Abstract

Toll-Like receptor (TLR) signalling is initiated by the recruitment of one or more adaptor proteins to the activated receptor complex. At present, four of these proteins are identified, namely MyD88, Mal, Trif and Tram and their selective usage by different TLRs in part accounts for TLR-specific transcriptional responses. Recent findings described unique biochemical properties for each of these TIR-domain containing adaptors and revealed that these adapters are subjected to post-translational modification. We used MAPPIT (Mammalian Protein-Protein interaction Trap), a two-hybrid technique that functions in a mammalian cell context, to study the molecular interactions downstream of TLR activation. We demonstrate pathway walking from TLR4 to IRAK-1 and identified Mal as a bridging adaptor, linking MyD88 to the activated TLR4.

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Introduction

The innate immune system provides first-line protection against the continuous threat of invading pathogens. The Toll-Like receptor (TLR) family, a group of germ-line encoded, type I transmembrane proteins, plays a key role in this immediate defence mechanism. TLRs sense a diverse pattern of pathogen structures like LPS (TLR4), dsRNA (TLR3), flagellin (TLR5) and bacterial lipoproteins (TLR2). TLR triggering leads to the production of pro-inflammatory cytokines including TNF-α, IL-12 and type I interferons (IFNs) and primes the adaptive immune system. The signalling cascade originating from these TLRs is based on an intracellular Toll/ Interleukin-1 Receptor (TIR) domain. TLRs use a limited set of four TIR-domain containing adaptors to convert an extracellular signal into an intracellular response, which in part accounts for ligand-specific responses. These four adaptors are myeloid differentiation primary response gene 88 (MyD88), MyD88-adaptor-like (Mal, also referred to as TIR-domain–containing adapter protein (TIRAP)), TIR-domain-containing adaptor inducing interferon (Trif, also known as TIR-containing adaptor molecule (Ticam-1)) and Trif-related adapter molecule (Tram, or also called TIR-containing adapter molecule-2 (Ticam-2) or TIR-domain containing adaptor (Tirp)). We here review the characteristics and functions of these adaptor proteins in TLR-proximal signalling events. Moreover, applications of the mammalian protein-protein interaction trap (MAPPIT) two-hybrid system for studying these signalling cascades will be discussed.

MyD88

MyD88 was the first TLR adaptor to be described. It was identified originally as a gene activated in M1D+ myeloid precursors following IL-6-induced terminal differentiation and growth arrest [1]. Only several years later, its central function in IL-1R[2-5] and TLR[6] signalling became apparent. Consequently, MyD88-deficient mice exhibit impaired responses to IL-1 and agonists of all TLRs except TLR3 [7-12].

MyD88 is composed of a C-terminal TIR domain and an N-terminal Death Domain (DD) linked together by a short intermediate domain (Fig.1). Signalling is initiated by interaction of MyD88 and the intracellular domain of an activated TLR through TIR-TIR association and this event results in the recruitment of the downstream serine/threonine IL-1 receptor-associated kinase-4 (IRAK-4) to MyD88 via a homotypic Death Domain association [13;14]. The intermediate domain is also crucial since an alternative splice variant of MyD88 lacking this region, MyD88$_s$, failed to recruit IRAK-4 and therefore acts as a negative regulator of TLR signalling [15]. Next, IRAK-4 interacts with IRAK-1, resulting in the phosphorylation and activation of the latter. Activated IRAK-1 autophosphorylates itself, leading to its dissociation of the MyD88/IRAK-4 complex and the association with TNF receptor-associated factor-6 (TRAF-6). Activation of TRAF6 ultimately leads to the activation of NF-κB and MAPKs through a series of phosphorylation and ubiquitination steps (Fig.2, reviewed in [16]).

In addition to IRAK recruitment, MyD88 was also shown to associate with members of the interferon regulatory factor (IRF) family. Two-hybrid screenings identified IRF7 as a MyD88 interacting protein, and this interaction is essential in TLR9-induced type I IFN production [17;18]. Moreover, MyD88 interacts with IRF5 and IRF1, leading to the activation of pro-inflammatory cytokines [19;20]. The specific function and regulation of IRFs in TLR signalling is reviewed elsewhere [21].

Mal

LPS stimulation of MyD88$^{-/-}$ cells still results in a significantly delayed NF-κB activation. Furthermore, IRF3 activation and concomitant IFN-inducible gene induction is unaffected [8;22]. These findings suggested the existence of a "MyD88-independent" pathway. Therefore the human genome was screened for TIR containing molecules that could fill this gap in the TLR4 signalling cascade. The first adaptor to be identified was Mal [23;24]. Overexpression studies using a dominant negative variant of Mal revealed a function for Mal in TLR2 and TLR4, but not in TLR9 or IL-1R signalling [23;24]. Analysis of Mal$^{-/-}$ mice confirmed this restricted role

for Mal, as early-phase NF-κB activation, and concomitant TNF-α, IL-6 and IL12p40 production was severely impaired following TLR2 and TLR4 stimulation [25;26]. However, LPS-induced late-phase NF-κB induction and IRF3 phosphorylation remained intact. Thus, Mal is not the adaptor involved in MyD88-independent signalling but acts as a bridging adaptor linking MyD88 to the activated TLR2 or TLR4.

In line with these observations, Mal was shown to possess a phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain in its N-terminus [27]. This domain mediates Mal recruitment to PIP2-rich areas in the plasma membrane and was shown to be essential in facilitating MyD88 delivery to an activated TLR4.
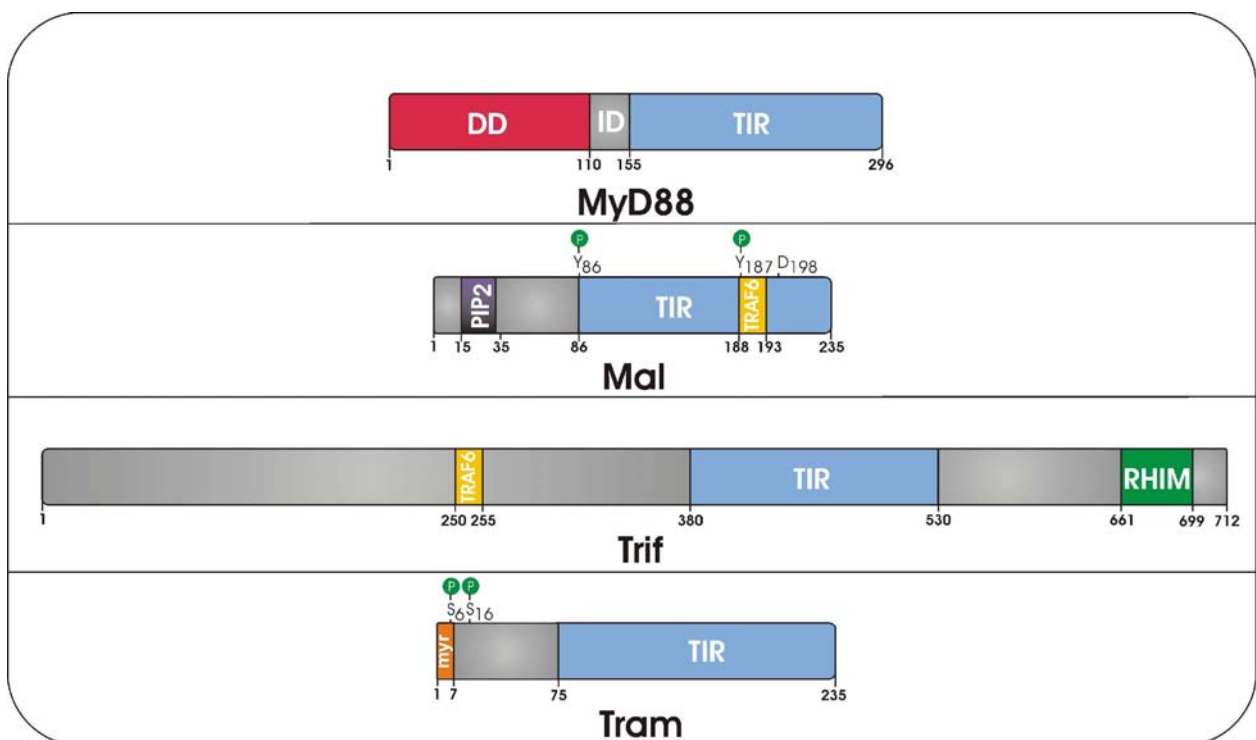
Figure 1: **Overview of interaction domains of TIR-containing adaptor molecules**. All TLR adaptors contain a Toll-IL-1R (TIR) interaction domain. In addition, MyD88 consists of a small intermediate domain and an N-terminal Death Domain (DD), by which it recruits downstream kinases. Mal is phosphorylated by Bruton's tyrosine kinase on tyrosine 86 and 187. An N-terminal PIP2-binding motif ensures recruitment of Mal to the plasma membrane. A TRAF6-binding motif in its TIR domain accounts for Mal-induced NF-κB activation. Mal is cleaved by caspase-1 at position 198. The N-terminus of Trif contains several TRAF6-binding regions, of which motif 248-255 is thought to be essential in TRAF6 recruitment. Moreover, Trif contains a C-terminal RIP homotypic interaction motif (RHIM). The N-terminus of Tram is myristoylated, which in analogy with Mal, directs Tram to the cell membrane and PKCε phosphorylates serine residues 6 and 16.

In addition, other biochemical properties of Mal were described over the past years. Upon TLR2 and TLR4 stimulation, Mal is phosphorylated by Bruton's tyrosine kinase (Btk). Phosphorylation of $Y_{86,}$ and Y_{187} was shown to be crucial for LPS-induced NF-κB activation[28]. In addition, Btk phosphorylates the p65 subunit of NF-κB, which results in transactivation of gene expression [29]. Phosphorylated Mal is a target for proteasomal degradation, mediated by SOCS-1. Thus, Mal phosphorylation is an essential step in TLR2/TLR4 signalling, not only crucial for activation of the NF-κB pathway, but also preventing a prolonged and possible harmful TLR signal.

Mal also comprises a TRAF6-binding motif in its TIR domain and Mal was shown to coimmunoprecipitate with TRAF6 [30]. Abrogation of this TRAF6-recruitment site leads to the loss of NF-κB activation upon TLR2- or TLR4-stimulation, suggesting that Mal links TRAF6 to TLR2 and TLR4. However, this putative regulatory role for Mal in NF-κB induction contrasts with the observation that Mal overexpression in MyD88$^{-/-}$ did not lead to NF-κB activation, whereas overexpression of MyD88 in Mal$^{-/-}$ cells did result in induction of NF-κB [31].

Interaction of PKCδ and the TIR domain of Mal was also demonstrated [32]. siRNA-mediated depletion op PKCδ in RAW264.7 cells abolished TLR2- and TLR4-mediated phosphorylation of p38 MAPK, IKK, and IκB, implying a essential role for PKCδ in Mal signalling.

Additionally, yeast two-hybrid experiments revealed the interaction between Mal and Caspase-1 [33]. Moreover, Mal was shown to be cleaved by caspase-1 after position D_{198} and this process is required for downstream signalling.

Mal is the most polymorphic TLR adaptor and various Mal polymorphisms, in contrast to polymorphisms of other TLR adaptors, can be linked to an altered susceptibility for various diseases. A study by Hawn et al. described 4 Mal polymorphisms present in Vietnamese tuberculosis patients [34]. One synonymous mutation (C558T; Ala186Ala) was associated with increased susceptibility for tuberculosis and TLR2, but not TLR4 agonist stimulation of lymphocytes of patients homozygous for this mutation, resulted in a decrease in IL-6 production. It remains unclear how this variant, of which the aa sequence is not altered, can exhibit a different phenotype and linkage with other functional mutations therefore cannot be excluded. A study performed by Khor and colleagues described a S180L Mal variant,

of which heterozygous carriage is associated with protection against tuberculosis, bacteremia, malaria and pneumococcal disease [35]. Biochemical analysis revealed that MalS180L still interacts with MyD88 and Mal, but is not capable of TLR2 binding. Since homozygous MalS180L individuals are rarely found, and homozygosity is associated with increased susceptibility for invasive pneumococcal disease, Khor et al. hypothesized that MalS180L heterozygosity results in an optimal intermediate immune response.

Trif

The observation that Mal-deficiency did not lead to the abrogation of IRF3 and late phase NF-κB activation upon LPS stimulation, suggested the existence of other TLR adaptor proteins. Further database screenings were preformed and this resulted in the identification of Trif [36]. Overexpression of Trif leads to the activation of NF-κB, although at a lower extent compared to Mal and MyD88. However, Trif activates the IFNβ-promoter at much higher level then MyD88 and Mal. Trif was also shown to coimmunoprecipitate with IRF3 and TLR3, and a dominant negative variant of Trif inhibited TLR3-mediated IRF3 activation. Concurrently, a yeast two-hybrid study identified Trif as a TLR3 interacting protein, and likewise characterized it as a mediator of TLR3-induced IRF3 activation [37]. In line with these findings, a chemically induced mutation in the Trif gene was described and this *Lps2* mutation abolished TLR3- and TLR4-mediated responses [38].

Analysis of Trif$^{-/-}$ cells established Trif as an adaptor involved in the MyD88-independent pathway, since IRF3 activation upon TLR3- or TLR4-stimulation was completely lost in these cells[12]. Trif-deficiency resulted also in a severely impaired NF-κB activation in response to TLR3 agonist stimulation, pointing at an essential and unique role for Trif in TLR3-mediated IRF3 and NF-κB induction. Activation of NF-κB upon LPS stimulation on the other hand was not affected in Trif-deficient cells. Mice deficient in both MyD88 and TRIF showed complete loss of NF-κB activation following TLR4 triggering, suggesting that Trif accounts for the MyD88-independent late phase NF-KB activation.

Distinct regions of Trif are involved in NF-κB and IRF3 activation. The N-terminal part of Trif is involved in IRF3 activation by complex formation with NF-κB-activating kinase-associated protein 1 (NAP1) [39], TRAF family member-associated NF-κB activator (TANK)-binding kinase (TBK1) [40], TRAF3 [41] and TANK [42], of which TBK1 is the crucial kinase for IRF3 activation (Fig.1).

In analogy with Mal, several putative TRAF6 binding sites were found in the N-terminal part of Trif [40;43]. Mutation of the motif at position 248-255, reduced NF-κB activation by Trif. However, the importance of TRAF6 in Trif-mediated NF-κB induction is somewhat unclear, as TLR3 signalling was not impaired in TRAF6^{-/-} macrophages [44], in contrast with the drastic effect observed in TRAF6^{-/-} mouse embryonic fibroblasts (MEFs) [43]. These findings indicate that the role of TRAF6 in NF-κB activation seems to be cell type-specific.

In addition to TRAF6 interaction, another mechanism of Trif-mediated NF-κB activation was described. Trif contains a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) at its C-terminus by which it recruits RIP1 [45;46]. RIP1 was shown to be critical in the Trif-NF-κB pathway since TLR3-induced activation of NF-κB, but not JNK or IRF3, was abolished in MEFs lacking RIP1 [45;46].

Next to its role in NF-κB activation, RIP1 is also involved in Trif-induced apoptosis, through its association with FADD and caspase-8 [47].

Tram

Database searches identified a fourth TIR-containing adapter, Tram. Concurrently, two groups revealed a function of Tram in TLR4 signalling [48;49]. Based on two-hybrid and coimmunoprecipitation studies, Tram was shown to bind to TLR4 and Trif, but not to other TLRs or MyD88. Overexpression of Tram induces the activation of NF-κB and IRF3 and both overexpression of a dominant negative variant of Tram as well as siRNA knockdown of Tram, resulted in an impaired TLR4-induced NF-κB and IFN-β induction. TLR3 responses on the other hand remained unaffected upon Tram knockdown. In addition, overexpression of a dominant negative Trif mutant or Trif-knockdown completely suppressed Tram-induced activation of the

IFN-β promoter. These data suggested a role for Tram in TLR4 signalling upstream of Trif.

The role of Tram in TLR4 signalling was further underscored by analysis of Tram^{-/-} mice [50]. In contrast to the findings of Bin and colleagues [51], IL-1 signalling was unaffected in Tram^{-/-} MEFs. However, TLR4- but not TLR2-, TLR3-, TLR7- or TLR9-induced cytokine production was severely suppressed in knock-out macrophages. Taken together, these data suggest a limited function for Tram in TLR signalling, being the bridging adaptor linking Trif to the activated TLR4.

Recently, two post-translational modifications of Tram were described, both capable of modulating Tram's activity. Tram contains an N-terminal myristoylation site, which is essential for membrane targeting of Tram. Disruption of the myristoylation leads to impaired LPS signalling [52]. Tram is also phosphorylated on serine 6 and serine 16 by PKCε upon LPS stimulation [53]. Disruption of the modification site again results in abrogated signalling. Phosphorylation of Tram leads to the translocation from the nucleus, but the exact consequence of this process is still elusive. Given the fact that phosphorylation of Tram is indispensable for signalling, translocation of Tram could facilitate the interaction with downstream signalling molecules.
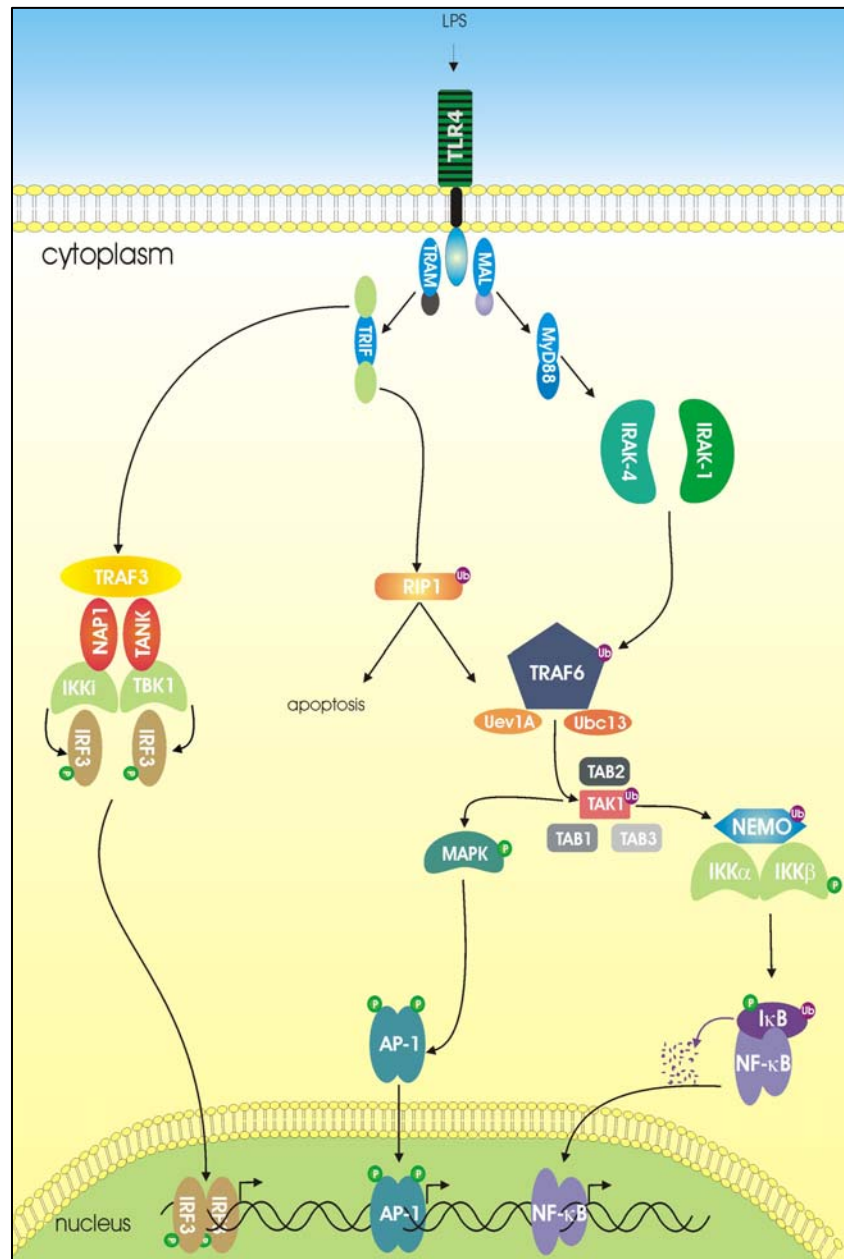
Figure 2: TLR4 signalling pathways. TLR signalling commonly is divided in a MyD88-dependent and Trif-dependent branch. MyD88-dependent signalling: TLR4 activation leads to the recruitment of adaptor molecules Mal and MyD88 to the receptor, through TIR-TIR domain interactions. Next, IRAK-4 interacts with MyD88, leading to the association and phosphorylation of IRAK-1. Phosphorylated IRAK-1 dissociates from the MyD88/IRAK-1 complex and binds TRAF6, which in turn activates TAK1 in an ubiquitin-dependent manner. Subsequently, TAK1 activates the IKK complex and the MAPK pathway, ultimately leading to the activation of respectively NF-κB and AP-1. Trif-dependent signalling: Triggering TLR4 leads to the recruitment of Tram and Trif to the activated receptor. Next, Trif recruits the IKKi/TBK1 complex, leading to the phosphorylation and nuclear translocation of IRF3. Moreover, Trif signalling leads to the activation of NF-κB and MAPKs, mediated by the association of Trif with RIP1 and TRAF6.

Applications of the MAPPIT Toolbox in TLR signalling

Contrary to the growing insight in the function and regulation of the adaptor molecules in TLR signalling, reports biochemically linking these adaptors to the TLRs remain scarce. Moreover, the applied methods are generally limited to yeast two-hybrid and coimmunoprecipitation [54]. These techniques suffer from some intrinsic limitations. As discussed above, the TLR adaptors are subjected to post-translational modifications, which are hard to reproduce in yeast. Coimmunoprecipitation on the other hand leads to disruption of the cell architecture, which can result in false positive interactions between proteins that normally localise in separate cellular compartments.

To circumvent these problems, we developed MAPPIT (MAmmalian Protein-Protein Interaction Trap), a mammalian two-hybrid method based on type I cytokine signalling [55]. Ligand-induced cytokine receptor clustering results in cross-phosphorylation and activation of receptor-associated Janus kinases (JAKs) (Fig.3A). Thereupon, these activated JAKs phosphorylate conserved tyrosine residues in the cytokine receptor tails, thus forming recruitment sites for signal transducer and activator of transcription (STAT) molecules. The STAT molecules subsequently are phosphorylated by the JAKs and translocate as dimers to the nucleus where they induce specific gene transcription.

The MAPPIT technique exploits this JAK/STAT signalling pathway. In brief, we made a C-terminal fusion of a given 'bait' protein with the transmembrane and intracellular part of a class I cytokine receptor that is deficient in STAT3 recruitment. The extracellular domain of both the erythropoietin and the leptin receptor can be used. The 'prey' protein on the other hand is linked to a series of 6 functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand stimulation leads to STAT3 activation and induction of a STAT3–responsive luciferase reporter (rPAPI-Luci). The stimulation of the reporter gene is used as a measure of the interaction between the bait and prey proteins (Fig.3B). An additional advantage of the MAPPIT technique is the separation of interaction (cytosol) and effector (nucleus) zone since signal readout is mediated by endogenous STAT molecules. That way bait and prey proteins cannot interfere with reporter transcription, thereby

reducing autoactivators and false positives. The standard MAPPIT assay makes use of the human embryonic 293T (HEK293T) cells, but it can be used in other cell types, as long as these cells sufficiently express STAT3. Cells expressing STAT5 can also be applied, through the use of the β_C-MAPPIT variant [56]. Examples of applications of the MAPPIT technique are given in references 57-60.
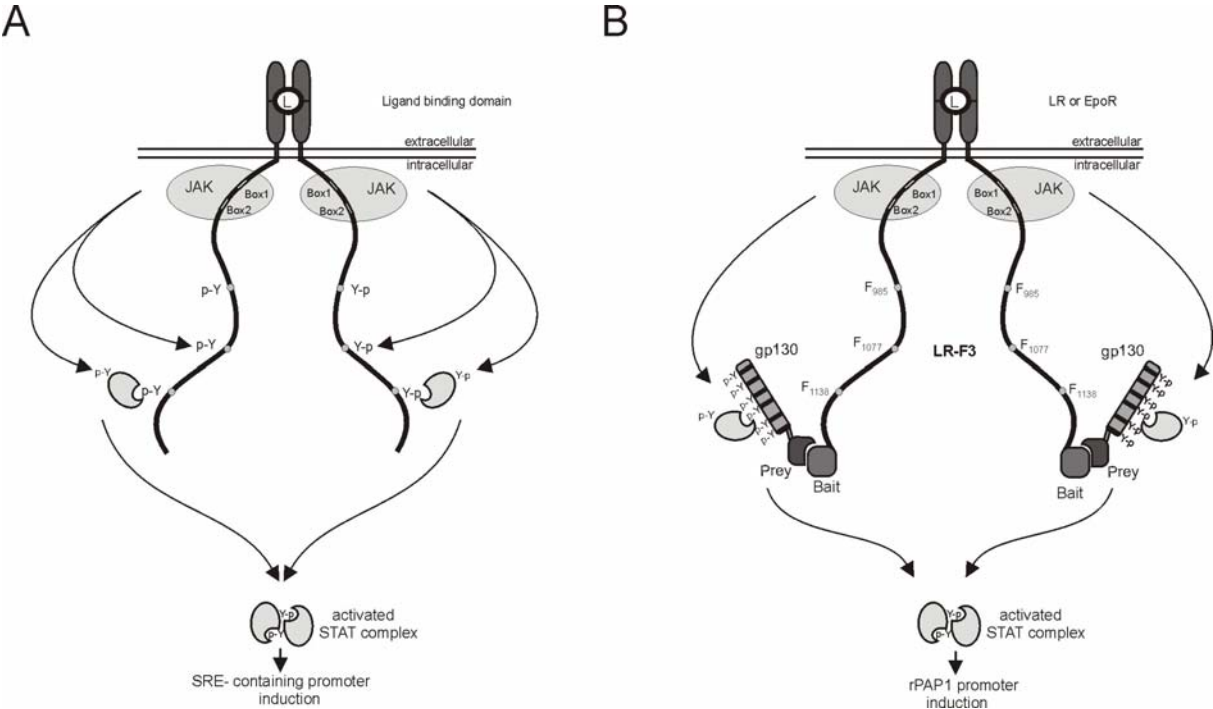
Figure 3: (A) Schematic overview of the JAK-STAT signal transduction pathway: ligand-induced cytokine receptor clustering results in the cross-phosphorylation and activation of associated JAK kinases. Next, these activated JAK kinases phosphorylate conserved tyrosine motifs in the cytoplasmic tails of the cytokine receptor. These phosphorylated tyrosine motifs form docking sites for STAT molecules, which in turn get phosphorylated. Those activated STATs dissociate from the receptor and translocate as dimers to the nucleus, where they induce transcription of specific genes. (B) MAPPIT principle: a given 'bait' is C-terminally fused with the transmembrane and intracellular part of a leptin receptor that is deficient in STAT3 recruitment. The extracellular domain of either the erythropoietin (EpoR) or of the leptin receptor (LR) can be used. The 'prey' protein is linked to a series of 6 functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand (L) stimulation leads to STAT3 activation and induction of a STAT3–responsive luciferase reporter (rPAPI-Luci).

Using MAPPIT we were able to reconstruct the signalling steps from TLR4 to IRAK-1. Therefore, the intracellular part of TLR4, the TLR adaptors and downstream kinases were cloned as either bait or prey. TLR signalling is initiated by ligand-induced receptor dimerisation, resulting in the recruitment of adaptor molecules. Using MAPPIT, the TLR4 homodimerisation could clearly be detected (Fig.4A). Moreover, recruitment of Tram or Mal could also be monitored (Fig.4B-C). Much in contrast, no association of TLR4 and MyD88 was detected, although the role of MyD88 in TLR4 signalling is well documented [8]. However, co-transfection of a Mal expression vector together with TLR4ic-bait and MyD88-prey resulted in a clear MAPPIT signal (Fig.4E). These data are consistent with the recent definition of Mal as a bridging adaptor, linking MyD88 to TLR4 [27] and prove that indirect interactions can be detected using the MAPPIT assay. In addition, heterodimerisation of Mal and MyD88 was readily observed (Fig.4D) and interaction of MyD88 and the downstream kinase IRAK-4 was also detected (Fig.4F). Again, we could observe the indirect association of Mal and IRAK-4, with a bridging role for MyD88 (Fig.4G). Finally, using IRAK-4 as a bait interaction with the IRAK-1-prey was demonstrated (Fig.4H).
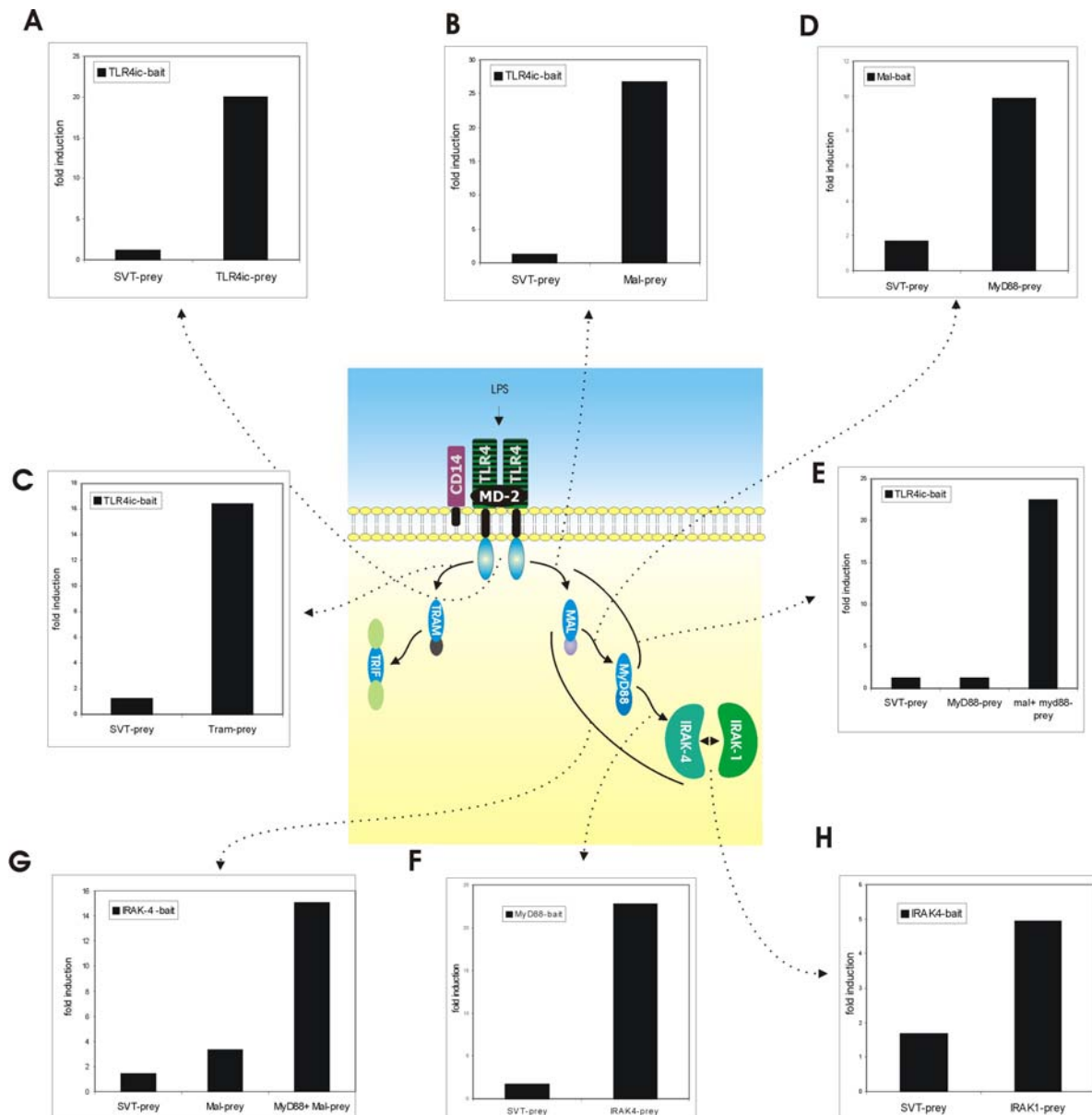
Figure 4: (A) Homodimerisation of TLR4. Hek293T cells were transiently co-transfected with the MAPPIT TLR4ic-bait plasmid, the TLR4ic-prey or a SVT-prey as negative control, and the STAT3-responsive rPAPI-luci reporter. Twenty-four hours after transfection the transfected cells were stimulated with leptin (100ng/ml) for another 24 h or were left untreated. Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (ratio stimulated/untreated). A similar experimental set-up was used in all following experiments. **(B-C) Adaptor recruitment by TLR4ic.** Using the TLR4ic-bait and either the Mal-prey (B) or the Tram-prey (C) recruitment of these adaptors to TLR4 was monitored. **(D) Heterodimerisation of Mal and MyD88.** Hek293T cells were transiently co-transfected with the Mal-bait and the myd88-prey vectors together with the rPAPI-luci reporter. **(E) Mal links MyD88 to TLR4.** The effect of Mal expression on MyD88 association was assayed by co-transfection of a Mal expression vector, together with the MyD88-prey and TLR4ic-bait. **(F) MyD88 and IRAK-4 association.** Interaction of MyD88 and IRAK-4 was assayed using the MyD88-bait and the IRAK4-prey vectors. **(G) MyD88 bridges IRAK-4 to Mal.** Co-transfection of a MyD88 expression vector mediates the association of IRAK-4 and Mal. **(H) Heterodimerisation of IRAK-1 and IRAK-4.** Using IRAK-4 as a bait and IRAK-1 as a prey, heterodimerisation of both kinases was monitored.

As discussed above, MyD88 is a universal adaptor used by all TLRs except TLR3. In contrast to the numerous reports discussing the impaired TLR responses of MyD88-deficient mice, biochemical data linking MyD88 to these TLRs remains limited. Therefore, we evaluated the interactions between the TIR domains of additional TLRs (TLR2, TLR3, TLR5, TLR7 and TLR 9) and MyD88 or Mal using MAPPIT (Table 1). Next to the previously described association with TLR4, interaction between Mal and TLR2 was also detected. These data are consistent with the observation that Mal-deficient mice exhibit severely impaired TLR2 and TLR4 responses. Clearly, neither of these TLRs directly interacts with MyD88, but moreover, we also failed to detect an interaction between MyD88 and all other TLRs examined. In analogy with our data, no direct interaction between TLR9 and MyD88 could be detected using yeast two-hybrid [54]. Of note, TLR7 and TLR9 are expressed on acidic endosomes whereas TLR2, TLR4 and TLR5 signal from the cell membrane. How MyD88 is recruited to those TLRs residing in distinct cellular compartments is still unclear. Our findings suggest that TLR5, TLR7 and TLR9, in analogy with TLR2 and TLR4, may also use accessory proteins, although we cannot rule out at present that these interactions cannot be detected using MAPPIT. However, such failure cannot be attributed to a defective prey or bait since the MyD88-prey is fully functional (see above) and functional interaction of all TLR baits with a JAK2-interacting prey can be detected, ruling out some intrinsic inhibition of the MAPPIT signal (data not shown).

| | | bait | | | | | |
		TLR2ic	TLR3ic	TLR4ic	TLR5ic	TLR7ic	TLR9ic
prey	MyD88	2,28	1,01	1,22	1,2	0,9	1,1
	Mal	**8,71**	4,01	**26,69**	1,5	1,9	2,7

Table 1: MAPPIT analysis of MyD88 and Mal recruitment by different TLRs. The intracellular part of several TLRs were used as bait whereas MyD88 or Mal were used as prey. Experimental setup was as in Figure 4.Positive MAPPIT signals are depicted in bold.

Taken together, these data illustrate that MAPPIT is a valuable tool to study TLR signalling in a near-optimal physiological context in mammalian cells. MAPPIT can also be used to screen for new interactors [57]. To this end, a STAT-induced surface tag is used as a selectable marker. Cells stably expressing the bait chimera and this reporter vector can be infected with a prey cDNA-library. Upon ligand administration, cells expressing interacting preys are sorted and deposited in single wells allowing identification of the prey-encoding cDNAs. Efficiencies up to 40% of cell clones expressing interactor preys have been obtained (unpublished results).

Uncontrolled activation of TLR signalling can be observed in autoimmune diseases, chronic inflammation and bacterial sepsis. Because of their specific role in TLR signalling, TLR adaptor molecules are considered as legitimate therapeutical targets. Based on the MAPPIT concept, a reverse two-hybrid system was developed (Reverse MAPPIT) [58] to screen for molecules that disrupt protein-protein interactions. Assays are currently being optimised to use this Reverse MAPPIT set-up to search for proteins or small molecules that interfere with TLR signalling.

Conclusion

Initial TLR signalling events are based on the recruitment of a limited set of TIR-containing adaptor molecules. The differential use of these adaptors by different TLRs contributes to a ligand-specific response. These adaptor molecules are subjected to post-translational modifications and their function is strictly regulated. Underscoring the importance of controlling TLR activation is the observation that unrestrained TLR signalling can result in excessive inflammation, autoimmune disorders and septic shock. Given the fundamental role of the adaptor molecules in TLR signalling, they are justifiable targets for drug development. Possible strategies include interfering with the relevant TIR/TIR interactions or with the critical post-translational modifications of the adaptors. MAPPIT, with its near optimal physiological context, might be a helpful tool for analysing the molecular principles underlying TLR signalling and also as a screening tool for compounds interfering in this pathway.

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2. MyD88-dependent signalling

Activation of TLRs by their ligands ultimately leads to the activation of NF-κB and IRFs. The initial steps of these signalling pathways are mediated by four TIR-domain containing adaptors: MyD88, Mal, Trif and Tram. Different TLRs make use of different adaptor combinations, which results in the activation of distinct signalling pathways. These signalling cascades can be divided in a MyD88-dependent and a Trif-dependent branch (Fig.5). Association of MyD88 and the intracellular domain of an activated TLR results in the recruitment of serine/threonine IL-1 receptor-associated kinase-4 (IRAK-4) via a homotypic Death Domain interaction (Janssens and Beyaert, 2003; Suzuki et al., 2002). Next, IRAK-4 interacts with IRAK-1, resulting in the phosphorylation and activation of the latter. Activated IRAK-1 autophosphorylates itself, leading to its dissociation of the MyD88/IRAK-4 complex and the association with TNF receptor-associated factor-6 (TRAF-6). TRAF-6, a RING domain protein with E3 ligase activity, interacts with other E3 ubiquitin ligase proteins like ubiquitin-conjugating enzyme 13 (Ubc13) and Ubc-like protein Uev1A, resulting in the lysine 63 polyubiquitination of TRAF-6 (Deng et al., 2000; Lamothe et al., 2007). Ubiquitinated TRAF6 then forms a complex with transforming growth factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 1, 2 and 3 (TAB1, TAB2 and TAB3), resulting in the ubiquitination and activation of TAK1 (Kanayama et al., 2004; Besse et al., 2007). TAK1 is the branching point resulting in the activation of two pathways (Wang et al., 2001; Sato et al., 2005). On the one hand, TAK1 activates the IKK complex, composed of IKKα, IKKβ and IKKγ/NEMO, which results in the phosphorylation and subsequent degradation of IκB protein. Proteasomal degradation of IκB is followed by nuclear translocation of NF-κB, resulting in gene induction of pro-inflammatory cytokines including TNF-α, IL-6 and IL-12p40 (Hayden and Ghosh, 2004; Chen, 2005). On the other hand, TAK1 phosphorylates mitogen activated protein kinase (MAPK) kinase 6 (MKK6) and MKK7, resulting in the activation of p38 MAPK and c-Jun N-terminal kinase (JNK). These MAPKs subsequently phosphorylate activator protein-1 (AP-1), resulting in the nuclear translocation and concomitant gene induction (Wang et al., 2001; Hanafusa et al., 1999). The role of TAK1 in TLR signalling was further underscored by analysis of TAK1$^{-/-}$ embryonic fibroblasts and B cells, which display reduced activation of NF-κB,

JNK and p38 upon TLR stimulation (Sato et al., 2005). Moreover, extracellular signal-regulated protein kinase 1 (ERK1) and ERK2 activation was as well impaired in TAK1$^{-/-}$ cells, further broadening the TAK1-activated MAPKs (Sato et al., 2005).

Next to TAK1, other MAPKKK are involved in TLR4-induced JNK, ERK and p38 activation. MAPK-ERK kinase kinase (MEKK3)- deficient macrophages exhibit impaired activation of NF-κB, JNK and p38, but not of ERK, upon LPS treatment (Huang et al., 2004). Contrary, Tumor progression locus 2 (Tpl2) kinase-deficient macrophages failed to activate ERK in response to LPS , whereas normal JNK and p38 activation is observed (Dumitru et al., 2000). Finally, apoptosis signal-regulating kinase 1 (ASK1) was shown to be involved in LPS-induced p38 activation (Matsuzawa et al., 2005), further diversifying the TLR4-induced MAPK pathway.
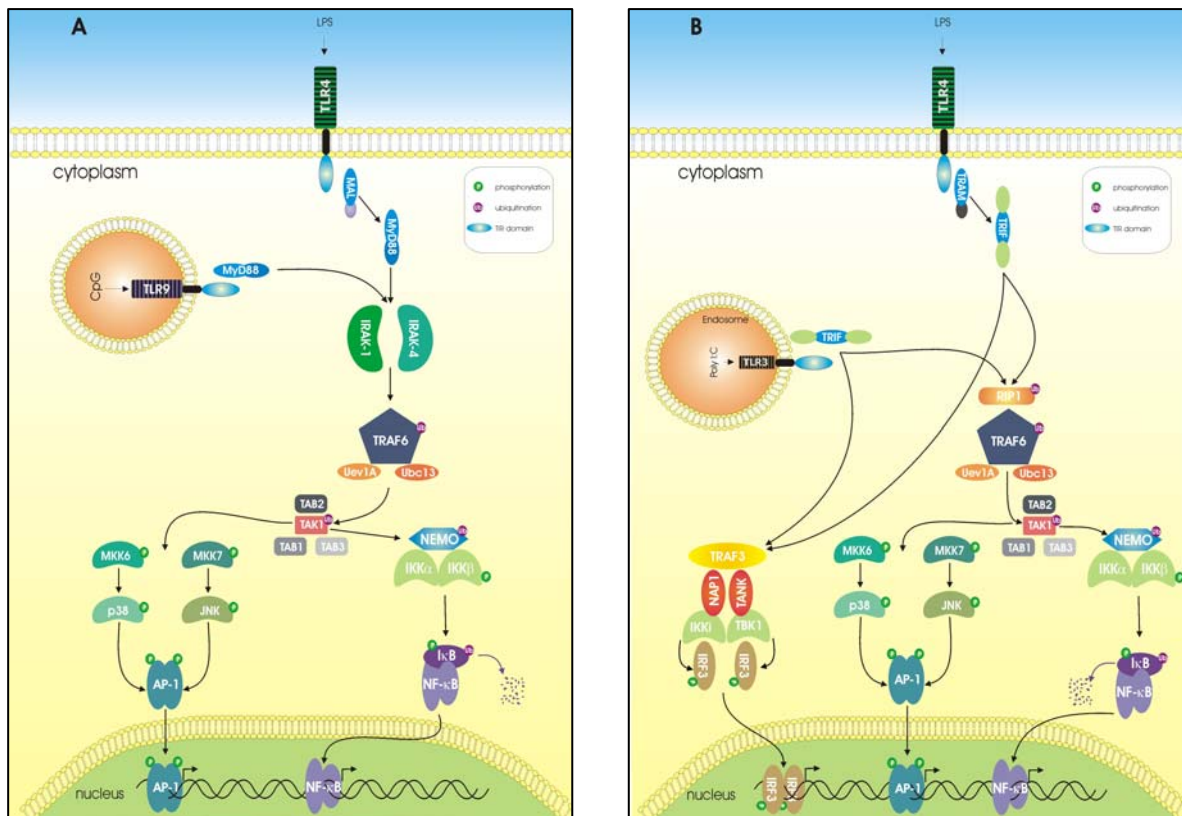
Fig.5: Toll-Like receptor signalling pathways. (A) MyD88-dependent signalling. TLR activation leads to the recruitment of adaptor molecules MyD88 or Mal/MyD88 to the receptor, through TIR-TIR domain interactions. Next, IRAK-4 interacts with MyD88, leading to the association and phosphorylation of IRAK-1. Phosphorylated IRAK-1 dissociates from the MyD88/IRAK-1 complex and binds TRAF6, which in turn activates TAK1 in an ubiquitin-dependent manner. Subsequently, TAK1 activates the IKK complex and the MAPK pathway, ultimately leading to the activation of respectively NF-κB and AP-1. (B) Trif-dependent signalling. Triggering TLR3 and TLR4 leads to the recruitment of respectively Trif or Tram/Trif to the activated receptor. Next, Trif recruits the IKKi/TBK1 complex, leading to the phosphorylation and nuclear translocation of IRF3. Moreover, Trif signalling leads to the activation of NF-κB and MAPKs, mediated by the association of Trif with RIP1 and TRAF6.

3. Trif-dependent signalling

Triggering of TLR3 and TLR4 leads, next to MyD88-dependent signalling, to the activation of the Trif-dependent pathway. Trif associates either directly (TLR3) or through the bridging adaptor Tram (TLR4) with the activated TLR, again via TIR-TIR domain interactions. Next, two non-canonical IKKs, inducible IKK (IKKi/IKKε) and TRAF family member-associated NF-κB activator (TANK)-binding kinase (TBK1), together with TRAF3 are recruited to the N-terminal part of Trif (Fitzgerald et al., 2003; Oganesyan et al., 2006; Sharma et al., 2003). This complex also involves TANK and NF-κB-activating kinase-associated protein 1 (NAP1) (Guo and Cheng, 2007; Sasai et al., 2005). Subsequently, IKKi/TBK1 phosphorylate IRF3, leading to the nuclear translocation of the latter and corresponding induction of IFNβ transcription (Hemmi et al., 2004; Perry et al., 2004; McWhirter et al., 2004). Next to IRF3, IRF7 is also phosphorylated by the IKKi/TBK1 kinase complex, although, in contrast with IRF3, IRF7 is poorly expressed in unstimulated cells. Following TLR stimulation, IRF7 expression is upregulated, and therefore IRF7 is thought to account for a sustained type I IFN response, rather then having a role in the initial induction of IFNβ (Sato et al., 1998; Levy et al., 2002).

Activation of NF-κB and MAPK following LPS stimulation, although delayed, is still observed in MyD88-deficient cells. Moreover, triggering of TLR3, which only makes use of Trif as adaptor, also leads to pro-inflammatory cytokine production (Hoebe et al., 2003; Yamamoto et al., 2002b; Yamamoto et al., 2003a). Thus, next to production of type I IFNs, Trif-dependent signalling also leads to the activation of NF-κB and MAPKs. This activation is mediated by receptor-interacting protein 1 (RIP1), which interacts with the Rip homotypic interacting protein (RHIM) motif in the C-terminal part of Trif. Consequently, TLR3-induced activation of NF-κB, but not JNK nor IRF3, was abolished in MEFs lacking RIP1 (Meylan et al., 2004; Cusson-Hermance et al., 2005). Moreover, Trif was shown to interact with TRAF6, mediated by three TRAF6-binding motifs in its N-terminal region (Sato et al., 2003). Disruption of the TRAF6-binding motifs in Trif lead to impaired NF-κB activation and overexpression of a dominant negative TRAF6 mutant results in the abrogation of poly(I:C)-induced NF-κB activation, suggesting a major role for TRAF6 in Trif-

dependent NF-κB activation. Taken together, recruitment of RIP1 and TRAF6 by Trif results in the activation of TAK1 leading to NF-κB and MAPK activation.

However, this model is somewhat questioned. In contrast, Gohda and colleagues observed no impaired NF-κB activation in TRAF6^{-/-} macrophages upon poly(I:C) stimulation (Gohda et al., 2004), indicating that the role of TRAF6 in NF-κB activation seems to be cell type-specific. Moreover, delayed activation of TLR4/Trif-induced NF-κB was shown to be based on IRF3 activation rather then on the Trif/RIP1/TRAF6 pathway and requires de novo synthesis of TNFα (Covert et al., 2005; Werner et al., 2005). Consistently, suppression of IRF3 expression leads to a reduced late-phase NF-κB activation, suggesting that Trif-activated IRF3 induces TNF-α expression. Next, TNF-α activates the TNF receptor via an autocrine loop, resulting in activation of IKKs and concomitant late-phase NF-κB activation. In contrast, TLR3-induced NF-κB activation still occurs in RIP^{+/-} TNFR^{-/-} fibroblasts, arguing against the existence of an autocrine NF-κB activation loop (Meylan et al., 2004). Hence, further studies are necessary to elucidate the exact roles of RIP, TRAF6, TBK1, and IRF3 in Trif-mediated NF-κB activation.


4. Interferon regulatory factors in TLR signalling

Next to IRF3/7, several other members of the IRF family (IRF1→ IRF9) are involved in TLR signalling (Fig.6). IRF1, IRF3, IRF5, IRF7 and IRF8 activate transcription of type I IFNs and pro-inflammatory cytokines, whereas IRF4 has a role in negative feedback (see below). An additional role for IRF7 in TLR signalling arose by the identification of a novel IFN-inducing pathway in plasmacytoid DC (pDC). This subset of DCs produces high amounts of IFNα following viral infection, and therefore is also known as type I IFN-producing cells (Colonna et al., 2004). Surprisingly, these cells do not express TLR3 nor TLR4, but instead produce high levels of TLR7 and TLR9. Moreover, IFNβ expression upon TLR9 stimulation was completely abrogated in MyD88^{-/-} DCs. These findings suggested the involvement of the MyD88-dependent pathway instead of Trif-dependent signalling in CpG-induced IFNβ production (Hoshino et al., 2002; Hemmi et al., 2003). Two-hybrid screening identified IRF7 as a MyD88-interacting protein, and the MyD88-IRF7 complex also involves IRAK-1, IRAK-4 and TRAF6 (Kawai et al., 2004; Honda et al., 2004). Overexpression of IRF7

leads to the activation of IFNα and IFNβ–inducible promoters and CpG stimulation of pDC derived from IRF7^{-/-} mice resulted in a severely reduced IFN induction (Kawai et al., 2004; Honda et al., 2005). IRAK1 was shown to phosphorylate IRF7 and consistently, IRAK1-/- pDC fail to phosphorylate IRF7 in response to TLR9 agonists (Uematsu et al., 2005). Recently, a similar role as IRAK-1 was described for IKKα, although it remains unclear whether or not IKKα cooperates with IRAK-1 (Hoshino et al., 2006). Taken together, these findings establish IRF7 and IRAK- 1 as key factors in viral-induced IFN response in pDC.

In contrast with pDC, bone-marrow-derived DCs depend on IRF1, and not IRF7, for efficiënt CpG-induced IFN production (Negishi et al., 2006). In addition, it was shown that IRF1 expression is upregulated by IFN-γ stimulation and gene induction of IL-12p35 and iNOS is severely impaired in IRF1^{-/-} DC stimulated with IFN-γ and CpG. Since IL-12p35 and iNOS are genes under control of a NF-κB inducible-promoter, this suggests that IRF1 cooperates with NF-κB, forming an enhanceosome complex resulting in efficient transcription of the latter genes. Mechanistically, CpG stimulation induces association of IRF1 with the intermediate domain of MyD88, followed by phosphorylation of IRF1 and translocation to the nucleus.

In analogy with IRF1, IRF5 was shown to interact with the intermediate domain of MyD88 (Takaoka et al., 2005). Analysis of IRF5^{-/-} mice revealed the critical role of IRF5 in TLR signalling. Pro-inflammatory cytokine production upon TLR3, -4, -5, -7, -9 agonist stimulation was severely reduced in macrophages and conventional DCs derived from IRF5^{-/-} mice and consistently, these mice were highly resistant against endotoxin shock. Following CpG stimulation IRF5 gets activated by a so far unidentified kinase and translocates to the nucleus where it was shown to bind to interferon-stimulated response element (ISRE) motifs of the IL-12p40 promoter. In the nucleus, IRF5, in coordination with NF-κB, induces transcription of IL-12p40, TNF-α and IL-6.

Finally, IRF8 was shown to be involved in TLR9-dependent gene induction. IRF8^{-/-} pDC failed to produce pro-inflammatory cytokines upon TLR9 activation (Tsujimura et al., 2004). IRF8 was shown to activate IKKα/β, essential kinases for NF-κB activation.
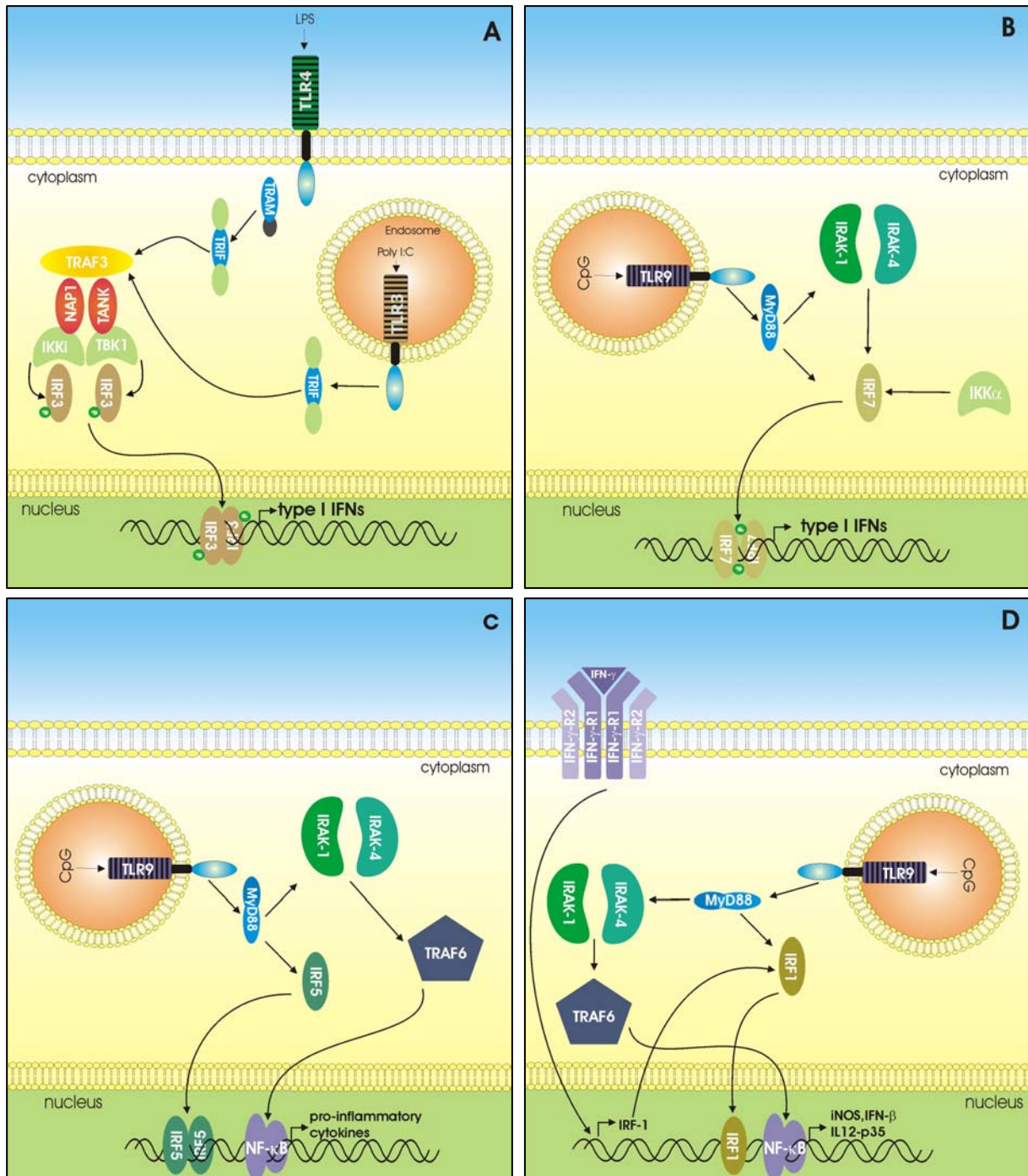
Fig.6: IRFs in TLR signalling. (A) Trif-dependent signalling. Activation of TLR4 and TLR3 leads to the recruitment of Trif, ultimately leading to phosphorylation and nuclear translocation of IRF3. (B) pDCs produce vast amounts of typeI IFNs upon viral infection. Activation of TLR7/8 or TLR9, leads to the recruitment of IRF7 to MyD88, followed by IRAK-1 (and IKKα)-dependent phosphorylation. Next, activated IRF7 translocates to the nucleus to induce IFN type I gene transcription. (C) IRF5 as well associates with MyD88 but is involved in inflammatory cytokine induction by TLRs. Upon stimulation, IRF5 translocates to the nucleus, where it binds ISRE motifs in promoter regions of pro-inflammatory cytokines like IL-12p40, TNF-α, and IL-6, and is thought to induce gene transcription in coordination with NF-κB. (D) IFNγ stimulation upregulates IRF1 expression. Upon TLR9 stimulation, IRF1 interacts with MyD88, resulting in activation and nuclear translocation of IRF1. In the nucleus, IRF1 induces IFN-β and cooperates with NF-κB to induce iNOS and IL-12p35.

Taken together, these findings prove that IRF proteins are essential components of TLR-mediated responses. The use of a whole spectrum of IRFs provides a basis for TLR specificity, which is based on cell type specific expression of IRFs, different activation pathways and differences in induced genes.

VI.   References

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CHAPTER 3: Negative regulation of Toll-Like receptor signalling

I.   Introduction

TLR signalling leads to the production and secretion of pro-inflammatory cytokines, resulting in a potent immune reaction against invading pathogens. However, uncontrolled TLR activation and concomitant excessive inflammation is extremely harmful for the host. Deregulation of TLR signalling is observed in several autoimmune diseases, chronic inflammation and bacterial sepsis. Hence, TLR signalling balances on the tight rope, hovering between inordinate inflammation and potent pathogen clearance.

Mammals have evolved several mechanisms that dampen the innate immune response, preventing sustained and toxic inflammation. Attenuation of TLR signalling is achieved either by inhibiting ligand binding, by downregulation of TLR expression or by interference of downstream signalling (Fig.1). Thus, this multileveled regulation ensures an efficient attenuation of excessive TLR responses.

Decoy receptors

Extracellular modulation of TLR signalling is mediated by soluble decoy Toll-Like receptors (sTLRs), directly inhibiting ligand recognition. Two sTLRs have been reported, sTLR4 and sTLR2. sTLR4 is generated by alternative splicing and is upregulated upon LPS stimulation (Iwami et al., 2000). Overexpression of sTLR4 in macrophages drastically reduced LPS-induced TNF-α production and NF-κB activation. sTLR4 attenuates signalling by inhibiting the interaction of TLR4 with MD-2 and CD14, resulting in a loss of LPS sensing (Hyakushima et al., 2004).

sTLR2, on the other hand, is formed by ectodomain shedding of TLR2. Blood monocytes constitutively produce sTLR2, but the kinetics of sTLR2 release increase upon cell stimulation (LeBouder et al., 2003). sTLR2 reduces lipopeptide-induced NF-κB activation and IL-8 secretion through competition with membrane-anchored TLR2 for ligand (Iwaki et al., 2002).
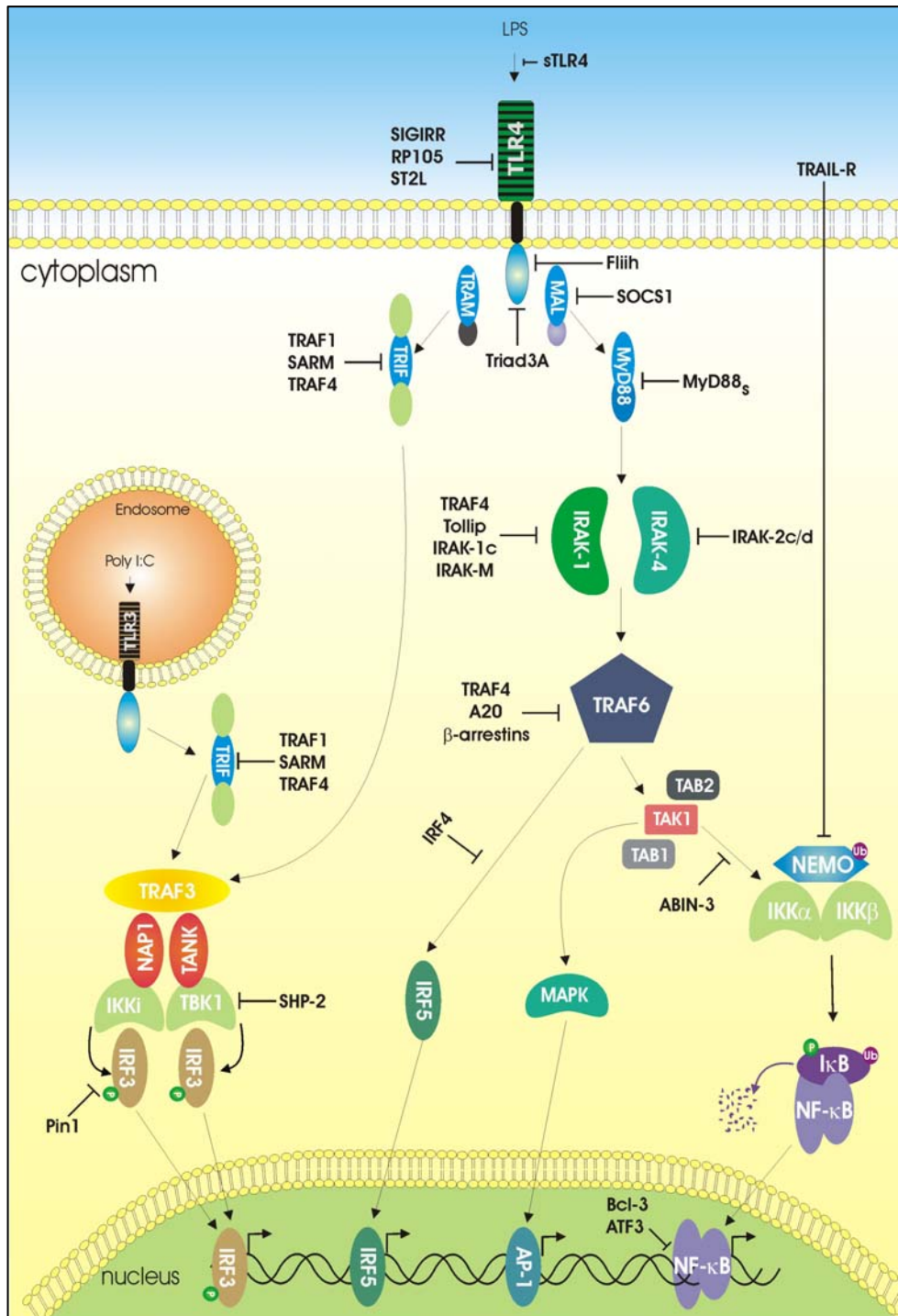
Fig.1: Negative regulators of Toll-Like receptor signalling. TLR signalling is attenuated at multiple levels. Decoy receptors (sTLR2/sTLR4) prevent ligand sensing, whereas Triad3a and TGF-β downregulate TLR expression. The transmembrane molecules RP105, SIGIRR, ST2L and TRAIL-R inhibit TLR signalling by inhibiting ligand binding, sequestering adaptor molecules or direct interference of downstream signalling respectively. TLR adaptors are also targeted for negative regulation. Mal is downregulated by SOCS-1, Trif by Sarm and by TRAF1/4 and MyD88 by Fliih and MyD88$_s$. Downstream IRAK kinases are inhibited by splice variants IRAK1c/2c/2d and by IRAK-M, Tollip and TRAF4. Activity of TBK1, a kinase involved in the MyD88-independent signalling pathway is modulated by SHP-2. TRAF6 is subjected to negative feedback by association of A20, TRAF4 and β-arrestins whereas ABIN-3 acts downstream of TRAF6. Finally, transcription factors NF-κB and IRF3 are inhibited by respectively Bcl-3/ATF3 and Pin-1.

Inhibition of TLR expression

Overexpression of the RING finger protein Triad3A, a E3 ubiquitin-protein ligase, enhances proteolytic degradation of TLR4 and TLR9 and results in significant reduction of membrane expression and signalling (Chuang and Ulevitch, 2004). Recently, Triad3A was shown to target some intracellular signalling components, like RIP1 and the TLR adaptor molecules Mal and Trif to the proteasome, expanding its radius of action (Fearns et al., 2006).

Moreover, TLR expression can be modulated by anti-inflammatory cytokines like transforming growth factor β1 (TGF-β1). Elevated levels of TLR4 mRNA accompanied with hyperresponsiveness to LPS was observed in TGF-β1$^{-/-}$ mice (Cartney-Francis et al., 2004). Following TLR stimulation, TGF-β1 suppresses NF-κB activation, by facilitating proteasomal degradation of MyD88, further highlighting its role in TLR regulation (Naiki et al., 2005).

Membrane-anchored regulators

Over the past years several transmembrane TLR modulators were described. These proteins attenuate TLR signalling either by sequestering adaptor molecules, by inhibiting ligand binding or by direct interference of downstream signalling. One of them, single immunoglobulin IL-1R-related protein (SIGIRR), is composed of a single immunoglobulin domain and a cytoplasmic TIR domain. SIGIRR is expressed in epithelial cells and DCs, but not on macrophages, endothelial cells or fibroblasts (Wald et al., 2003). SIGIRR-deficient mice are more susceptible to endotoxic shock and DCs derived from these mice show enhanced activation in response to IL-1, LPS, CpG DNA but not to poly(I:C). Upon IL-1 treatment, SIGIRR interacts with IL-1R, IRAK-1 and TRAF6, hinting SIGIRR inhibits IL-1R signalling by sequestering downstream signalling molecules. In addition, SIGIRR was also shown to interact directly with TLR4, which might point to an analogous inhibitory mechanism.

In analogy, another member of the TIR superfamily, ST2L, negatively regulates IL-1R and TLR4 signalling. ST2L was shown to sequestrate MyD88 and Mal trough its TIR domain, thereby inhibiting interaction of these TLR adaptors with TLR4 (Brint et al., 2004).

Radioprotective 105 (RP105) consist of a LRR homologous to TLR4 and a short intracellular domain. It was originally identified as a B cell-specific protein, but is also expressed in monocytes and myeloid DCs. RP105 associates with MD-1, and this RP105/MD-1 complex interacts with the TLR4 signalling complex, thereby sequestering LPS binding sites (Divanovic et al., 2005a; Divanovic et al., 2005b).

Finally, TNF-related apoptosis-inducing ligand receptor (TRAIL-R), a member of the TNFR superfamily, was shown to be involved in TLR regulation. TRAIL-R$^{-/-}$ mice were less susceptible to MCMV infection which corresponded with augmented levels of IL-12, IFN-α, and IFN-γ. Moreover, stimulation of TLR-2, -3, and -4 upregulated the expression of TRAIL, the transmembrane ligand of TRAIL-R, and enhanced cytokine production in TRAIL-R$^{-/-}$ macrophages. TRAIL-R stabilizes IκBα, preventing nuclear translocation of NF-κB and as a result inhibiting TLR-induced cytokine production (Diehl et al., 2004). Since both TRAIL and its receptor are transmembrane proteins, cell-cell contact is required for TRAIL-R function, adding an additional dimension to negative feedback mechanisms of TLR signalling.

Intracellular regulators

The importance of a tight control of TLR signalling is probably most reflected by the number of different intracellular regulators. For each step in the signal cascade, one or more inhibitors are described. A first group of regulating molecules interferes with the stability or function of TLR adaptors. MyD88$_{s(hort)}$, a LPS-induced splice variant of MyD88 which lacks the intermediate domain bridging the DD to the TIR domain, was shown to inhibit LPS-induced NF-κB activation (Burns et al., 2003). MyD88$_s$ fails to recruit IRAK-4, thereby preventing phosphorylation and thus activation of IRAK-1. However, overexpression of MyD88$_s$ still resulted in JNK phosphorylation and activator protein 1 (AP-1)-dependent reporter gene induction, indicating that alternative splicing could play a role in the fine-tuning of TLR responses (Janssens et al., 2003).

Sterile alpha and HEAT-Armadillo motifs containing protein (Sarm), is a 690aa TIR-domain containing molecule. The *C. elegans* ortholog of Sarm, TIR-1, has a critical function in fungal defence. Recently however, human Sarm has been assigned a role in negative regulation of Trif-dependent responses (Carty et al., 2006). Overexpression of Sarm inhibits Trif-dependent activation of NF-κB and IRF3

while silencing of endogenous Sarm expression resulted in elevated cytokine and chemokine production following LPS and poly(I:C) stimulation. Sarm expression is highly upregulated by LPS stimulation, leading to a fast downregulation of Trif-dependent signalling. The hypothesis for this negative feedback loop was further supported by the enhanced association of Trif and Sarm after LPS stimulation.

Proteomic analysis of lipid A –stimulated macrophages identified Flightless I homolog (Fliih) as a possible MyD88-interacting molecule. Overexpression of Fliih exerted an inhibitory effect on LPS and Lipid A-induced NF-B activation and siRNA studies in Hek293 cells confirmed this regulatory role of Fliih in TLR4 signalling. Two-hybrid analysis and co-immunoprecipitation studies further revealed that Fliih interacts with TIR domains of both MyD88 and TLR4, thereby directly interfering with TLR4-MyD88 complex formation in a LPS-dependent way (Wang et al., 2006a).

The SOCS protein family consists of eight members: SOCS1 to SOCS7 and CIS. These proteins regulate JAK-STAT signalling, activated by cytokines. Cytokine receptor activation induces SOCS expression, thereby preventing prolonged activation (Starr et al., 1997). All SOCS members are composed of a central Src homology (SH2)-domain, involved in interaction with phosphotyrosine motifs in receptors and signalling molecules and a C-terminal SOCS-box, essential for the recruitment of the ubiquitin machinery. Next to its regulatory role in cytokine signalling, SOCS proteins also regulate some JAK-STAT-independent pathways like insulin, TNF-α and TLR signalling (Morita et al., 2000; De Sepulveda et al., 1999; Kawazoe et al., 2001). SOCS1-deficient mice were highly susceptible to LPS-induced sepsis and no endotoxin tolerance was observed (Kinjyo et al., 2002; Nakagawa et al., 2002). Additionally, SOCS1$^{-/-}$ macrophages produce increased levels of nitric oxide and pro-inflammatory cytokines in response to LPS and SOCS1 overexpression suppresses LPS-induced NF-κB activation in macrophages. Moreover, SOCS expression is induced upon TLR stimulation (Stoiber et al., 1999; Dalpke et al., 2001; Baetz et al., 2004). Taken together, these data suggested a role for SOCS1 as negative regulator of TLR signalling, although no direct target of SOCS1 could yet be identified. However, two groups reported that SOCS1 has an indirect inhibitory effect on TLR signalling, targeting the secondary type I IFN signalling pathway and not the main NF-κB pathway (Baetz et al., 2004; Gingras et al., 2004). Recently, a molecular basis for TLR regulation by SOCS1 was described. Upon TLR2 or TLR4 activation, Mal is phosphorylated by Bruton's tyrosine kinase,

leading to its association with SOCS1. Subsequently, SOCS1 induces polyubiquitination of Mal, leading to its proteasomal degradation (Mansell et al., 2006).

Yeast two-hybrid analysis revealed interaction of the C-terminal domain of TRAF1 with the TIR domain of Trif. Overexpression of Trif caused caspase-8-dependent cleavage of TRAF1. The cleaved N-terminal but not the C-terminal Trif-binding part of TRAF1 inhibits TLR3- and Trif-mediated NF-κB and IRF3 activation. Thus, Trif-induced cleavage of TRAF1 seems required for abolishing Trif signalling (Su et al., 2006). Another member of the TRAF family, TRAF4, interacts with Trif, IRAK1 and TRAF6 and overexpression of TRAF4 leads to the specific inhibition of TLR-induced NF-κB and IRF3 activation, but did not affect TNFR signalling (Takeshita et al., 2005). These results indicate that next to the crucial role of TRAF6 and TRAF3 in activation of NF-κB, other TRAF proteins are involved in negative regulation.

Downstream kinases are targeted for negative regulation as well. IRAK-M, a member of the IRAK serine/threonine kinase family, is upregulated following TLR activation, alluding a regulatory function (Kobayashi et al., 2002). Contrary to IRAK-1 and IRAK-4, IRAK-M lacks kinase activity and its expression is limited to monocytes and macrophages (Wesche et al., 1999). IRAK-M$^{-/-}$ cells responded much stronger to TLR stimulation and similarly, IRAK-M$^{-/-}$ mice exhibited a lowered endotoxin tolerance compared to wild type littermates (Kobayashi et al., 2002). Mechanistically, IRAK-M prevents dissociation of IRAK-1 and IRAK-4 from the activated TLR complex, and as such inhibits IRAK-1-TRAF6 complex formation.

Analogous to MyD88$_s$, some splice variants of IRAK-1 and IRAK-2 are also involved in TLR regulation. Contrary to IRAK-1, IRAK-1c lacks kinase activity and cannot be phosphorylated by IRAK-4 (Rao et al., 2005). IRAK-2c and IRAK-2d on the other hand lack a DD, preventing MyD88 association (Hardy and O'Neill, 2004).

Toll-interacting protein (Tollip) was the first described intracellular regulator of TLR signalling. Originally, it was identified as an IL-1R accessory protein-binding protein using a two-hybrid screen and appears to be involved in recruitment of IRAK-1 to the activated IL-1R complex (Burns et al., 2000). Moreover, a role for Tollip in endosomal targeting of the IL-1R is described (Brissoni et al., 2006). Overexpression

of Tollip also downregulates TLR2 and TLR4-induced NF-κB activation (Zhang and Ghosh, 2002). Next to TLR2 and TLR4 binding, it also interacts with IRAK-1. This association is dependent on the phosphorylation status of both proteins: TLR-induced autophosphorylation of IRAK1 leads to the phosphorylation of Tollip, resulting in a dissociation of the Tollip/IRAK1 complex and facilitating ubiquitination and degradation of IRAK1.

Downstream kinases of the MyD88-independent pathway are also subjected to negative regulation. Recently, SH-2 containing protein tyrosine phosphatase-2 (SHP-2), a widely used tyrosine phosphatase, was described as a specific regulator of TLR3 and TLR4 signalling (An et al., 2006). SHP-2 inhibits TLR3-induced pro-inflammatory cytokine production, independently of its phosphatase activity. Moreover, SHP-2 deficiency results in upregulation of TNFα and IFNβ expression upon poly(I:C) and LPS treatment. Immunoprecipitation studies revealed that SHP-2 directly binds the kinase domain of TBK-1, which could account for the SHP-2-dependent inhibition.

Next to TRAF4, some other inhibitors of TRAF6 are described. These regulators interfere in the polyubiquination of TRAF6, an indispensable step for TLR-induced NF-κB activation. A20, a TNF- and LPS-inducible de-ubiquitinating protein, binds TRAF6, thereby directly removing ubiquitin moieties from TRAF6 and inhibiting its function. In vivo experiments in A20$^{-/-}$ mice established the importance of A20 in endotoxin tolerance (Boone et al., 2004).

Recently, a new member of the A20-binding inhibitors of NF-κB (ABINs) was identified (Wullaert et al., 2007). In analogy with ABIN-1 and ABIN-2, ABIN-3 inhibits TNF and IL-1 induced NF-κB activation. However, much in contrast with other ABINs, ABIN3 expression is enhanced upon LPS stimulation, suggesting a role as negative regulator of TLR4 pathway. In vitro, overexpression of ABIN-3 resulted in a decrease of LPS-induced NF-κB activation and co-expression studies further revealed that ABIN-3 inhibits TLR4-dependent gene expression downstream of TRAF6 but upstream of IKKβ. Moreover, adenoviral gene transfer of ABIN-3 in mice partially protected them against a lethal dose of LPS.

β-arrestins are proteins involved in endocytosis of G-protein coupled receptors (GPCRs), leading to signal attenuation. In addition, they function as scaffold proteins for GPCR-induced activation of MAP kinases. Two members of the β-arrestin family,

β-arrestin-1 and -2, are involved in TLR signalling, exerting somewhat ambiguous functions. Both arrestins were shown to directly bind TRAF6, thereby preventing autoubiquitination of TRAF6 and as a result inhibiting NF-κB and AP-1 activation (Wang et al., 2006b). Much in contrast with this negative regulatory function, β-arrestin-2 was shown to positively regulate ERK-1/2 activation and IL-6 production. Moreover, both β-arrestins upregulated LPS-induced IL-8 expression. Thus, further investigations are needed to clarify the precise functions of β-arrestins in TLR signalling pathways.

Likewise, transcription factors are susceptible to negative feedback. Activation of one of them, IRF3, depends on phosphorylation induced by TLR3 or TLR4 stimulation. Phosphorylation of the Ser339-Pro340 motif results in the association with peptidyl-prolyl-isomerase (Pin1). This interaction induces polyubiquitination and subsequent proteasomal degradation of IRF3, thereby blocking IFN responses (Saitoh et al., 2006).

Expression of IRF4, another member of the IRF family, is upregulated by TLR activation, suggesting a regulatory function in TLR signalling. TLR-induced production of pro-inflammatory cytokines is enhanced in peritoneal macrophages derived from IRF4-/- mice and these mice are hypersensitive to DNA-induced shock. At a molecular level, IRF4 was shown to compete with IRF5, but not with IRF7 for MyD88 association, resulting in inhibition of IRF5-dependent pro-inflammatory gene expression (Negishi et al., 2005).

System biology approaches led to the identification of activating transcription factor 3 (ATF3), a member of the ATF/CREB family of transcription factors, as a negative regulator of TLR signalling (Gilchrist et al., 2006). Transcription and production of IL-6 and IL-12 was markedly enhanced in ATF3^{-/-} macrophages stimulated with LPS. ATF3 inhibits IL-6 and IL-12 transcription by altering chromatin structure in their promoter region, thereby preventing access of NF-κB and AP-1.

Bcl-3, a nuclear member of the IκB family, interacts and stabilizes transcriptional inactive p50 homodimers. A recent study revealed that Bcl3^{-/-} mice were hyperresponsive to TLR stimulation and septic shock (Carmody et al., 2007). Moreover, Bcl-3 was shown to be induced upon LPS stimulation, further adding to a possible regulatory role for Bcl-3 in TLR signalling. Mechanistically, Bcl-3 prevents

ubiquitination and degradation of promoter-associated p50 homodimers, thereby blocking access for transcriptional active NF-κB heterodimers.

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CHAPTER 4: TLR signalling in infectious and non-infectious diseases

Toll-Like receptors are crucial components of the first-line defence against invading pathogens. It is therefore not surprising that aberrant TLR signalling leads to severe disease conditions. Uncontrolled TLR activation will lead to a massive production of pro-inflammatory cytokines, and could eventually lead to septic shock, whereas reduced signalling could lead to a higher prevalence of bacterial or viral infection. However, due to its central position in the interface between innate and adaptive immunity, TLRs are also involved in pathologies unrelated to host defence. These diseases include severe autoimmune diseases like systemic lupus erythematosus or rheumatoid arthritis, asthma, several cardiovascular diseases and even some neuropathologies. The following part summarizes the genetic evidence linking TLRs with the abovementioned diseases. The role of TLR signalling in atherosclerosis, a common disease in western society, is further enlightened. In addition, an overview is given how TLR signalling can be exploited to generate therapeutics for these diseases.

I.   TLR Polymorphisms associated with pathogenesis

Single nucleotide polymorphisms (SNPs) are found in almost every TLR gene and in several downstream mediators, and some of these can be linked with increased or in some cases reduced susceptibility to infectious as well as non-infectious diseases (Table 1). These SNPs can be located either in the promotor region, the introns or the exons of the gene. SNPs affecting the aa sequence of the TLR are found in both the TLR ectodomain as well as the intracellular TIR domain. One of the most extensively studied polymorphism is a co-segregating missense mutation (D299G and T399I) in the extracellular domain of TLR4, which can be found in approximately 5- 10 % of the total population. Patients bearing these patients were shown to be hyporesponsive to inhaled endotoxin and an increased susceptibility to septic shock was observed (Arbour et al., 2000). Over the years, susceptibility to

several diseases was shown to be associated with these TLR4 polymorphisms. A well-documented disease condition affected by these SNPs is respiratory syncytial virus (RSV) infection. For long time it was known that RSV fusion protein also triggers TLR4 (Kurt-Jones et al., 2000). Moreover, it has been demonstrated that a functional TLR4 is indispensable for efficient clearance of RSV in mice (Kurt-Jones et al., 2000). Therefore, it came not as a surprise that several studies demonstrated a clear association of these TLR4 SNPs with an increased risk for RSV infection in infants (Awomoyi et al., 2007; Tal et al., 2004; Tulic et al., 2007). Others disease conditions linked to this TLR4 D299G and T399I polymorphism include a higher susceptibility towards malaria (Mockenhaupt et al., 2006), resistance to Legionnaires' disease (Hawn et al., 2005a), increased risk of prostate cancer (Cheng et al., 2007) and systemic inflammatory response syndrome (Child et al., 2003).

A recently described polymorphism in TLR1, TLR1I602S, exhibits some interesting characteristics. This TLR1 polymorphism is associated with aberrant trafficking of the receptor to the cell surface (Johnson et al., 2007). As a result inflammatory responses to bacterial lipopeptides are impaired (Hawn et al., 2007; Johnson et al., 2007). Surprisingly, a lower incidence of leprosy is observed in persons carrying the I602S allele (Johnson et al., 2007). These findings indicate that recognition of *Mycobacterium leprae* by TLR1/TLR2 is an essential step in the immune evasion of this pathogen.

Two polymorphisms in the TIR domain of TLR2, R753Q and R677W, respectively are associated with an increased susceptibility to tuberculosis and a higher risk of lepromatous leprosy (Ogus et al., 2004; Kang et al., 2002).

In addition, polymorphisms in TLR3, TLR5, TLR6, TLR7, TLR9 and CD14 are associated with several disease states (Verstak et al., 2007).

Gene	SNP	Associated condition/disease	Reference
TLR1	Pro315Leu	Impaired response to bacterial cell wall components	(Omueti et al., 2007)
	Ile602Ser	Impaired cell surface trafficking and functional response / Protection against leprosy	(Johnson et al., 2007; Hawn et al., 2007)
TLR2	T-16933A	Increased prevalence of Gram-positive bacterial cultures and sepsis	(Sutherland et al., 2005)
	Arg677Trp	Increased susceptibility to lepromatous leprosy	(Kang et al., 2002; Bochud et al., 2003)
		Increased susceptibility to tuberculosis in Tunisian patients	(Ben-Ali et al., 2004)
	Arg753Gln	Predisposition to staphylococcal infection	(Lorenz et al., 2000)
		Association with urinary tract infection in children	(Tabel et al., 2007)
		Enhanced risk of developing tuberculosis	(Ogus et al., 2004)
		Association with acute rheumatic fever in children	(Berdeli et al., 2005)
		Association with susceptibility to recurrent bacterial infections	(Kutukculer et al., 2007)
TLR3	C2593T/C2642A/A2690G (intron 3)	Association with type I diabetes in South African blacks	(Pirie et al., 2005)
	Asn284Ile/Leu412Phe	Reduced expression and activity	(Ranjith-Kumar et al., 2007)
	T299698G (exon 3)	Association with Stevens-Johnston syndrome in Japanese population	(Ueta et al., 2007)
TLR4	Asp299Gly	Decreased risk of carotid artery atherosclerosis	(Kiechl et al., 2002)
		Premature birth	(Lorenz et al., 2002a)
		Increased severity of systemic inflammatory response syndrome	(Child et al., 2003)
		Lower susceptibility to myocardial infarction and cardiovascular events	(Holloway et al., 2005; Boekholdt et al., 2003)
		Higher prevalence of asthma	(Fageras Bottcher et al., 2004)
		Possible protection against late-onset Alzheimer's disease	(Minoretti et al., 2006)
	Asp299Gly/Thr399Ile	Association with symptomatic respiratory syncytial virus infection in infants	(Awomoyi et al., 2007; Tal et al., 2004)
		Endotoxin hyporesponsiveness	(Arbour et al., 2000; Lorenz et al., 2002b)
		Association with ulcerative colitis and Crohn's disease	(Torok et al., 2004a; Franchimont et al., 2004)
		Association with periodontal disease	(Schroder et al., 2005)
		Reduced prevalence of diabetic neuropathy in patients with type 2 diabetes	(Rudofsky, Jr. et al., 2004)
		Resistance to Legionnaires' disease	(Hawn et al., 2005a)
		Increased risk of prostate cancer	(Cheng et al., 2007)

TLR5	Arg392STOP	Association with susceptibility to Legionnaires' disease	(Hawn et al., 2003)
		Resistance to systemic lupus erythematosus	(Hawn et al., 2005b; Demirci et al., 2007)
		Heterozygous carriage protects against Crohn's disease in Jewish subjects	(Gewirtz et al., 2006)
TLR6	Ser249Pro	Decreased risk for asthma	(Tantisira et al., 2004; Hoffjan et al., 2005)
TLR7	c.1-G120T (intron 1)	Protection against advanced inflammation and fibrosis in chronic male HCV patients	(Schott et al., 2007)
TLR9	T-1237C (promotor)	Association with Crohn's disease	(Torok et al., 2004b)
		Putative association with atopic eczema	(Novak et al., 2007)
	T-1237C /T-1486C (promotor)	Creation of new potential transcription factor binding sites	(Hamann et al., 2006)
	A1174G (intron 1)	Predisposition to systemic lupus erythematosus in humans homozygous to G allele	(Tao et al., 2007)
	A1174G (intron 1)/ A1635G (exon 2, Pro545Pro)	Rapid progression of HIV-1 infection	(Bochud et al., 2007)

Table 1: TLR receptor polymorphisms and associated conditions.

Downstream signalling adaptors are in a varying degree polymorphic. Mal is the most polymorphic adaptor, and no amino acid altering variations are described for Tram and Trif (Sheedy and O'Neill, 2007). A study by Hawn et al. described 4 Mal polymorphisms present in Vietnamese tuberculosis patients (Hawn et al., 2006). One synonymous mutation (C558T; A186A) was associated with increased susceptibility for tuberculosis. Additionally, TLR2, but not TLR4 agonist stimulation of blood cells of patients homozygous for this mutation, resulted in a decrease in IL-6 production. However, it remains unclear how this variant, of which the aa sequence is not altered, can exhibit a different phenotype and linkage with other functional mutations therefore cannot be excluded. Another study, performed by Khor and colleagues, described a S180L Mal variant, of which heterozygous carriage is associated with protection against tuberculosis, bacteremia, malaria and pneumococcal disease (Khor et al., 2007). Biochemical analysis revealed that MalS180L still interacts with

MyD88 and Mal, but is not capable of TLR2 binding. Since homozygous MalS180L individuals are rarely found, and homozygosity is associated with increased susceptibility for invasive pneumococcal disease, Khor et al. hypothized that MalS180L heterozygosity results in an optimal intermediate immune response.

In addition, IRAK4 and NEMO/IκBα mutations are linked to respectively increased Gram-positive infections and X-linked anhidrotic ectodermal dysplasia with immunodeficiency (Picard et al., 2003; Courtois et al., 2003; Medvedev et al., 2003).

II.   Involvement of Toll-Like receptor signalling in atherosclerosis

Atherosclerosis is characterized by a chronic inflammatory response in the walls of arteries, resulting in the accumulation of lipids and inflammatory cells in the subendothelial space (Libby, 2002). Atherosclerosis is initiated by the retention and aggregation of circulating lipids onto the vessel wall (Skalen et al., 2002). This event results in inflammatory cells infiltration and unregulated lipid uptake by macrophages. Next, these lipid loaded macrophages or foam cells accumulate and mature into lesions characterized by smooth muscle cell infiltration, extracellular matrix formation, calcification and necrosis. Rupture of these lesions can result in an artery-blocking thrombus which can provoke a myocardial infarction or stroke (Bjorkbacka, 2006). Elevated levels of plasma lipids are fundamental for the development of atherosclerosis. In addition, a growing number of reports link Toll-Like receptors signalling to disease development. These recent findings will be summarized throughout the following section.

The first evidence linking Toll-Like receptors and atherosclerosis arose from the observation that various TLRs were expressed in atherosclerotic lesions, mostly on infiltrated macrophages and endothelial cells (Edfeldt et al., 2002). Recent bone-marrow transplantation experiments in mice suggested that only TLRs expressed on cells not of bone-marrow origin contribute to lesion progression (Mullick et al., 2005). In addition it was shown that vascular endothelial cells do not express the adaptor Tram (Harari et al., 2006). These data suggest that TLR-induced progression of

atherosclerosis primary depends on the MyD88-dependent pathways in endothelial cells.

The role of TLR signalling in atherosclerosis was further highlighted by the connection of a TLR4 polymorphism which abrogates signalling, TLR4 Asp299Gly, with decreased susceptibility to atherosclerosis and a lower risk to myocardial infarction and cardiovascular events (Kiechl et al., 2002; Holloway et al., 2005).

The most compelling evidence arose from knockout experiments in atherosclerosis mouse models. Targeted deletion of MyD88 or TLR4 in ApoE$^{-/-}$ mice resulted in a clear reduction of aortic plaques (Michelsen et al., 2004). Additionally, knockout experiments with the atherosclerosis-prone mouse strain Ldlr$^{-/-}$ revealed a role for TLR2 in disease progression (Mullick et al., 2005).

The exact nature of TLR ligands inducing atherosclerosis remains unclarified since both exogenous, derived from pathogens such as HSV or *Chlamidia pneumoniae* as well as endogenous TLR ligands, like minimally modified LDL (MM-LDL), were shown to influence disease onset (summarized in Michelsen and Arditi, 2006). At a molecular level these ligands induce a variety of cell-type dependent responses. These include pro-inflammatory cytokine and chemokine secretion, upregulation of adhesion molecules, secretion of matrix metalloproteases, involved in the degradation of the extracellular matrix and intitiating the adaptive immune response (Bjorkbacka, 2006).

Another TLR-dependent process based on inhibition of Liver X receptor (LXR) activity could as well influence atherosclerosis progression. LXRs are transcriptional regulators of genes like ABCA1 and ABCG1 that promote cholesterol efflux in foam cells via reverse cholesterol transport. Synthetic LXR ligands were shown to reduce atherosclerosis and loss of LXR expression dramatically contributes to disease progression (Naik et al., 2006; Tangirala et al., 2002; Joseph et al., 2002). Various bacterial and viral pathogens inhibit LXR function and cholesterol efflux in foam cells through a Trif-dependent mechanism (Castrillo et al., 2003). This inhibition could trigger lipid accumulation and as a result accelerate atherosclerosis.

These data provide conclusive evidence for the connection between TLR signalling and the progression of atherosclerosis and validates TLR signalling components as possible therapeutic targets for atherosclerotic drug development.

III. Therapeutic Targeting of TLR signalling

During the past years, lots of effort is put in the development of therapeutics which modulate the innate immune system. The clinical applications of these drugs are versatile as discussed above: cancer, allergic diseases, infections and autoimmune diseases. The Toll-Like receptor family is keystone of this innate immunity and as a consequence forms a major target of this research. Both TLR agonists as well as TLR antagonists are assayed for their therapeutic potential.

TLR agonists as vaccine adjuvants

Activation of the innate immune system is primordial for efficient vaccination. Therefore vaccine adjuvants are co-administered that enhance antigen uptake by DCs, resulting in a long-lasting antigen-specific adaptive immune response. Recent findings revealed a clear link between innate and adaptive immune responses, which suggested the possibility of TLR agonists as vaccine adjuvants. Currently, the adjuvant effect unmethylated CpG DNA (TLR9), LPS analogues (TLR4) and imidazoquinoline compounds (TLR7) in HBV, influenza and HIV vaccines are evaluated (Daubenberger, 2007; Wille-Reece et al., 2005; Dupont et al., 2006). Vaccination efficiency can further be enhanced by linkage of antigen to the TLR agonist, which increases the antigen uptake by DCs and by delivery in lipid emulsions, microparticles or virus-like particles (Kanzler et al., 2007).

TLR agonists in treatment of allergic diseases

Asthma and other allergic diseases are characterised by an inappropriate TH_2-response. Therefore, the effect of TLR4 and TLR9 agonist, which induce a potent TH_1-response, was analysed in diverse asthmatic and allergic model systems. TLR induction resulted in a shift of TH_2/TH_1 ratio and consequently diminished allergic symptoms (Creticos et al., 2006; Gauvreau et al., 2006). Drugs based on TLR4/TLR9 agonist combined with modified allergens successfully passed clinical trials and will be marketed soon.

TLR agonists and antiviral therapy

Agonists of TLR3, TLR7/8 and TLR9 are currently being evaluated for the treatment of viral infections. Imiquimod, a TLR7 agonist, is already used in the treatment of genital warts caused by human papilloma virus (Chang et al., 2005). In addition, the therapeutic applicability of these agonists in HCV and HIV treatment is being assayed (Fletcher et al., 2006).

TLR agonists in cancer therapy

As mentioned above, TLR7 agonists are used for the treatment of some virus-induced cancers. Additionally, innate immune activation by CpG DNA results in antitumor activity in rodent models of cancer. This activity is based on activated DC which boosts both NK cells and tumor-specific T cell responses. Promising results of these TLR9 agonists are obtained in clinical trials for colorectal cancer, lung cancer and non-Hodgkin's lymphoma (Kanzler et al., 2007; Paul, 2003).

TLR antagonists as therapeutic tool

In contrast to the therapeutic use of TLR agonists, studies concerning the appropriateness of TLR antagonists in the treatment of inflammatory disorders did not lead to drug development so far. However, the potency of some TLR antagonists is apparent. Currently, only the extracellular part of TLRs is targeted either by the use of neutralising antibodies or by the use of structural analogues of TLR agonists, capable of binding the receptor but inhibiting further signalling (Meng et al., 2004; Bennett-Guerrero et al., 2007). At present, two antagonistic analogues of Lipid A, the TLR4-activating part of LPS, are currently in phase 3 trials for bacterial sepsis (Rossignol and Lynn, 2005). Moreover, excessive non-bacterial inflammation, observed in some autoimmune diseases like Systemic lupus erythematosus, can be dampened by TLR7/TLR9 antagonists (Barrat et al., 2005). Growing evidence predict a therapeutic use of TLR antagonists in other inflammatory diseases, including atherosclerosis, asthma, rheumatoid arthritis and inflammatory bowel disease (Gearing, 2007).

Next to targeting the TLRs, inhibition of downstream signalling molecules is also considered as a therapeutic target (Bartfai et al., 2003). Several peptides based on

the BB loop of the TIR domain of MyD88 were shown to block further signalling (Bartfai et al., 2003; Loiarro et al., 2007). However, the potency of these types of inhibitory components was not yet assayed in a clinical trial at present.

IV. References

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CHAPTER 5: Other pattern-recognition receptors

I.   Dectin-1

Control of fungal infection is mediated by TLR2 and TLR4, which respectively recognize the yeast cell wall components zymosan/phospholipomannan and mannan (Jouault et al., 2003; Netea et al., 2006; Tada et al., 2002; Underhill et al., 1999). Recently however, other 'fungi-sensing' PRRs were identified (Brown, 2006; Dennehy and Brown, 2007). One of these, Dectin-1 is a member of the lectin-receptor family and was shown to detect β-1,3-glucan, which represents up to 50% of the fungal cell wall (Brown and Gordon, 2001; Palma et al., 2006). Extracellularly it is composed of a ligand binding single C-type lectin-like domain while an intracellular ITAM (immunoreceptor tyrosine-based activation)-like motif is responsible for further signal transduction. Originally, Dectin-1 expression was thought to be restricted to dendritic cells (Ariizumi et al., 2000), but high levels of Dectin-1 can also be found on macrophages, B and T lymphocytes (Taylor et al., 2002; Willment et al., 2005). Fungal recognition by Dectin-1 results in the production and secretion of inflammatory cytokines, phagocytosis and oxidative burst (Rogers et al., 2005; Underhill et al., 2005; Herre et al., 2004).

Signal transduction starting from Dectin-1 can be divided into a Syk (Spleen tyrosine kinase)-dependent and Syk-independent pathway (Fig.1). Fungal triggering of Dectin-1 leads to tyrosine phosphorylation of the ITAM-like motif, mediated by Src kinases, and the recruitment of Syk kinase through one of its SH2-domains. This activated Syk kinase is thought to activate phospholipase C-γ (PLC-γ), which produces diacylglycerol (DAG). Protein kinase C (PKC) is activated by this DAG and in turn activates Card 9 (Caspase-recruitment domain 9) (Dennehy and Brown, 2007; Gross et al., 2006; Hara et al., 2007). Phosphorylated Card 9 forms a docking site for the Bcl-10/MALT1 complex. This complex activates the IkB kinases leading to the activation of NF-κB and the induction of IL-10 and TNF-α. However, Dectin-1 mediated phagocytosis of zymosan, a β–glucan rich particle of the yeast cell wall, does not require Syk kinase, but the exact mechanism remains to be elucidated (Underhill et al., 2005; Herre et al., 2004).

The importance of Dectin-1 in fungal control was further underscored by the generation of Dectin-1$^{-/-}$ mice, which were more susceptible to *Candida albicans* and *Pneumocystis carini* infections than their wild type littermates (Taylor et al., 2007), although these data could only partially be confirmed by another group (Saijo et al., 2007).

Next to Dectin-1, TLR signalling pathways are also involved in the mammalian control of fungal infection. Moreover, efficient production of pro-inflammatory cytokines including TNF-α and IL-12 requires both Dectin-1 and TLR2 (Gantner et al., 2003; Brown et al., 2003). Although the exact mechanisms of this synergy remain unknown, the adaptor molecule TRAF6 likely provides a link between both signalling pathways (Dennehy and Brown, 2007).
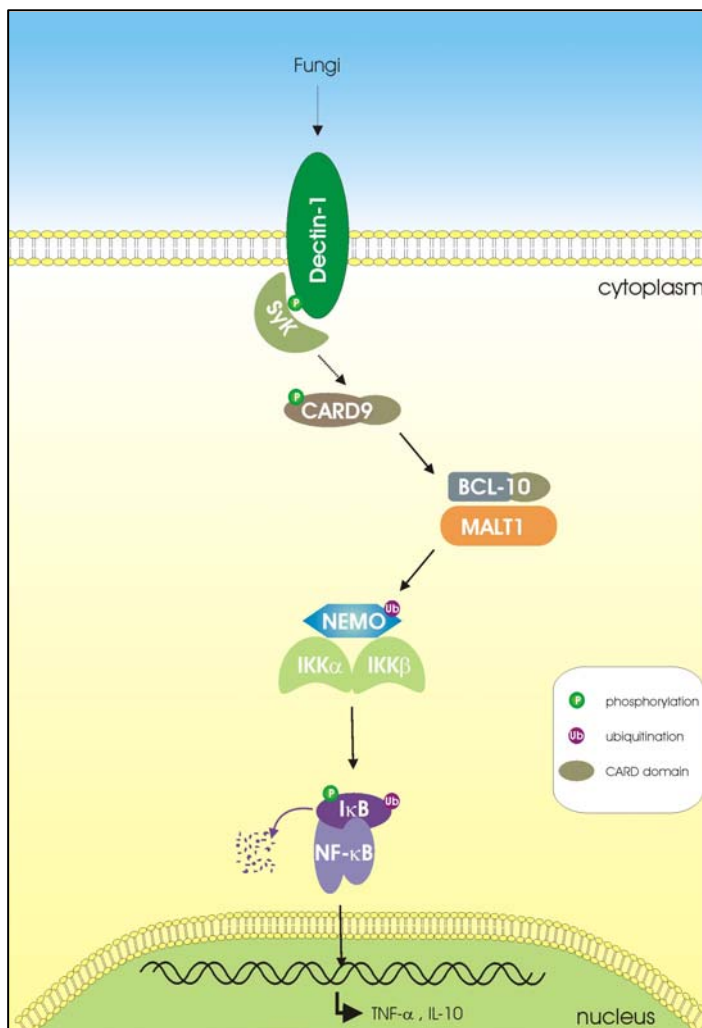
Fig.1: The Syk-dependent Dectin-1 signalling pathway. β-1,3-glucan recognition by Dectin-1 leads to the phosphorylation of its intracellular ITAM-like motif, forming a docking site for Syk kinase. Subsequent activation of Syk kinase leads to the phosphorylation of Card9, which in turn recruits the Bcl-10/MALT1 complex, resulting in the activation of NF-κB en the production of TNF-α and IL -10.

II.   RIG-I-Like receptors

The role of TLRs in antiviral response is well defined. TLR2 and TLR4 detect viral PAMPs at the cell surface whereas TLR3, TLR7/8 and TLR9 recognise different viral PAMPs in endosomes. Viral triggering of these TLRs results in the induction of type I interferons. However, replication of viruses in the cytoplasm can not be sensed by the TLR system. This void is filled by two cytoplasmic PRRs, retinoic-acid-inducible protein I (RIG-I) and Melanoma differentiation associated gene 5 (Mda5) or Helicard (Yoneyama et al., 2004; Andrejeva et al., 2004). Both RIG-I and Mda5 are able to detect cytoplasmic dsRNA. N-terminally they are composed of two CARD domains, necessary for downstream signalling, and a C-terminal DExD/H box helicase domain, involved in dsRNA binding. The RIG-I homologue Lgp2 also contains this helicase domain but lacks the CARD domains, and acts as a negative regulator of RIG-I and Mda5 (Rothenfusser et al., 2005; Komuro and Horvath, 2006; Saito et al., 2007).

Overexpression of RIG-I blocks replication of Vesicular Stomatitis Virus (VSV) and ECMV and conventional dendritic cells and fibroblasts of RIG-I$^{-/-}$ mice are unable to produce inflammatory cytokines and type I IFNs upon NDV, VSV and Sendai Virus infection (Kato et al., 2005; Kato et al., 2006; Yoneyama et al., 2005). Mda5 on the other hand seems to be important in the response against picornaviruses, like Encephalomyocarditis Virus (EMCV) and the synthetic dsRNA analogue poly(I:C) (Kato et al., 2006; Gitlin et al., 2006). RIG-I detects and directly binds the 5'-end of certain viral RNA genomes, in particular a 5'-triphosphate group. Such 5'-triphosphates are generally removed from or capped in host RNA species, enabling discrimination between self and non-self RNA (Pichlmair et al., 2006; Hornung et al., 2006). The binding properties of Mda5 on the other hand still remain elusive.

RIG-I and Mda5 signal transduction starts with the recruitment through homotypical CARD domain interactions of the mitochondrion-associated protein 'interferon promoter stimulator' (IPS-1), also known as virus-induced signalling adaptor (VISA), mitochondrial antiviral signalling protein (MAVS) or CARD adaptor inducing IFNβ (Cardif) (Kawai et al., 2005; Meylan et al., 2005; Xu et al., 2005; Seth et al., 2005) (Fig.2). Recently it was shown that this RIG-I/IPS-1 association, but not

the Mda5/IPS-1 interaction, depends on virus-induced ubiquitinylation of the RIG-I CARD domain by the ubiquitin ligase TRIM21 (Gack et al., 2007). The IPS-1 forms a branching point between two pathways either resulting in the induction of IRFs or NF-κB. On the one hand, IPS-1 interacts with TRAF3, which in turn engages the TBK1/IKKi complex, resulting in the phosphorylation and activation of IRF3 and IRF7 and ultimately leading to the production of type I IFNs (Saha et al., 2006; Kawai et al., 2005). On the other hand, IPS-1 is also able to interact with FADD and RIP-1 (Kawai et al., 2005; Balachandran et al., 2004). This results in the activation of IKK/NF-κB pathway and the production of inflammatory cytokines. Recently, a crucial role for caspase-8 and caspase-10 in dsRNA-induced NF-κB activation was described, although the mechanistics of their involvement remain to be elucidated (Takahashi et al., 2006).

In analogy with viral dsRNA, viral dsDNA is also detected in the cytoplasm in a TLR-independent manner. The subsequent induction of type I IFNs seems to be dependent on IPS-1 and the IKKi/TBK1 complex but not on RIG-I, which suggested the existence of a cytoplasmic viral DNA-sensing PRR (Ishii et al., 2006). However, another group observed an IPS-1 independent IFN induction following viral DNA triggering (Sun et al., 2006). Recently, such a cytoplasmic DNA sensor was described (see below) (Takaoka et al., 2007), but further experiments are still needed to clarify the role of IPS-1 in cytoplasmic DNA-induced IFN production.
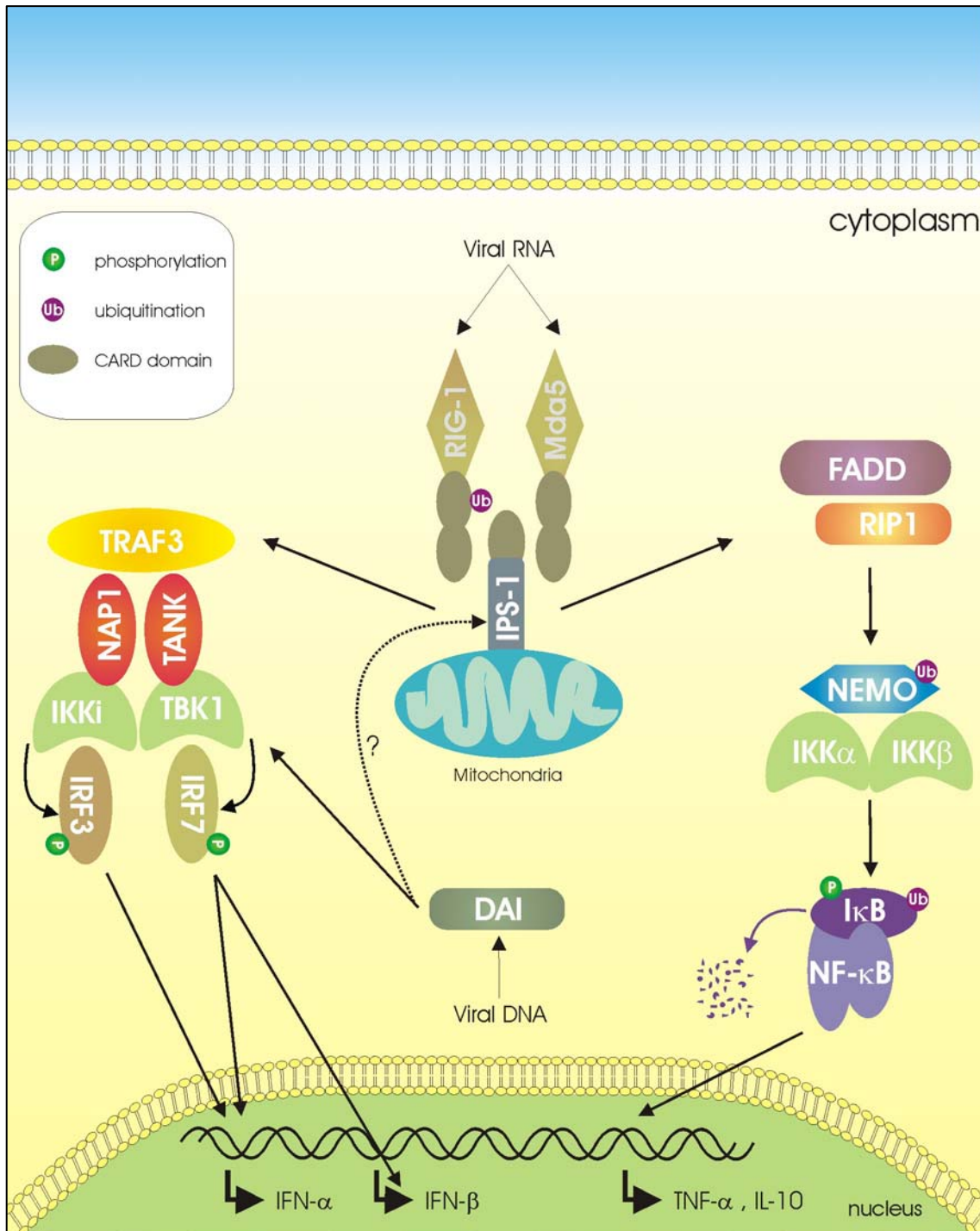
Fig.2: Cytoplasmic detection of viral RNA and DNA through RIG-I-Like receptors and IPS-1. Upon viral RNA sensing, RIG-I and Mda5 associate with the mitochondrion-associated molecule IPS-1 through homotypical CARD domain interactions. Starting from IPS-1, two signalling cascades are activated ultimately leading to the activation of IRFs and NF-κB respectively. Activated IPS-1 engages TRAF3 and the TBK1/IKKi complex, resulting in phosphorylation and activation of IRF3 and IRF7. Additionally, IPS-1 activates the IKK/NF-B pathway, a proces mediated by the FADD/RIP1 complex. Viral DNA is sensed by DAI, which results in the association with TBK1 and the activation of IRF3.

III. DAI

Over the past years, several reports described the immunogenic nature of cytosolic DNA. Detection of this intracellular pathogenic DNA was shown to be TLR9-independent and resulted in type I IFN induction (Hochrein et al., 2004; Ishii et al., 2006; Stetson and Medzhitov, 2006). These findings suggested the presence of one or more cytosolic DNA sensors. Recently, Takaoka and colleagues described DNA-dependent activator of IFN-regulatory factors (DAI) as a candidate DNA sensor (Takaoka et al., 2007). DAI was first reported as a gene induced in host tissues in response to tumours (Fu et al., 1999) and was later reported to bind Z-form DNA (Ha et al., 2006), although the functional relevance hereof remained elusive. FRET analysis and pull-down assays revealed a direct interaction between B-form DNA and DAI and this association induces DAI/TBK1/IRF3 complex formation, resulting in the activation of the latter (Takaoka et al., 2007). Consistently, knock-down of DAI abolished IRF3 activation and concomitant IFNβ gene induction in response to viral, bacterial, mammalian and B-form DNA, establishing the importance of DAI in DNA-induced innate immune responses.

IV. NOD-Like receptors

Intracellular pathogen invasion is, next to the RIG-I-like family of receptors, also monitored by a second group of cytosolic 'pathogen-sensing' proteins, the NOD-Like receptor (NLR) family (Martinon and Tschopp, 2005; Inohara et al., 2005). NLRs can be split up in two major sub-classes: the NOD (nucleotide-binding oligomerisation domain) group of proteins (NOD1-5) and the NALP (NACHT-, LRR-, and pyrin-domain-containing proteins) series of proteins (NALP1-14). Three other proteins, IPAF, CIITA and NAIP5 complete the NLR family. All 22 NLR members contain C-terminal LRRs, a nucleotide-binding and oligomerisation domain (NACHT domain) and an N-terminal effector domain (Fig.3). NOD receptors signal through CARD domains, while NALPs contain a Pyrin motif. NAIP5 is the only NLR using a string of 3 baculoviral inhibitor of apoptosis repeats (BIRs) as a signalling domain. Ligand binding to these NLRs induces NACHT-dependent oligomerisation, ultimately leading to the production of inflammatory cytokines and activation of caspase-1 and the inflammasome (Meylan et al., 2006).
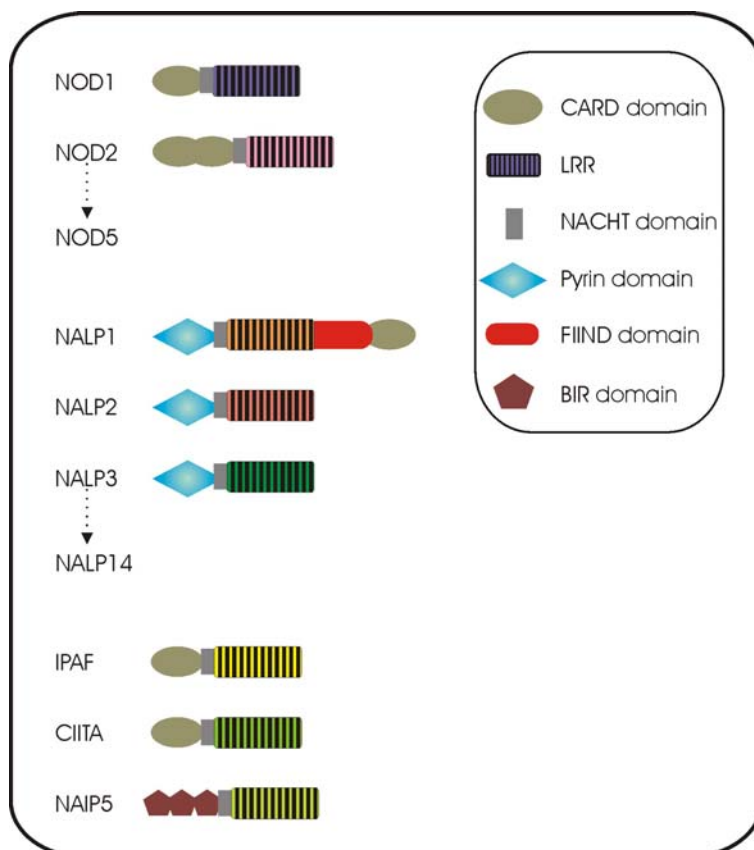
Fig.3: NOD-Like receptor family
(adapted from (Meylan et al., 2006)).
Based on structural features, NOD-Like receptors are divided in two subclasses: NOD proteins (NOD1→5) and the NALP family (NALP1→14). IPAF, CIITA and NAIP5 complete the NLR family.

1. NOD proteins

NOD1 and NOD 2 both sense bacterial peptidoglycan (Girardin et al., 2003b; Girardin et al., 2003a; Viala et al., 2004), although the exact nature of their ligands differs. NOD2 detects muramyl dipeptide (MDP), which is found in the cell wall of both Gram-positive and Gram-negative bacteria. NOD1, on the other hand, exerts a more restricted pathogen recognition pattern, as it responds to meso-diaminopimelic acid (DAP), which is limited to Gram-negative peptidoglycan. Recognition of these ligands through their LRRs results in homo-oligomerisation and activation of NOD proteins and leads to a CARD-dependent recruitment of the serine-threonine kinase RIP2 or CARD9 and the subsequent activation of respectively NF-κB and MAPKs (Fig.4) (Viala et al., 2004; Hsu et al., 2007; Inohara et al., 2003).

The importance of NOD proteins in innate response becomes apparent by the growing number of autoimmune diseases linked to NOD polymorphisms. Mutations in the NOD2 gene are linked with severe inflammatory diseases such as Crohn's disease, Blau syndrome and early-onset sarcoidosis. Moreover, predisposition to inflammatory bowel disease and asthma is observed in patients with NOD1 mutations (McGovern et al., 2005; Hysi et al., 2005). The precise role of NOD proteins in these disorders is far from understood, as contradictory effects of these mutations are observed (see box1).
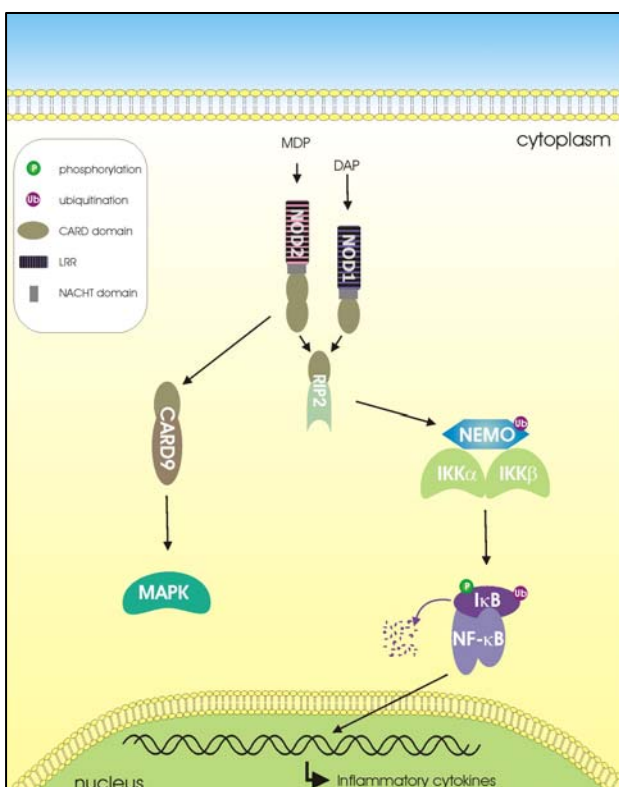
Fig.4: NOD signalling. NOD proteins recognize different parts of the bacterial cell wall component peptidoglycan; NOD1 binds meso-diaminopimelic acid (DAP) while NOD2 senses muramyl dipeptide (MDP). Upon ligand recognition, NOD proteins associate with RIP2 and CARD9 through homotypic CARD domain interactions, leading to the activation of MAPK and NF-κB.

Text box: Role of NOD2 in Crohn's disease

Crohn's disease is, next to ulcerative colitis, one of the two major forms of inflammatory bowel disease. It is characterised by severe intestinal inflammation, associated with increased production of TNF-α and IL-1β in the lamina propria. As a result, patients suffer from fever, abdominal pain, diarrhoea and weight loss.

More than 60 NOD2 variants are reported, of which 3 mutant alleles (R702W, G908R and L1007fs) account for 50% of all patients (*1*). All three mutations affect the C-terminal LRR region of NOD2 (a single amino acid is altered for R702W and G908R, whereas the L1007fs allele bears a frame-shift mutation, resulting in a truncated form of NOD2, of which the last 33 amino acids are missing. Heterozygosity for one of these mutated alleles, results in a 2- to 4 –fold increased risk for Crohn's disease, whereas a up to 40-fold increase in risk is observed with homozygous patients (*2*). However, NOD2 mutations are neither sufficient nor necessary to develop Crohn's disease, as mutated NOD2 alleles are found in healthy individuals and 60%-70% of Crohn's disease patients do not have a mutated form of NOD2. So, next to NOD2 mutations, other genetic and environmental factors are involved in Crohn's disease pathogenesis (*3*).

Cultured macrophages and monocytes from Crohn's disease patients, homozygous for the $NOD2^{L1007fs}$ mutation, do not respond to muramyl dipeptide (MDP), the common NOD2 ligand (*4*). Moreover, macrophages of $NOD2^{-/-}$ mice neither produce IL-1β or TNF-β upon MDP stimulation (*5*). These data conflict with the severe inflammation observed in Crohn's disease patients. However, macrophages derived from $NOD2^{L1007fs}$ knock-in mice, exhibit an increased level of NF-κB activation and pro-inflammatory cytokine production following MDP administration (*6*). Apparently, the molecular basis of NOD mutations as a predisposing risk factor for Crohn's disease is far from elucidated, and interpretation is further complicated by the clear synergy between NOD2/NOD1 and NOD2/TLR signalling pathways (*5, 7*).

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2. NALP proteins

NALP proteins are major constituents of the multiprotein inflammasome complex. At present, fourteen human NALPS have been identified, but only two types of 'NALP-inflammasomes' are characterised (Martinon et al., 2002; Agostini et al., 2004). The NALP1 inflammasome consists, next to NALP1, of Caspase-1/Caspase-5 and Asc. (Apoptosis-associated spec-like protein containing a CARD), whereas the NALP3 inflammasome is composed of NALP3, Asc, Cardinal and Caspase-1. Asc is a central adaptor molecule in the inflammasome linking NALP with Caspase-1 through homotypic CARD and Pyrin domain interactions. NALP3 misses, compared to NALP1, a C-terminal CARD and FIIND domain. This lack is by-passed through Cardinal, a molecule with an analogous domain structure as the C-terminal region of NALP1 (Fig.5).

Not only PAMPs, like MDP from bacterial PG or bacterial and viral RNA, but also host danger signals can activate the NALP3 inflammasome (Martinon et al., 2004; Kanneganti et al., 2006b; Kanneganti et al., 2006a). These danger-associated molecular patterns or DAMPs include K^+ efflux, induced by high concentrations of ATP or the *Staphylococcus aureus* α-toxin, and monosodium urate crystals, a danger signal from dying cells (Martinon et al., 2006; Mariathasan et al., 2006). Anthrax lethal toxin, on the other hand, is the only activator of the NALP1 inflammasome known thus far (Boyden and Dietrich, 2006). Activation of the inflammasome complex leads to the caspase-1-dependent processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18 and, at least in some cell types, to cell death (Petrilli et al., 2005; Martinon et al., 2002).

Excessive or uncontrolled release of IL-1β results in systemic inflammation and periodic fever (Aganna et al., 2002; Agostini et al., 2004; Hoffman et al., 2001; Feldmann et al., 2002). Therefore, production, processing and secretion of IL-1β are strictly controlled processes (Burns et al., 2003). To elicit an efficient IL-1β release, at least two distinct danger signals are required. At first, TLR activation induces the production of pro-IL-1β, whereas a second stimulus, e.g. K^+ efflux, activates the inflammasome. Thus, both an external as well as an internal stimulus are indispensable for IL-1β secretion, avoiding innate responses against commensals. In addition, different proteins, like COP, ICEBERG, INCA and pyrin, inhibit caspase-1

function, either by interference of caspase-1 recruitment to the inflammasome or through direct inhibition of caspase-1 activity (Lee et al., 2001; Lamkanfi et al., 2004; Humke et al., 2000; Chae et al., 2006). Recent findings describe a role for NOD proteins in this two-step model, as MDP-induced IL-1β release is dependent on both NOD2 and NALP3, although the exact modalities of this synergy are yet to be unravelled (Pan et al., 2007).
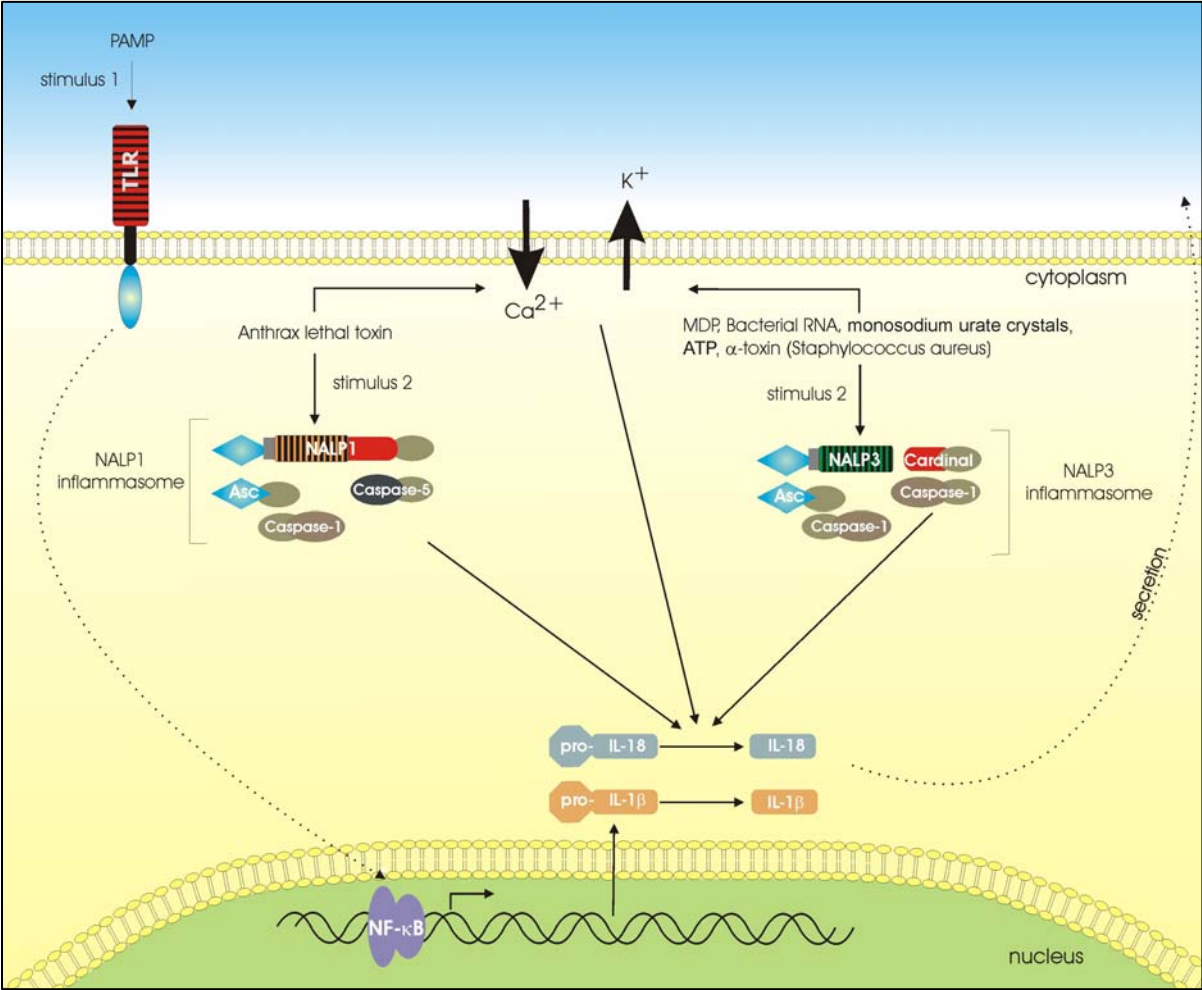
Fig.5: Inflammasome mediated IL-1β release. At least two disctinct stimuli are required for efficient IL-1β secretion. At first, an extracellular stimulus results in the expression of pro-IL-1β, a proces often mediated by TLR ligands. Next, an intracellular DAMP activates the inflammasome and induces K+ efflux, resulting in the maturation and secretion of IL-1β.

3. Other NLRs

Genetic studies using the A/J mouse strain revealed the importance of neural apoptosis inhibitory protein 5 (NAIP5, also called birc1e) in pathogen clearance. Polymorphisms in the NAIP5 locus of these mice results in a greater susceptible to *Legionella pneumophila* infection (Wright et al., 2003; Diez et al., 2003). Recently it was shown that NAIP5 recognises flagellin, resulting in the induction of caspase-1-dependent cell death in macrophages (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). In addition, IL-1β converting enzyme (ICE) protease activating factor (IPAF) is also required for *Legionella pneumophila* growth restriction. NAIP5 and IPAF physically interact, supporting the hypothesis that NAIP5/IPAF cooperate in a complex (Zamboni et al., 2006). The observation that IL-1β release but not bacterial growth is impaired in Asc-deficient macrophages following *Legionella* infection, reveals an unique function of the NAIP5/IPAF complex in pathogen clearance, but the exact modalities remain to be elucidated (Zamboni et al., 2006; Mariathasan et al., 2004).

V.   References

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Scope of the thesis

The Toll-Like receptor family is an essential component of the vertebrate innate immune system. Not only are TLRs important in the activation of innate immune cells like macrophages and neutrophils, but TLR signalling also activates and shapes the subsequent antigen-specific adaptive response. The clinical importance of TLRs is reflected by a number of disease states associated with aberrant TLR signalling including endotoxic shock, bacterial sepsis and autoimmune diseases like systemic lupus erythematosus. Thus, TLR activation is a double-edged sword, balancing between effective pathogen clearance and excessive inflammation. Therefore, several mechanisms have evolved that dampen TLR responses, preventing prolonged and harmful inflammation. TLR signalling is attenuated either by preventing ligand binding, by downmodulation of TLR expression or by inhibition of downstream signalling. The cardinal role of TLRs in mammalian innate immunity makes of them valuable therapeutic targets. Currently, TLR agonists as well as TLR antagonists are tested for their therapeutic potential in cancer, infections, allergic and autoimmune diseases.

The MAPPIT system is a mammalian two-hybrid technique that was developed in our laboratory. Its mammalian context is a major asset for unravelling signalling pathways as reflected by several studies on different type I cytokine receptors. Several aspects of erythropoietin, leptin and growth hormone receptor signalling pathway were successfully studied. The main objective of this thesis was the adaptation of the MAPPIT technique into a TLR context. This work mainly focuses on TLR adaptors and downstream kinases, which both are involved in the initial steps of TLR signalling. Throughout this thesis we aimed at providing a better insight in the adaptor recruitment by various TLRs and as such provide a molecular basis for some questions raised by adaptor knock-out studies. In addition, the role for the SOCS protein CIS in TLR adaptor protein regulation was also assayed. The rationale of this research was the growing evidence that SOCS proteins are involved in negative regulation of TLR signalling.

Using MAPPIT we could demonstrate a 'signalling interaction cascade' from TLR4 to the downstream IRAK kinases (Part II, chapter 1). Moreover, we generated a TLR-adaptor interaction map (Part II, chapter 2). In that way, we could confirm several well-documented interactions and we also found some new interactions. We showed that MAPPIT can detect indirect associations and these MAPPIT data could be confirmed by coimmunoprecipitation studies (Part II, chapter 2).

Secondly, we identified CIS, a member of the SOCS protein family, as a MyD88-interacting protein (Part II, chapter 3). The role of SOCS proteins in attenuation of cytokine receptor signalling is well established and recently SOCS1 was identified as a negative regulator of TLR signalling. Our data suggest a role for CIS in the regulation of MyD88-dependent signalling. We also observed a differential binding modus of CIS between cytokine receptor association and MyD88 interaction, reflected by a selective dependency of a conserved tyrosine in the SOCS-box domain of CIS.

PART 2: Results

CHAPTER 1: Mammalian protein-protein interaction Trap (MAPPIT)

I. Tools to study Protein-Protein interactions

Interactions between proteins form the basis of most cellular processes. Protein complexes can form stable structures such as ribosomes or the nuclear scaffold. However, a particular cellular context results in a different cellular response. The protein-protein interactions involved in the regulation and control of these cellular processes are therefore temporary and often dependent on the modification of one or more interaction partners.

The generation of an interaction profile of a given protein substantially contributes to its functional characterisation in a particular cellular context. Therefore, a wide spectrum of both biochemical and genetic methods for studying protein complexes has been developed. Biochemical techniques rely on the co-purification and co-precipitation of interaction complexes. Classical examples are coimmunopreciptiation, chromatography-based methods and tandem affinity purification (TAP). The latter technique makes use of a bait protein linked to a dual affinity-tag. Following cellular expression of the bait protein, cell lysis and a two-stage purification step, protein complexes are separated by gel electrophoresis. Subsequent identification is based on mass spectrometry and database searches (Puig et al., 2001; Rigaut et al., 1999). TAP analysis has been successfully applied to purify yeast, plant as well as mammalian protein complexes (Gavin et al., 2006; Rohila et al., 2004; Bouwmeester et al., 2004). Over the years, several adaptations of the original protocol were described, which significantly improved identification efficiency either by optimizing the dual-affinity tag (Burckstummer et al., 2006) or by RNAi knockdown of the endogenous bait protein (Forler et al., 2003). A major asset of the technique is that it allows detection of protein complexes in whole. However, the indispensable lysis step can result in the loss of weak interactions. Moreover, this removal of proteins from their cellular environment allows interaction between differentially localised proteins, which could lead to a number of false positive interactions.

Genetic approaches rely on hybrid bait and prey molecules designed in such a way that their interaction leads to a detectable signal. The yeast two-hybrid method is based on reconstitution of a transcription factor (Fields and Song, 1989). Herein, bait

and prey are genetically fused to either the DNA-binding domain or the transcription activation domain of a transcription factor. Interaction of bait and prey restores transcriptional activity, resulting in the activation of a reporter gene. The yeast-two hybrid system is the most widely used genetic assay to identify protein interactions and has been applied for high-throughput interaction mapping. A proteome-wide interactome has been accomplished for *Saccharomyces cerevisiae* (Ito et al., 2001), *Drosophila melanogaster* (Giot et al., 2003) and *Caenorhabditis elegans* (Reboul et al., 2003) and a human interaction map is underway (Rual et al., 2005; Stelzl et al., 2005). However, the technique suffers from some major draw-backs. Functional complementation is carried out in the nucleus. Hence, failure in nuclear localisation of either bait or prey results in false negatives. Moreover, the yeast environment is not favourable for interactions of higher eukaryotes, which often depend on post-translational modifications. These limitations can in part be circumvented by the use of mammalian cell systems.

In analogy with the original yeast protocol, the mammalian two-hybrid strategy is based on transcription factor complementation and accordingly is dependent on a nuclear localisation of bait and prey (Dang et al., 1991). In the past years several other mammalian systems have been described allowing detection of protein interactions in their natural environment. These methods include the mammalian Ras recruitment system, split-ubiquitin approaches, protein-splicing based assays, MAPPIT and reporter enzyme complementation systems (reviewed in Eyckerman and Tavernier, 2002; Lievens et al., 2006). In the latter technique, distinct non-functional protein fragments of reporter enzymes like β-galactosidase, luciferase or dihydrofolate reductase, are fused to bait and prey. Upon bait-prey interaction, the reporter protein is reassembled, leading to its functional reconstitution.

Some techniques can be used to visualize interactions in real time, of which fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) are the principle ones (Jares-Erijman and Jovin, 2003; Hu et al., 2002). FRET detection is based on the energy transfer between two fluorophores that are brought in close proximity by association of the fused proteins, whereas BiFC depends on the formation of a bimolecular fluorescent complex, facilitated by the interaction of proteins linked to the non-fluorescent constituents of this complex.

Mammalian Protein-Protein interaction Trap (MAPPIT) is a type I cytokine-receptor based mammalian two-hybrid technique that was developed in our

laboratory (Eyckerman et al., 2001). The following section overviews the MAPPIT technique and its analytical application in TLR signalling.

II. References

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III.   MAPPIT

MAPPIT (Mammalian Protein-Protein Interaction Trap) analysis of early steps in TLR signalling

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running head: MAPPIT analysis of TLR signalling

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Abstract

The mammalian protein-protein interaction trap (MAPPIT) is a two-hybrid technique founded on type I cytokine signal transduction. Thereby, bait and prey proteins are linked to signalling deficient cytokine receptor chimeras. Interaction of bait and prey and ligand stimulation restores functional JAK-STAT signalling, which ultimately leads to the transcription of a reporter or marker gene under the control of the STAT3-responsive rPAP1 promoter. In the subsequent protocol we describe the use of MAPPIT to study early events in TLR signalling. We here demonstrate a 'signalling interaction cascade' from TLR4 to IRAK-1.

Key words

MAPPIT, two-hybrid, JAK-STAT, cytokine signal transduction, TLR

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1. Introduction

Monitoring interaction partners of a given protein is often a major step in revealing its biological role. Therefore, a wide spectrum of biochemical and genetic methods for studying protein-protein interactions has been developed. For a recent general review, we refer to Lievens *et al.* (1). The Mammalian Protein-Protein Interaction Trap (MAPPIT) (2) was used in this protocol to map TLR signalling from TLR4 to IRAK-1. MAPPIT is a two-hybrid assay based on type I cytokine signal transduction. Ligand-induced activation and reorganisation of type I cytokine receptors leads to cross-phophorylation and activation of receptor-associated cytosolic Janus Kinases (JAKs). Subsequently, these activated JAKs phosphorylate conserved tyrosine motifs in the cytoplasmic tail of the receptor, which become docking sites for signalling molecules like Signal Transducers and Activators of Transcription (STATs). Upon recruitment, STATs are phosphorylated by the JAKs and those activated STATs translocate as dimers to the nucleus, where they induce specific gene transcription (**Fig 1A**). In MAPPIT, a C-terminal fusion of a given 'bait' protein with a leptin receptor that is deficient in STAT3 recruitment is made. The 'prey' protein on the other hand is linked to a series of 4 functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand stimulation leads to phosphorylation of the prey chimeras resulting in STAT3 recruitment, activation and translocation to the nucleus leading to induction of a STAT3–responsive luciferase reporter (rPAPI-Luci) (**Fig 1B**).

The mammalian cell context of the assay is a major asset compared to the classical yeast-two hybrid method. This near-optimal physiological environment encompasses the need for posttranscriptional modification, often crucial in mammalian protein-protein interactions. For example, JAK2 target sites in the bait protein were found to be phosphorylated upon receptor activation, further underscoring the relevance of using MAPPIT to study signalling pathways. Serine phosphorylation-dependent interactions could also readily be detected using heteromeric MAPPIT (3), whereby kinase and substrate were fused to one of both heteromeric receptor chains.

Intrinsic to the MAPPIT setup, interactor and effector zones are physically separated respectively in the cytosol and the nucleus since signal readout is mediated by endogenous STAT molecules. In that way, direct interference of bait and prey chimeras with transcription of the reporter gene is avoided. Moreover, signals are cytokine-stimulation dependent, allowing us to discriminate for ligand-independent false positives.
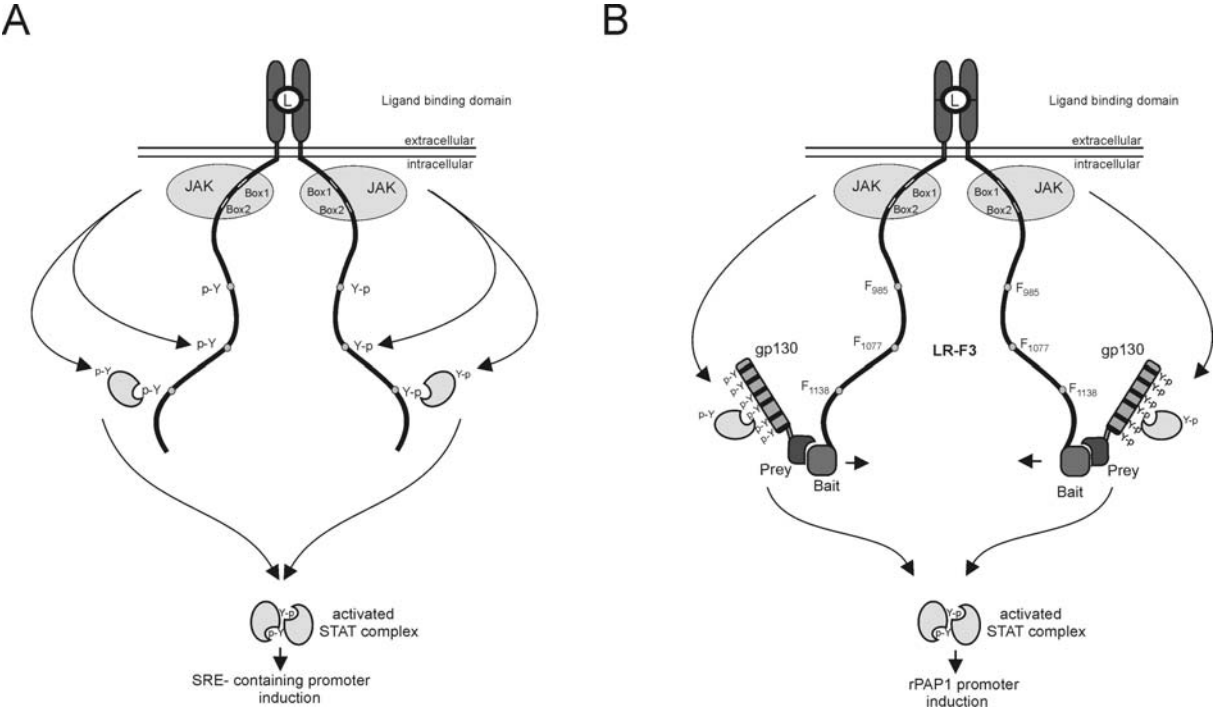
Figure 1:
(A) Schematic overview of the JAK-STAT signal transduction pathway (see main text). L, Ligand. SRE, STAT-responsive element.
(B) MAPPIT principle. A given 'bait' is C-terminally fused with a leptin receptor that is deficient in STAT3 recruitment. The extracellular domain of either the erythropoietin (EpoR) or of the leptin receptor (LR) can be used. The 'prey' protein is linked to a series of 6 functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand stimulation leads to STAT3 activation and induction of a STAT3–responsive luciferase reporter (rPAPI-Luci).

The potency of the analytical use of the MAPPIT technology is reflected by a growing number of studies on different type I cytokine receptors. For instance, aspects of erythropoietin (4), leptin (5;6) and growth hormone (7) receptor signalling have been extensively studied. Next to these type I cytokine receptors, this method can also be used to study some aspects of Toll-Like Receptor signalling (8), as described in this protocol.

Based on this MAPPIT technology, a reverse two hybrid system has been developed (reverse MAPPIT) (9), which allows relatively easy discovery and analysis of disruptor molecules. Using reverse MAPPIT in a TLR context could lead to the development of therapeutics alleviating the consequences of uncontrolled TLR stimulation like excessive inflammation, autoimmunity and septic shock.

2. Materials

2.1 MAPPIT vectors

1. pXP2d2-rPAP1-luci (pXP2d2 is a gift from Dr. S. Nordeen, Colorado Health Sciences Center, Department of Pathology, Denver, CO, 80262, USA, steve.nordeen@uchsc.edu).
2. pCEL4f, pCLL2f (derived from pcDNA5/FRT vector; Flp-In System, Invitrogen) and pMG2 vectors (derived from pMet7 (2)).

2.2 Analytical Application of MAPPIT

2.2.1 Seeding of cells.

1. Hek293T cell line (available from ATCC).
2. Growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf Serum
3. Gentamycin and Penicillin/Streptomycin Solution (Invitrogen).

2.2.2 Transient $Ca_3(PO4)_2$ transfection of Hek293T cells.

1. 2.5M $CaCl_2$. Prepare in dH_2O. Filter sterilize by passage through a 0.45 µM nitrocellulose membrane and store at -20 °C.

2. 2x Hepes-Buffered Saline (HeBS): 280 mM NaCl, 1.5 mM Na_2HPO_4, 50mM HEPES. Adjust pH to 7.05 with NaOH. Filter sterilize by passage through a 0.45 µM nitrocellulose membrane and store at -20 °C.

2.2.3 Cell Transfer and Stimulation

4. Ca^{2+}- and Mg^{2+}-free Phosphate Buffered Saline (Invitrogen) and Cell dissociation agent (Invitrogen).

5. Mouse leptin (R&D systems). Dilute to 20µg/ml in growth medium. Store aliquots at -20°C.

2.2.4 Luciferase Reporter Gene Assays

1. Cell culture lysis reagent: 25 mM Tris-phosphate (pH 7,8), 2 mM DTT, 2 mM 2,2 diaminocyclohexane-N,N,N',N'-tetra-acetate (DCTA), 10% glycerol, 1% Triton X-100.

2. Luciferase substrate: 20mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 33.3 mM DTT, 0.1 mM EDTA, 270 µM CoA, 530 µM ATP, 470 µM D-Luciferin.

3. Luminescence counter (e.g. Topcount, Packard).

2.3 Assaying prey expression

1. Modified RIPA lysis buffer: 200 mM NaCl, 50 mM Tris-HCl (pH 8), 0.05% SDS, 2 mM EDTA, 1% NP40, 0.5% Desoxycholate, 1 mM NaVanadate, 1 mM NaF, 20 mM b-glycerophosphate. Prepare 50 ml in dH$_2$O. Add 1 tablet of Complete$^®$ proteinase inhibitor cocktail just prior to use. Store at 4°C.

2. Separating buffer: 1.5 M Tris-HCl, pH 8.8, 0.1% SDS.

3. Stacking buffer: 0.5 M Tris-HCl, pH 6.8, 0.1% SDS.

4. Thirty percent acrylamide/bis solution (29:1) (Bio-Rad) (caution: this agent is a neurotoxin when unpolymerized).

5. N,N,N',N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).

6. Ammonium persulfate (10% solution).

7. Prestained molecular weight marker (e.g. All Blue Standards, Biorad).

8. Running buffer (5X): 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS.

9. Loading Buffer (2X): 5% beta-mercaptoethanol, 2% SDS, 8% glycerol, 62 mM Tris-HCL pH 6.8, 1% Bromine-phenolblue.

10. Nitrocellulose membrane (e.g. Hybond-C , Amersham Biosciences) and 3MM Whatman paper.

11. Transfer Buffer: 25 mM Tris base, 0.2 M Glycine, 20% Methanol.

12. 10X Tris-Buffered Saline (TBS): 0.2 M Tris-HCl, 1.37 M NaCl. Adjust pH to 7.6 with HCl.

13. Wash Buffer (1X TBS-T): 1X TBS, 0.1% Tween-20.

14. Blocking Buffer (LI-COR$^®$ Biosciences).

15. Antibody dilution buffer: Blocking Buffer (LI-COR$^®$ Biosciences), 0.1% Tween-20.

16. Primary antibody: anti-Flag M2 (Sigma).

17. Secundary antibody: goat anti-mouse IRDye$^®$ 680 or 800CW (LI-COR$^®$ Biosciences).

18. Odyssey$^®$ infrared imaging system (LI-COR$^®$ Biosciences).

3. Methods

The standard MAPPIT assay makes use of the human embryonic 293T (HEK293T) cells. Other cells lines can also be used. Examples include the haematopoietic TF1 (10) and the neuronal N38 cell lines (Wauman *et al.*, submitted). Briefly, the cells are transfected with the bait plasmid, the prey plasmid and a STAT-dependent reporter gene (pXP2d2-rPAP1-luci). The stimulation of the reporter gene is used as a measure of the interaction between the bait and prey proteins.

To study TLR signalling using MAPPIT we cloned the intracellular part of some TLRs as a bait and TLR adaptors and downstream signalling molecules both as bait and prey. As proof-of-principle we studied the signalling cascade starting from TLR4. Tested interactions are depicted in Figure 2 (**see Note 1**).
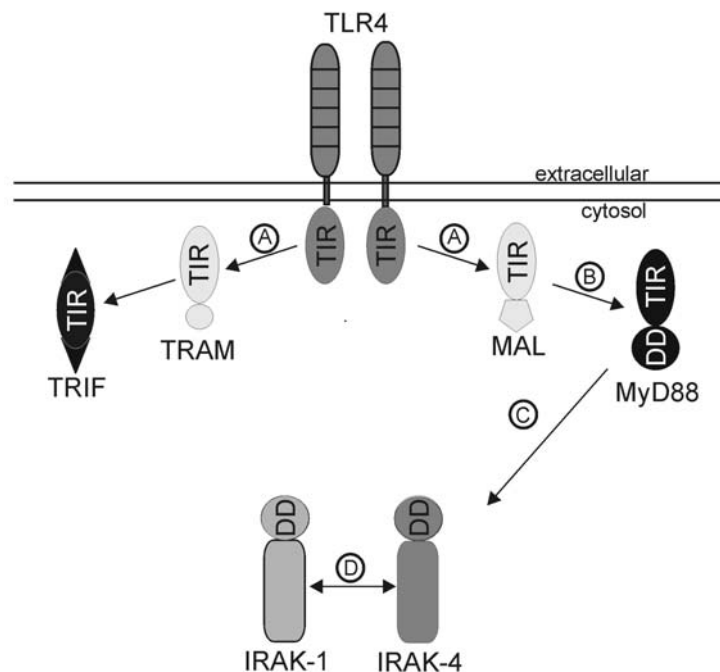
Figure 2: Initial steps in TLR4 signalling. All tested MAPPIT interactions, as shown in Figure 3, are annotated with an encircled capital.

We first examined the adaptor recruitment by TLR4, using the intracellular part of TLR4 as bait (TLR4ic) and different adaptors as prey (**Fig 3A**). Clear interaction could be detected between TLR4ic and Tram or Mal. However, co-transfection of the TLR4ic bait and the MyD88-prey did not lead to any luciferase induction, although the role of MyD88 in TLR4 signalling is well documented (11). We therefore examined if this interaction could be indirect. When co-transfecting a Mal expression vector together with TLR4ic-bait and MyD88-prey, a clear MAPPIT signal was detected, indicating that Mal bridges MyD88 to TLR4. These data are consistent with the phenotype of Mal-deficient mice, which is analogous to MyD88-deficient mice in terms of TLR2 and TLR4 signalling (12), and with a recent report, describing delivery of MyD88 to TLR4 as the primary function of Mal (13). These data prove that indirect interactions can be detected using the MAPPIT assay.

As expected, heterodimerisation of Mal and MyD88 was readily detected in a reciprocal way, using these adaptors either as bait or prey (**Fig 3B**). Further downstream, the interaction of MyD88 and IRAK-4 was also observed (**Fig 3C**) and association of Mal with IRAK-4 was strongly increased upon MyD88 co-expression, in analogy with the indirect TLR4-MyD88 binding (**Fig 3C**, **lower panel**). Finally, dimerisation of IRAK-1 and IRAK-4 could be demonstrated (**Fig 3D**). Taken together, these data illustrate that MAPPIT can be used to walk down the TLR signalling pathway from the receptor to the downstream IRAK kinases. MAPPIT thus provides a unique tool to study these molecular interactions in great detail in intact mammalian cells.

The design of prey and bait vectors and a typical protocol for an analytical MAPPIT assay are described below.
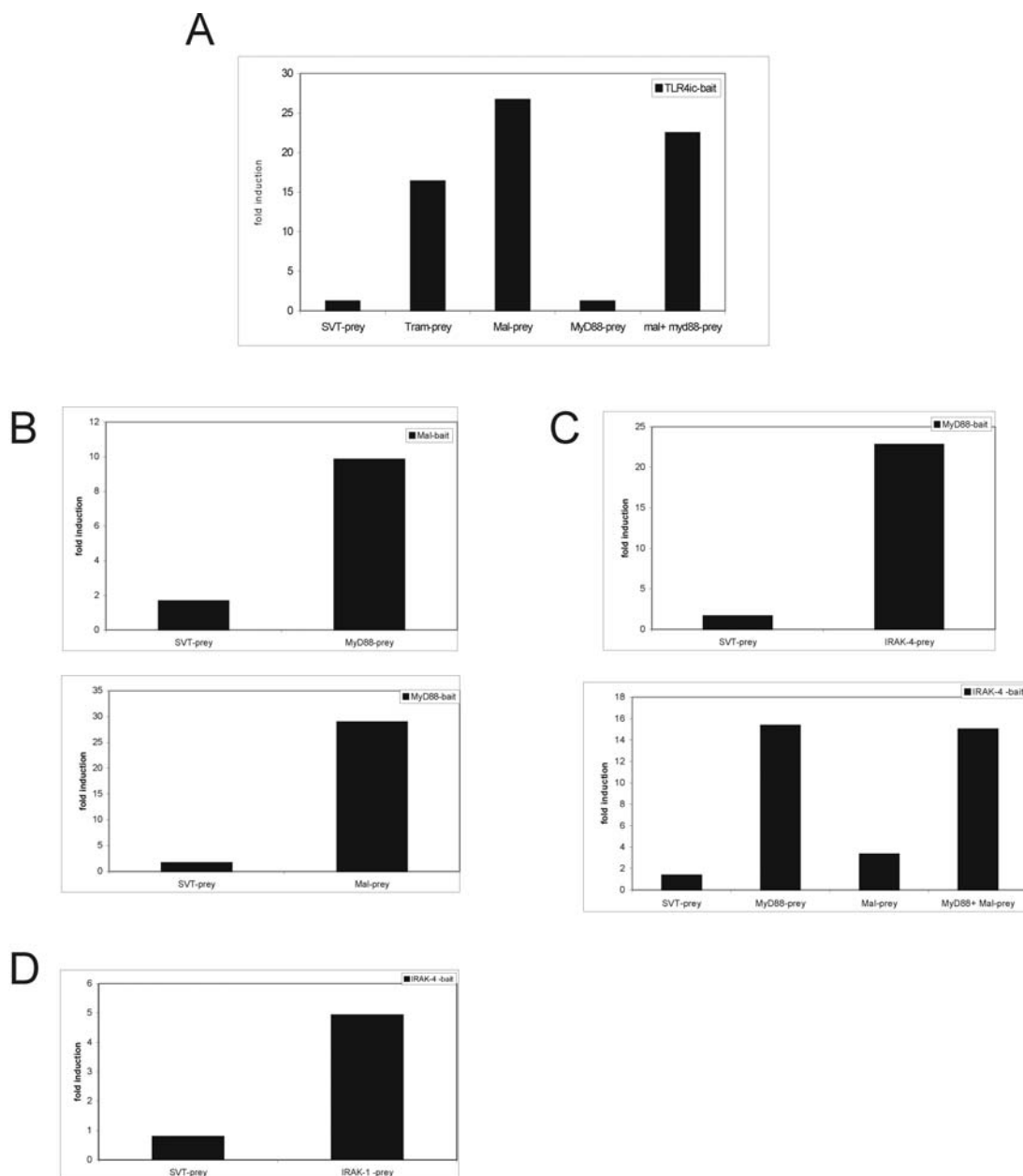
Figure 3: (A) Adaptor recruitment by TLR4. Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-TLR4ic, various TLR-adaptor prey constructs (or a SVT-prey as negative control (**see Note 6**)), and the STAT3-responsive rPAPI-luci reporter. The effect of Mal expression on MyD88 association was assayed by co-transfection of a Mal expression vector (pcDNA5-Mal). Twenty-four hours after transfection the transfected cells were stimulated with leptin (100 ng/ml) for another 24 h or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (ratio Stimulated/NS).

(B) Heterodimerisation of Mal and MyD88. Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-Mal and the prey vector pMG2-MyD88 (upper panel) or the reciprocal situation (lower panel) together with the rPAPI-luci reporter. Experimental setup was as in (A).

(C) MyD88 and IRAK-4 association. Interaction of MyD88 and IRAK-4 was assayed using the MyD88-bait plasmid pCLL-MyD88 and the pMG2-IRAK4 prey vector (upper panel) or with the reciprocal setup (lower panel). Indirect association of Mal and IRAK-4 was demonstrated by co-transfecting a MyD88 expression vector (pMet7-MyD88) (lower panel). Experimental setup was as in (A).

(D) Dimerisation of IRAK-1 and IRAK-4. Using IRAK-4 as a bait (pCLL-IRAK-4) and IRAK-1 as a prey (pMG2-IRAK-1), heterodimerisation of both kinases was monitored. Experimental setup was as in (A).

3.1 MAPPIT vectors

The plasmid vectors pMG2, pCEL4f and pCLL2f were developed for analytical MAPPIT applications. The structure of these vectors and of the protein chimeras they encode is shown in Figure 4. Subcloning is performed using standard methods.

The protein used as a prey is cloned C-terminal to the gp130 fragment. The pMG2 plasmid is derived from the mammalian expression vector pMET7, which contains the strong SRα promoter. This vector encodes the FLAG-tagged gp130 fragment (aa 760-918), of which aa 905-918 were duplicated, with a glycine-glycine-serine (GGS) amino acid linker region preceding the stuffer.

To exchange the stuffer with the prey-encoding sequence the following restriction sites can be used: EcoRI in combination with NotI, XhoI or XbaI (Fig. 4A).

The pCEL4f and pCLL2f vectors express the receptor-bait chimera. Both vectors originate from the pcDNA5-FRT plasmid (Flp-In system, Invitrogen). Bait expression is controlled by the human cytomegalovirus (CMV) promoter. The extracellular portion of either the human erythropoietin receptor (EpoR, pCel4f) or the murine leptin receptor (LR, pCLL2f) is used (**see Note 2**). Transmembranary and intracellularly, both bait-chimeras consist of the LR in which conserved tyrosine residues have been mutated to phenylalanine (LR-F3). Bait proteins can be cloned after the C-terminal GGS hinge sequence using SacI or BamHI and NotI restriction sites (Fig.4B). These vectors also contain a Flp Recombination Target (FRT) site followed by a hygromycin resistance cassette to permit Flp recombinase-mediated integration in suited cell types (Invitrogen).
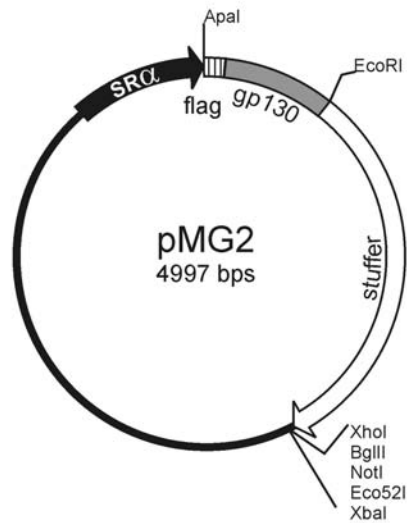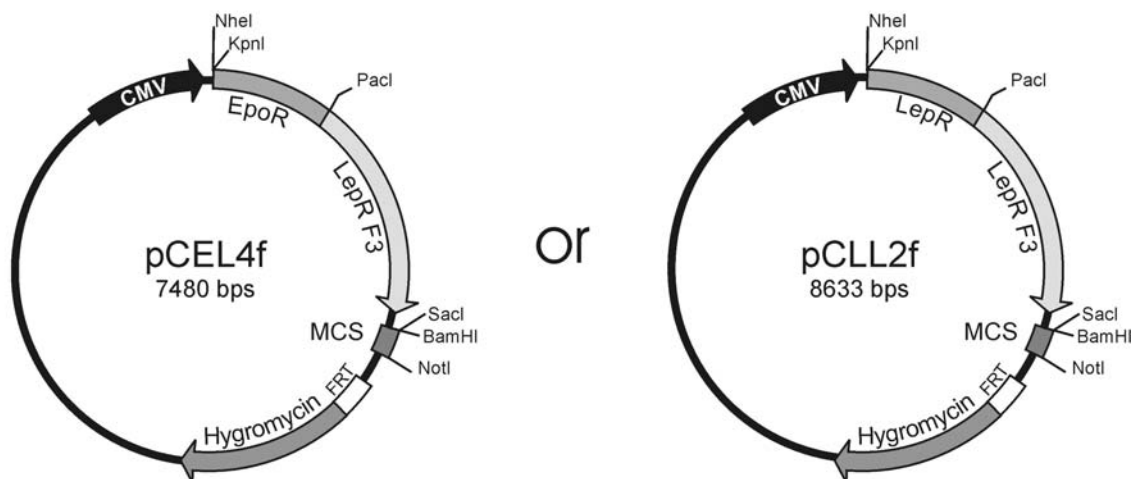
Figure 4: Diagrammic presentation of vectors used in an analytical MAPPIT approach.
(A) The pMG2 vector is used to express the prey. An N-terminal flag-tag was added to check for prey expression, which is controlled by the strong SRα promoter. The pMG2 vector contains aa 760-918 of the gp130 chain, of which aa 905-918 were duplicated.
(B) The pCEL4f and pCLL2f vectors encode the chimeric bait receptor under control of the CMV promoter. pCEL4f and pCLL2f contain the extracellular domains of the EpoR or LepR, respectively. Both vectors contain a FRT sequence which can be used for recombination-assisted integration into the genome of suited cell lines, e.g. T-Rex cell lines (Invitrogen).

3.2 Analytical Application of MAPPIT

3.2.1 Seeding of cells.

1. For each tested condition, plate 4×10^5 subconfluent HEK293T cells in one 10-cm^2 well (6-well plate). Sufficient cells should be plated to allow the experiments to be performed in triplicate and to monitor expression of the bait and prey proteins.

2. Grow overnight in a humidified atmosphere at 37°C and 8 to 10 % CO_2.

3.2.2 Transient $Ca_3(PO4)_2$ transfection of Hek293T cells.

1. For each well make a DNA/$CaCl_2$ mixture containing 0.5 µg of pCLL2f bait construct, 0.5 µg of pMG2 prey construct, 200 ng pXP2d2-rPAP1-luci and 15 µl 2.5 M $CaCl_2$ in a total volume of 150 µl dH_2O in a 1.5-ml tube (**see Note 3**). Gently drop 125 µl of the DNA mixture into 125 µl $2\times$ HeBS solution in a 1.5-ml tube, while vortexing

2. After vortexing the mixture for 5 additional seconds, leave precipitation mixture for 10-20 minutes at room temperature (**see Note 4**).

3. Add the precipitation mixture to one of the wells of a 6-well plate containing the HEK293T cells.

4. Leave precipitate on cells overnight in the incubator.

5. Remove medium and wash cells once with 1 ml PBS (**see Note 5**).

6. Add 2 ml fresh growth medium

7. Incubate the cells for 8 hours in a humidified atmosphere at 37°C and 8 to 10 % CO_2.

3.2.3 Cell Transfer and Stimulation

1. Remove the Growth Medium and wash once with 1 ml PBS

2. Add 200 µl Cell Dissociation Agent to each well and incubate for 5 minutes.

3. Gently tap plate to detach all cells.

4. Add 2 ml Growth Medium and triturate using a 1 ml pipette to break cell clusters.

5. Plate 50 µl of cell suspension in a black-well plate. We recommend 3 wells for every stimulation condition. The remainder of the cells are left in the 6-well and can be used for Western blot analysis to verify prey expression (see 3.3).

6. Add 50 µl Growth Medium or 50 µl Growth Medium containing leptin to each well of the black-well plate. Final leptin concentration should be 100 ng/ml.

7. Grow both the black-well plate and the 6-well plate with the remainder of the cells overnight in a humidified atmosphere at 37°C and 8 to 10 % CO_2.

3.2.4 Luciferase Reporter Gene Assays

1. Remove growth medium from the black 96-well plates.

2. Add 50 µl of cell culture lysis reagent to each well and incubate for 15 minutes. At this time point, plates can be stored at -20°C.

3. Add 35 µl of luciferase substrate to each well and instantly measure the plate in a luminescence counter.

3.3 Assaying prey expression.

For a correct analysis of the data generated with the MAPPIT technique, the expression of the prey chimera should be assayed. Using standard SDS-PAGE and Western Blotting techniques, prey expression can be checked using the N-terminal flag-tag present on every prey.

1. Discard Growth medium of the remainder of the transfected cells in the 6-well plate (after 3.2.3) and wash once with 1 mL PBS.

2. Add 150µl modified RIPA to each well and incubate on ice for 5 minutes.

3. Transfer lysates into a 1.5 mL tube and centrifuge at full speed to pellet the nuclei.

4. Use 15 µl of the supernatant and add 15 µl 2x loadingbuffer.

5. Boil samples for 5 minutes.

6. After cooling down to room temperature, samples are ready for SDS-PAGE.

7. Load samples on a 1.5 mm thick, 10 % polyacrylamide gel. The gel can be run at 80V through the stacking gel and at 120V through the separating gel until the dye fronts run off the gel.

8. Next, the separated samples are transferred to a nitrocellulose membrane electrophoretically. Transfer can be obtained at 120V for 1.5 hour or overnight at 30V.

9. After transfer, the membrane is incubated in 10 mL blocking buffer for 1 hour at 4°C on a rocking platform.

10. The blocking buffer is discarded and a 1:4000 dilution of the anti-Flag antibody (Sigma) is administered. Incubate for 1 hour at 4°C on a rocking platform.

11. Discard the primary antibody and wash the membrane 5 times for 5 min each with 10 mL TBS-T.

12. Incubate the membrane for another hour with a 1:5000 dilution of the secondary antibody.

13. Discard the secundary antibody and wash the membrane 5 times for 5 min each with 10 mL TBS-T.

14. Once the final wash is removed from the blot it can be stored at -20°C or scanned directly for prey expression with the Odyssey® infrared imaging system (LI-COR® Biosciences)

4. Notes

1. No MAPPIT signals were detected using Trif as bait or as prey. Expression of the Trif-prey was limited to a perinuclear compartment. Since MAPPIT measures interactions in the sub-membranary space, this finding provides a likely explanation for the lack of Trif-dependent signals in MAPPIT experiments. Of note, mutagenesis studies in conjunction with the MAPPIT read-out and immunofluorescence studies can be used to identify the localisation signals involved in targeting Trif to this cellular subcompartment.

2. Generally, a stronger signal is obtained when using the extracellular part of the LR, which can be explained by the fact that after binding of its ligand the LR undergoes higher order clustering, while the EpoR forms homodimers. Throughout this protocol only the pCLL-2f vector is used.

3. Different ratios of bait and prey plasmids may yield better results in some settings.

4. Do not let the mixture incubate much longer since this will reduce the transfection efficiency. The precipitate can be checked microscopically; the particles should look like small speckles (almost invisible at a 100× magnification) to obtain optimal transfection efficiencies.

5. Do not leave the PBS too long on the cells since this can cause detachment.

6. The pMG2-SVT vector is routinely used as a negative control. This vector encodes for a SV40 large T prey which lacks its nuclear localisation signal. Bait

expression can be monitored by using a prey capable of interacting with the LR-F3 portion of the bait, e.g. the SH2β-prey (data not shown).

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CHAPTER 2: MAPPIT analysis of TLR adaptor complexes

In this article we used the MAPPIT technique described in chapter 1 to study early events in TLR signalling. We cloned the intracellular part of some TLRs as bait and the TLR adaptor proteins both as bait and as prey. In this way, we generated a partial TLR-adaptor interaction map. The relevance of using MAPPIT to study TLR signalling was reflected by the ligand-independent activation of NF-κB following overexpression of the MyD88- or Mal-bait, indicating that MAPPIT allows the formation of functional signalling complexes at the cell membrane. Several known interactions could be confirmed including Tram or Mal association with TLR4 and homo-and heterodimerisation of Mal and MyD88. However, no interactions with the adaptor molecule Trif could be observed, possibly due to its perinuclear expression pattern. Also new interactions were found like the association of the TIR domain of Sarm with MyD88 and Tram. Using MAPPIT we could also show an indirect binding of MyD88 and TLR4 or TLR2. These interactions are mediated by Mal, consistent with the recent definition of Mal as a bridging adaptor, linking MyD88 to the activated TLR4 (Kagan and Medzhitov, 2006). These data were confirmed by coimmunoprecipitation. In addition, the dimerisation properties of MyD88 and Mal and the relative contribution of each of their subdomains in this process were further evaluated.

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MAPPIT analysis of TLR adaptor complexes

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Abstract Toll-like receptors (TLRs) are crucial components of the innate immune system, coupling pathogen recognition to a cellular response. We used the MAPPIT mammalian two-hybrid technique to investigate protein–protein interactions in the early steps in TLR signalling. A partial TLR-adaptor interaction map was constructed confirming several known but also documenting novel interactions. We show that the TLR adaptor Mal is critical for linking Myeloid Differentiation primary response protein 88 (MyD88) to TLR2 and TLR4. Analysis of the contributions of the different sub-domains of MyD88-adaptor-like protein (Mal) and MyD88 in adaptor homo- and hetero-dimerisation provides an initial mechanistic insight in this bridging function of Mal.
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Keywords: Toll-like receptor; MAPPIT; Signal transduction

1. Introduction

One of the first steps in pathogen clearance is the activation of signalling pathways by Toll-like receptors (TLRs). At present, 10 TLRs are identified in the human genome. Typical TLR ligands are bacterial lipopolysaccharide (LPS), recognised by TLR4 [1], bacterial lipopeptides (TLR2) [2], double stranded RNA (TLR3) [3], flagellin (TLR5) [4] and CpG motifs in DNA (TLR9) [5]. Next to ligand binding, adaptor molecules are recruited to the receptor through homotypic TIR–TIR (Toll/IL-1 receptor domain) domain interactions. MyD88 (Myeloid Differentiation primary response protein 88) was the first adaptor to be described [6], and is thought to be used by every TLR except TLR3. Subsequently, three more TIR-containing adaptors were found: Mal/TIRAP (MyD88-adaptor-like protein/TIR domain containing adaptor-like protein), which, based on knock-out studies, appears to be important in TLR2 and TLR4 signalling and exerts more

or less the same function as MyD88 [7–10], Trif/Ticam-1 (TIR-domain containing adaptor inducing interferon-β/TIR containing adaptor molecule), an adaptor recruited by both TLR3 and TLR4 and responsible for activation of IRF3 [11,12] and Tram/Ticam-2 (Trif-related adaptor molecule/TIR containing adaptor molecule-2), known to act as a bridging adaptor between TLR4 and Trif [13,14]. Recently, Sarm (sterile alpha and HEAT-Armadillo motifs containing protein), a potential fifth TLR adaptor molecule, was shown to negatively regulate Trif-dependent Toll-Like receptor signalling [15]. The usage of different adaptors by different receptors partially provides a molecular basis for the specificity of the immune reaction against a given pathogen. However, the exact modalities of this specificity are not yet fully understood [16].

Further downstream signalling can be primarily divided into a MyD88-dependent branch, which ultimately leads to activation of NF-κB and mitogen-activated protein kinases (MAPK) like p38 and Jun N-terminal Kinase (JNK) and a MyD88-independent branch resulting in phosphorylation of Interferon Regulatory Factors (IRFs) and expression of interferon-β (IFN-β). The MyD88-dependent pathway is initiated by recruitment of IRAK-4 (IL-1 receptor associated kinase-4) to the activated receptor [17] through a homotypic interaction with the death domain of MyD88. Next, IRAK-4 phosphorylates IRAK-1 [18] and activated IRAK-1 associates together with TRAF-6 (TNF-α receptor-associated factor 6) [19] into a complex with TAB (TAK-1 binding) proteins and TAK-1 (TGF-β-activated kinase-1) [20]. This complex is a branching point for the activation of NF-κB and induction of the MAPK pathway. Activation of TLR3 and TLR4 also engages a MyD88-independent pathway. Recruitment of Trif to the activated receptor leads to activation of TBK1 (TANK-binding kinase-1) and IKKε, which are essential for IRF-3 phosphorylation and nuclear translocation and subsequent induction of IFN-β [21]. In this report we make use of the mammalian two-hybrid method MAPPIT (MAmmalian Protein–Protein Interaction Trap) [22] to study the modalities of TIR–TIR interactions involved in the early steps of TLR signalling.

2. Material and methods

2.1. Constructs

Generation of the pMG2 and pMG2-SVT prey vectors were described earlier [23]. Human full-length MyD88 was amplified using primers 1 and 2 on the pCDNA3-MyD88-AU1 plasmid (gift of Dr. Muzio). After *Eco*RI–*Xba*I digestion, the fragment was cloned in the pMG2 vector, resulting in pMG2-MyD88. The TIR domain of MyD88 was cloned in an analogous manner into the pMG2 vector

using primers 3 and 4 and a *Bst*eII/*Xba*I digestion. The pMG2-Mal and pMG2-MalTIR plasmids were generated by amplification of Mal and the TIR domain of Mal from the pDC304–Mal vector (gift from Dr. O'Neill) with primers 5–6 and 7–8 respectively, and *Eco*RI/*Xba*I or *Bst*eII/*Xba*I digestion. The TIR domain of Sarm was amplified using primers 9–10 on HepG2 cDNA followed by *Eco*RI/*Xba*I digestion. This resulted in the pMG2-SarmTIR vector. The pMG2-Tram vector was generated by amplification of full-length Tram using primer 11–12 on Hek293 cDNA. After *Bst*eII/*Xho*I digestion, the fragment was cloned in the pMG2 vector.

Generation of the human LepR-bait constructs in the pcDNA5/FRT vector was described before [22], and this construct was named pCLL. The pCLL-MyD88 plasmid was generated by amplification of full-length MyD88 from the MyD88-AU1 vector using primer pairs 13–14. After *Bam*HI/*Not*I digestion, the fragments were cloned in the pCLL vector. The pCLL-Mal and pCLL-Tram plasmids were generated in an analogous manner using primers 15–16 and 17–18, respectively. The intracellular domains of Toll-Like receptor 2, 3, 4, 5, 7, 9 were amplified using primer sets 19–20, 21–22, 23–24, 25–26, 27–28, 29–30 on MRC5 cDNA (TLR2), the pFLAG CMV1 – TLR3 vector (gift from Dr. O'Neill), the pFLAG CMV1 – TLR4 vector (gift from Dr. O'Neill), and RZPD clones HU3_p983D0273D (TLR5), IR-ATp970B1256D (TLR7) IRATp970H1255D (TLR9) respectively. After *Bam*HI/*Not*I digestion, the fragments were cloned into the pCLL vector.

The MalP125H and MyD88P200H mutant vectors were generated by PCR based mutagenesis using primers 31–32 and 33–34, respectively (Quikchange™ site-directed mutagenesis method, Stratagene). The pcDNA5-TAP2-CIS vector was generated by amplification of TAP2-CIS using primers 35–36 on the pMET7-TAP2-flag vector, which was described earlier [24], followed by a *Kpn*I/*Not*I based ligation into the pCDNA5-FRT-TO vector (Invitrogen). Mal was amplified using primers 37–38 on the pDC304–Mal vector and *Eco*RI/*Not*I based ligation into the pcDNA5-TAP2-CIS vector resulted in the pcDNA5-Mal-flag plasmid.

The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was used as previously described [22]. The pNFconluc reporter was a gift of Dr. A. Israel.

2.2. Cell culture, transfection, reporter assays and expression controls

Cell culture conditions, transfection procedures and luciferase assays for Hek293T cells were previously described [25]. For a typical luciferase experiment, 4×10^5 cells were seeded in 6-well plates 24 h before transfecting them overnight with the desired constructs together with the luciferase reporter gene. Cells were left untreated (negative control NC) or were stimulated overnight with 100 ng/ml leptin followed by measurement of luciferase activity in cell lysates by chemiluminescence.

Prey expression was examined by Western Blot using anti-FLAG mouse monoclonal antibody (Sigma), on lysates of transfected cells. All results are representative for at least three independent transfection experiments.

2.3. Co-immunoprecipitation

Approximately 2×10^6 Hek293T cells were transfected with different combinations of MAPPIT bait vector pCLL-TLR4ic, MyD88-prey plasmid, and an expression vector encoding Mal. Cleared lysates (modified RIPA lysis buffer: 200 mM NaCl, 50 mM Tris–HCl PH8, 0.05% SDS, 2 mM EDTA, 1% NP40, 0.5% DOC, Complete Protease Inhibitor Cocktail (Roche)) were incubated with a mixture of two rat anti-mouse LR monoclonal antibodies (4 μg/ml) [26] and protein G-sepharose (Amersham Biosciences). After immunoprecipitation, SDS–PAGE and Western Blotting, interactions were detected using anti-flag-tag antibody (Sigma), anti-HA (HA-11, CRPinc) and anti-mouse-HRP (horseradish peroxidase) (Amersham Biosciences).

2.4. Fluorescence microscopy and immunocytochemistry

Hek293T cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (Invitrogen) for 20 min at room temperature. Next, cells were permeabilised in 0.1% Triton X-100, washed in phosphate-buffered saline, incubated with anti-flag antibody (Sigma) for 1 h at 37 °C and following several washes in phosphate buffered saline incubated with Alexafluor488-conjugated goat anti-mouse antibody (Molecular Probes) at room temperature. Nuclei were DAPI stained. Microscopic images were captured with a Zeiss Axiovert 200 epifluo-

rescence microscope equipped with an Axiocam cooled CCD camera and processed using AXIOVISION software.

3. Results

3.1. MAPPIT concept

MAPPIT (MAmmalian Protein–Protein Interaction Trap), a mammalian two-hybrid method [22], was used to study protein–protein interactions in the TLRs pathway (Fig. 1A). In brief, we made a C-terminal fusion of a given 'bait' protein with a leptin receptor that is deficient in STAT3 recruitment. The 'prey' protein on the other hand is linked to a series of four functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand stimulation leads to STAT3 activation and induction of a STAT3-responsive luciferase reporter (rPAPI-Luci). Expression of all the different preys used throughout this study was checked by Western blot analysis using an anti-FLAG antibody (Supplementary data). Bait expression was assayed by FACS analysis using an anti-LR antibody (Supplementary data) (see Table 1).

We cloned all TLR adaptors both as bait and as prey, and the intracellular part of selected TLRs as bait. An overview of all tested adaptor/adaptor and adaptor/TLR interactions can be found in Table 2. Well-documented interactions like homo- or hetero-oligomerisation of Mal and MyD88 or interaction of Tram with TLR4 were confirmed. In addition, new interactions were found, including the association of the TIR domain of Sarm with MyD88 and Tram. Other new findings will be discussed in more detail below. No MAPPIT signals were detected using Trif as bait or as prey. This cannot be explained by an a-specific effect of Trif on the MAPPIT readout since Trif over-expression did not influence an established interaction-dependent signal (data not shown). We also ruled out cytotoxic effects by the Trif-prey since its over-expression did not lead to increased annexin V binding, a marker for apoptosis (data not shown). We also constructed a Trif-prey with a C-terminal fusion of the gp130 tail to exclude interference of a N-terminal Trif fusion protein with protein–protein interactions, but again no positive signals could be observed (data not shown). Strikingly, the subcellular expression of the Trif-prey was limited to a perinuclear compartment, much in contrast to functional prey molecules (Fig. 1B). Since MAPPIT measures interactions in the sub-plasmamembranary space, this finding provides a likely explanation for the lack of Trif-dependent signals in MAPPIT experiments.

We also monitored the effect of bait expression on endogenous TLR signalling (Fig. 1C). The MyD88-bait and, to a lesser extent, the Mal-bait were able to activate the NF-κB pathway in a ligand independent manner. These data show that the MAPPIT setup forms functional signalling complexes at the cell membrane, underscoring its relevance to study TLR signalling.

3.2. Homo- and hetero-oligomerisation of Mal and MyD88

Mal and MyD88 both have a C-terminal TIR interaction domain (Toll/IL-1 receptor domain), a property shared with all TLRs and their adaptors. This TIR domain is thought to be the critical interaction domain for recruitment of TLR adaptors to their cognate receptors and for adaptor dimerisation. MyD88 bears an additional N-terminal Death Domain linked to the TIR domain by a short intermediate region.
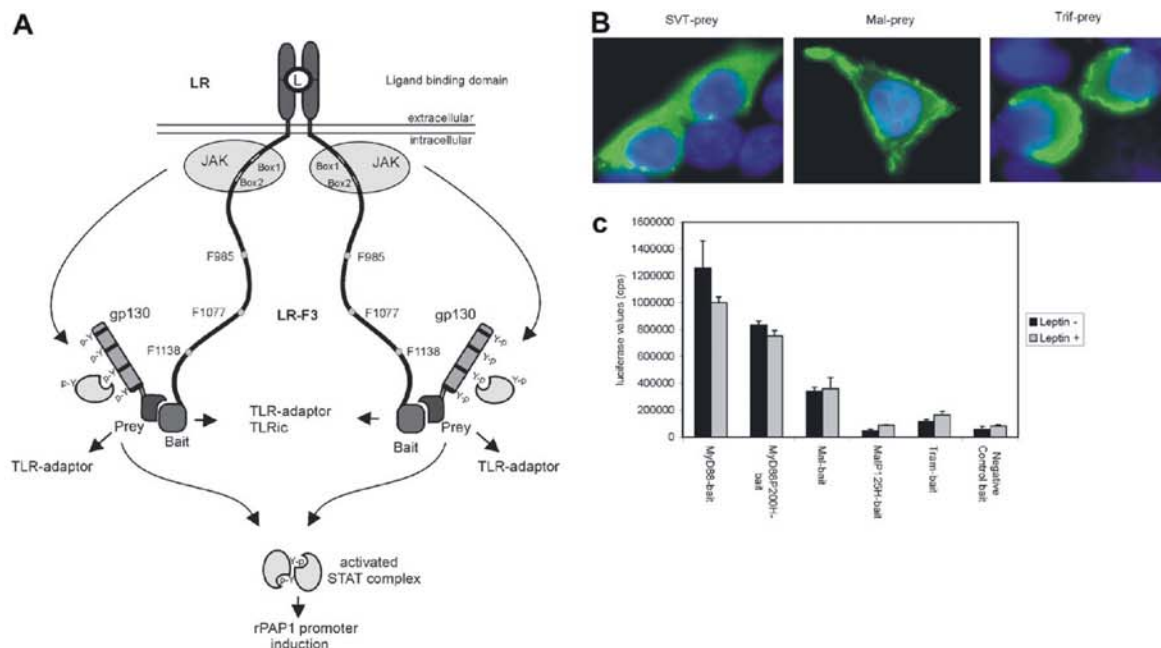
Fig. 1. (A) MAPPIT principle. MAPPIT, a cytokine receptor-based two hybrid method, makes use of a bait protein, C-terminally fused to a leptin receptor that is deficient in STAT3 recruitment by a Y1138F mutation, and a prey protein linked to a string of four functional STAT3 recruitment sites of the gp130 chain. Interaction of bait and prey leads to a ligand-dependent complementation of STAT3 activity that can be measured with a STAT3-responsive luciferase reporter (rPAPI-luci). TLR adaptors were used as bait or prey, while the intracellular parts of selected TLRs were used as bait. (B) Trif-prey localisation. Hek293T cells were transfected with either Trif-prey, Mal-prey or SVT-prey lacking its nuclear localisation sequence . Cells were fixed and incubated with anti-flag antibody. Prey expression was visualised with a fluorescent secondary antibody (anti-mouse IgG-alexa488). Nuclei were DAPI stained. (C) TLR adaptor bait expression activates NF-κB pathway. Hek293T cells were transfected with different TLR adaptor bait-constructs (1 μg) and a NF-κB responsive luciferase reporter (pNF-conluci). Twenty-four hours after transfection the transfected cells were stimulated with leptine (100 ng/ml) for another 24 h or left untreated (NS). Mean Luciferase activity + S.D. of triplicate measurements is plotted.

Mal on the other hand contains an N-terminal phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain, which mediates Mal recruitment to the plasma membrane [27]. As seen in Table 2, the TLR adaptors Mal and MyD88 clearly can form homo- and hetero-dimers, a well-established early event in TLR signalling. To elucidate which domains of these adaptors are essential in this dimerisation process we mutated a proline to histidine in the TIR domain of Mal (P125H) and MyD88 (P200H), a mutation known to abrogate TLR signalling [1]. We also generated a prey construct only containing the C-terminal TIR domain of MyD88 and Mal.

Strikingly, when analysing the interaction properties of the Mal-bait (Fig. 2A), we observed a drastic effect of the P → H mutation. Mutation of the TIR domain of either bait or prey is sufficient to abrogate interaction. These data confirm that the TIR domain of Mal and MyD88 is necessary and sufficient for heterodimerisation. These properties were confirmed using MyD88 as bait (Fig. 2B).

Much in contrast, Mal homodimerisation depended on its N-terminal part in addition to TIR-TIR binding. No interaction was observed when using the Mal-bait combined with a MalTIR-prey only containing the C-terminal TIR region (Fig. 2A). However, this prey is still functional since clear association of the MyD88-bait and the MalTIR-prey was observed (Fig. 2B). Mutation of a single TIR domain leads to complete loss of homodimerisation (Fig. 2A), pointing again at the importance of this proline residue.

We also checked the effect of the P → H mutation on MyD88 homodimerisation (Fig. 2B). When using full-length MyD88 as bait and prey, mutation of both TIR domains did not affect interaction, whereas binding of the MyD88TIR-prey was completely lost when using MyD88P200H as a bait. These results indicate that the death domain of MyD88 is not only important in recruiting downstream signalling molecules, but also for MyD88 homodimerisation.

3.3. Mal bridges TLRs to MyD88

We next examined the TLR4 interaction profile using the intracellular part of TLR4 as bait (TLR4ic) and different TLR adaptors as prey (Fig. 3A). As expected, clear interaction was seen between TLR4ic and Tram, confirming its unique function in TLR4 signalling [13,14]. Mal/TIRAP also bound to TLR4ic, an interaction which was reported before [8]. Co-transfection of the TLR4ic-bait and the universal TLR adaptor MyD88-prey did not result in any luciferase induction although the role of MyD88 in TLR4 signalling is very well documented [28,29]. Therefore, we examined if the MyD88-TLR4 interaction could be indirect (Fig. 3B). Co-transfection of a Mal expression vector together with TLR4ic-bait and MyD88-prey resulted in a clear luciferase induction, indicating that Mal bridges MyD88 to TLR4. This interaction was completely lost when using the TIR disrupting Mal (MalP125H) or MyD88 (MyD88P200H) mutations, pointing at the importance of the different TIR domains in this interaction.

Table 1
Overview of primers used in this study

Primer 1	5'-GCGAATTCATGGCTGCAGGAGGTCCCGGCG-3'
Primer 2	5'-GCGCTCTAGATCAGGGCAGGGACAAGGCCTTGG-3'
Primer 3	5'-GCGGTAACCGTGCCGCCATGGACCCCCTGGGGCATATGCC-3'
Primer 4	5'-GCTCTAGACAGTCAGGGCAGGGACAAGGCC-3'
Primer 5	5'-GCGAATTCATGGCATCATCGACCTCC-3'
Primer 6	5'-GCGCTCTAGACTCAAAGTAGATCAGATAC-3'
Primer 7	5'-GCGGTAACCGTGCCGCCATGGGCAGTAGTCGCTGGAGC-3'
Primer 8	5'-GCTCTAGACAGTCAAAGTAGATCAGATACTGT-3'
Primer 9	5'-GCGCGAATTCACTCCAGATGTCTTCATCAGC-3'
Primer 10	5'-GGCCTCTAGATTAGCGGCCCTGCAGGAAGCGG-3'
Primer 11	5'-GCGGTAACCGCCGCCATGGATTACAAGGATGACGACGATAAGGGTATC GGAAGTCTAAAATAAATTCC-3'
Primer 12	5'-GCCTCGAGTCAGGCAATAAATTGTCTTTGTACC-3'
Primer 13	5'-GCGGATCCATGGCTGCAGGAGGTCCCGGC-3'
Primer 14	5'-GCGCGGCCGCTCAGGGCAGGGACAAGGCC-3'
Primer 15	5'-GCGAGCTCCGGATCCATGGCATCATCGACCTCC-3'
Primer 16	5'-ATAGTTTAGCGGCCGCTCACCGTAGATCAGATAC-3'
Primer 17	5'-GCCGGGATCCATGGGTATCGGGAAGTC-3'
Primer 18	5'-GCCGATGCGGCCGCTCAGGCAATAAATTGTC-3'
Primer 19	5'-GCGGATCCTGCCACCGTTTCCATGG-3'
Primer 20	5'-GCGCGGCCGCCTAGGACTTTATCG-3'
Primer 21	5'-GCGAGCTCGTCTTTTTATTGGAATG-3'
Primer 22	5'-GCGCGGCCGCTTAATGTACAGAGTTTTTGG-3'
Primer 23	5'-GCGGATCCTGCATAAAGTATGGTA-3'
Primer 24	5'-GCGCGGCCGCTCAGATAGATGTTGC-3'
Primer 25	5'-GCGGATCCACAAAGTTCCGGGGCTTCTG-3'
Primer 26	5'-CCGGCCGCGGCCGCCTTAGGAGATGGTTGCTAC-3'
Primer 27	5'-GCGGATCCCACCTCTATTTCTGGGATGT-3'
Primer 28	5'-CCGGCCGCGGCCGCCCTAGACCGTTTCCTTGAACACC-3'
Primer 29	5'-GCGCGCGGATCCTGGCGGGGGCGGCAAAGTGG-3'
Primer 30	5'-CCGGCCGCGGCCGCCCTATTCGGCCGTGGGTCCC-3'
Primer 31	5'-GCAACTCCGGGATGCAACCCACGGCGGCGCTATAGTGTCC-3'
Primer 32	5'-GGACACTATAGCGCCGCCGTGGGTTGCATCCCGGAGTTGC-3'
Primer 33	5'-GTCTGACCGCGATGTCCTCCATGGCACCTGTGTCTGGTC-3'
Primer 34	5'-GACCAGACACAGGTGCCATGGAGGACATCGCGGTCAGAC-3'
Primer 35	5'-GCGAGGTACCGCCACCATGGCCCAGCACGA-3'
Primer 36	5'-GCAGCGGCCGCTTAGAGTTGGAAGGGGTACTG-3'
Primer 37	5'-GCGCGAATTCATGGCATCATCGACCTCCC-3'
Primer 38	5'-GCCGCTGCGGCCGCTCAAAGTAGATCAGATACTGTAGC -3'

Table 2
Overview of tested adaptor/adaptor and adaptor/TLR interactions

	Mal	MalTIR	MyD88	MyD88TIR	Tram	SarmTIR	SVT
Prey							
Mal	**8.53**	2.54	**19.19**	**11.55**	2.3	4.57	1.68
MyD88	**23.77**	**44.84**	**19.99**	**45.14**	1.05	**12.15**	2.30
Tram	2.59	2.01	1.33	1.25	**18.66**	**11.66**	2.46
Bait							
TLR2ic	**8.71**	**8.18**	2.28	0.92	1.06	2.79	1.04
TLR3ic	4.01	3.07	1.01	0.99	1.16	4.43	1.22
TLR4ic	**26.69**	**56.65**	1.22	1.05	**16.40**	1.70	1.22

Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid (0.5 µg), different TLR adaptor prey plasmids (0.5 µg) and the STAT3 responsive rPAPI-luci reporter (0.3 µg). Twenty-four hours after transfection the transfected cells were stimulated with leptin (100 ng/ml) for another 24 h or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (ratio Stimulated/NS). Positive interactions (fold induction >5) are highlighted in bold. SVT-prey was used as a negative control. Expression of all the different preys was checked by Western blot analysis using an anti-FLAG antibody.

These data were confirmed by co-immunoprecipitation (Fig. 3C). The extracellular portion of the leptin receptor was used to immunoprecipitate the MAPPIT TLR4ic-bait protein (using an anti-LR antibody) and only when Mal was co-expressed, the MyD88-prey protein could be co-immunoprecipitated.

In an analogous manner, the TLR2ic interaction profile was examined (Fig. 3D). In accordance with the TLR4ic interaction profile, no interaction between MyD88 and TLR2 could be observed. However, complementation with Mal gave rise to a clear luciferase induction, indicating that Mal also acts as bridging adaptor between MyD88 and TLR2.

4. Discussion

One of the first steps in pathogen recognition and clearance is the recognition of evolutionary conserved pathogen-associated molecular patterns whereby TLRs play a crucial role.
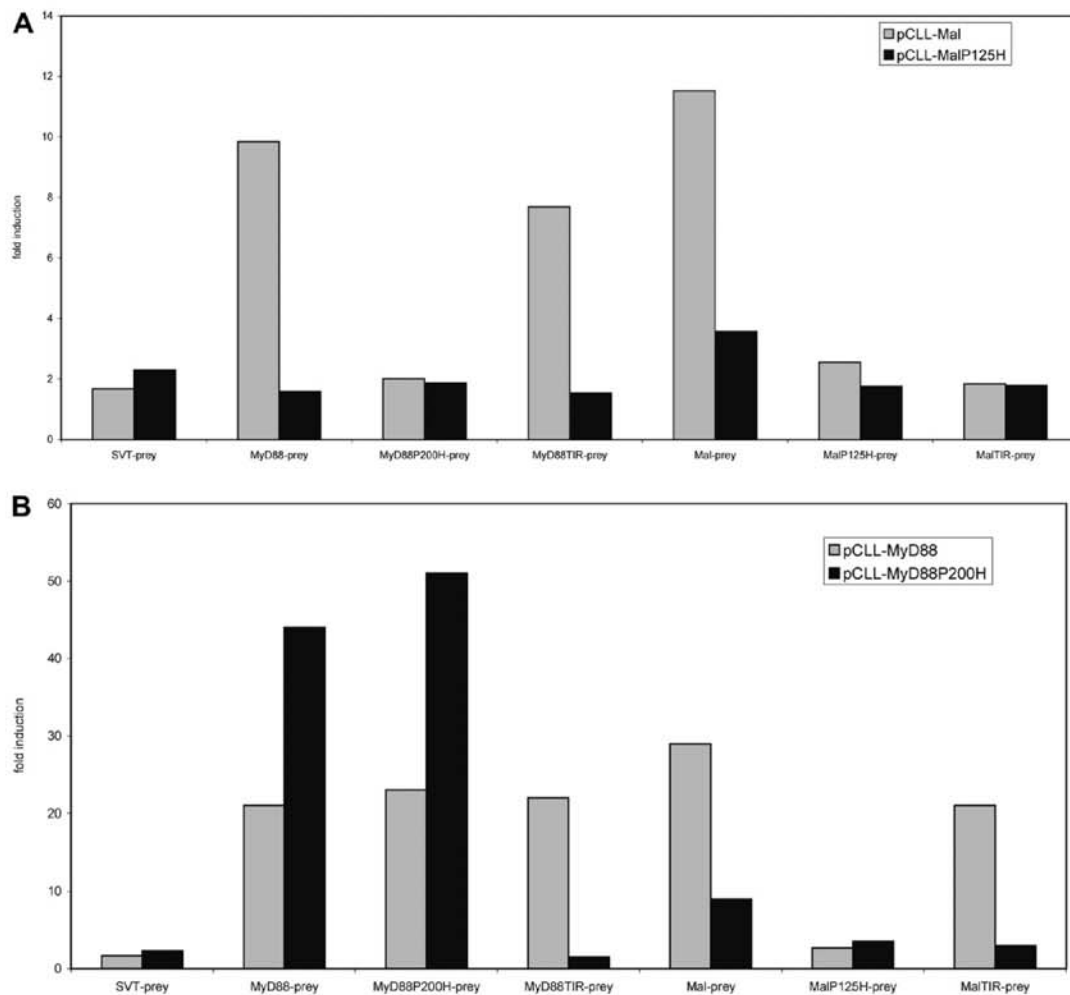
Fig. 2. Mal and MyD88 homo- and heterodimerisation. (A) Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-Mal or pCLL-MalP125H, different TLR adaptor prey plasmids and the STAT3 responsive rPAPI-luci reporter. Experimental setup was as in Table 2. (B) Interaction analysis of the MyD88 bait (pCLL-MyD88) or its mutant (pCLL-MyD88P200H). Experimental setup was as in Table 2.

Ligand binding to a given TLR activates complex signalling pathways, ultimately leading to pro-inflammatory responses. These responses vary between activated TLRs, which is in part explained by different adaptor usage. We here take a closer look at the mechanistics of TLR adaptor recruitment using MAPPIT, a mammalian two-hybrid strategy. We cloned all known TLR adaptors either as bait or as prey, and the intracellular part of selected TLRs as bait. As shown in Table 2, well-documented interactions like MyD88/Mal homo- and hetero-dimerisation, or recruitment of Tram to TLR4 could readily be detected. No interactions were observed using Trif as a bait or as a prey, probably due to its unique, non-cytoplasmic localisation that most likely interferes with the MAPPIT readout (Fig. 1B). Next to known interactions, some new observations were also found. Recently, Sarm was described as a negative regulator of Trif-dependent signalling pathways [15]. However, as seen in Table 2, we also observed a clear interaction with MyD88 or Tram when using the 'TIR' interaction domain of Sarm as a prey. These data suggest that Sarm, next

to inhibition of Trif , also could be involved in regulating the TLR adaptors MyD88 and Tram.

MyD88 is commonly referred to as a "universal" adaptor, used by every TLR except TLR3. However, biochemical data providing a direct link between MyD88 and a TLR are scanty. In this report we could not detect an interaction between MyD88 and TLR2, TLR4 (Fig. 3). However, the TLR4-bait is fully functional since a clear signal was obtained for the well-documented interactions with the Mal- or Tram-preys [13,14]. This inability of MyD88 to bind TLR4 in MAPPIT is in line with a recent report showing no MyD88/TLR4 binding when analyzed via the yeast two-hybrid method [30]. Significantly, co-expression of the TLR adaptor Mal resulted in a clear MAPPIT signal for the TLR4-bait/MyD88-prey combination. Since Mal binds both TLR4 and MyD88, this observation demonstrates its bridging role between MyD88 and TLR4, analogous to the Tram/Trif branch of TLR4 signalling [31] (Fig. 3B). These data were confirmed using co-immunoprecipitation (Fig. 3C). While this manuscript was in
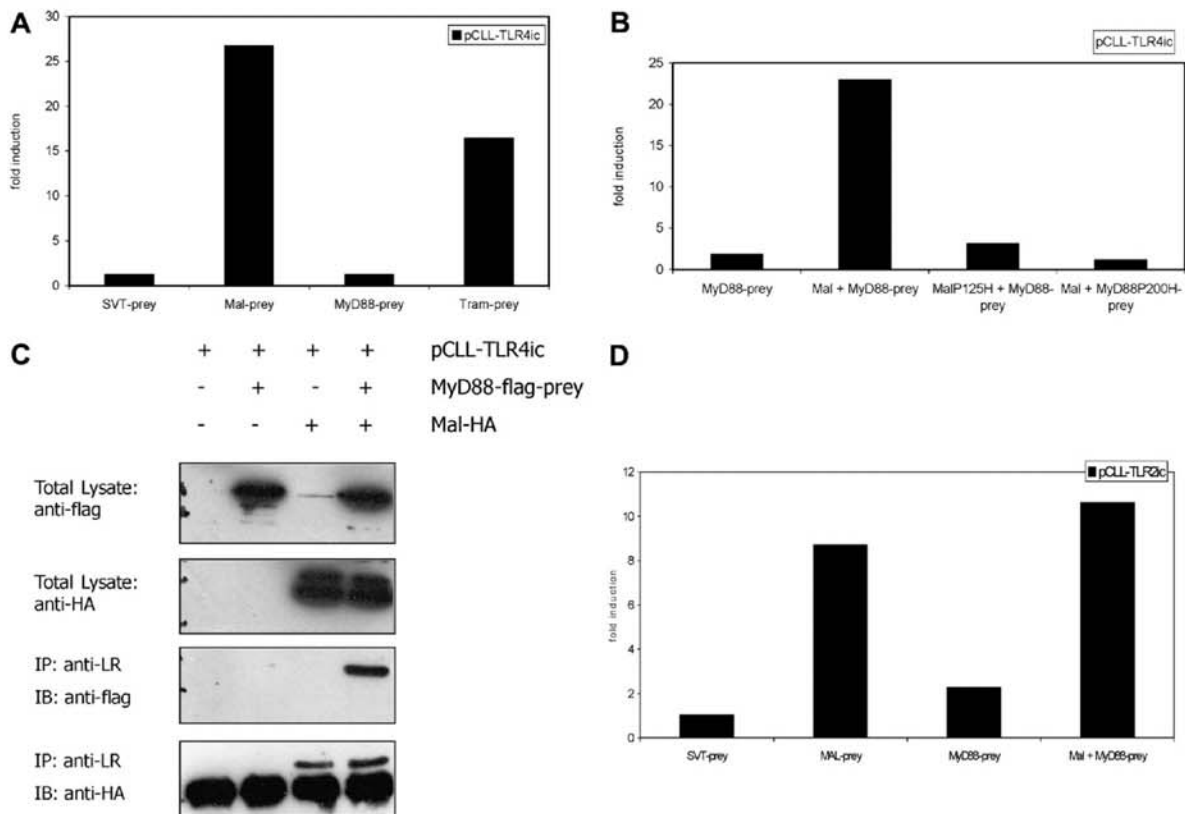
Fig. 3. TLR4ic interaction profile. (A) Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-TLR4ic, various TLR adaptor prey constructs (or a SVT-prey as negative control) and the rPAPI-luci reporter. Experimental setup was as in Table 2. (B) Effect of a Mal expression vector. Hek293T cells were transiently co-transfected with pCLL-TLR4ic, a MyD88-prey (pMG2-MyD88/pMG2-MyD88P200H), a Mal expression vector (pcDNA5-Mal/pcDNA5-MalP125H) and the rPAPI-luci reporter. Experimental setup was in Table 2. (C) Co-immunoprecipitation analysis. Hek293T cells were transfected with combinations of pCLL-TLR4ic, pMG2-MyD88, pDC304-Mal-HA. Cell lysates were immunoprecipitated with an a mixture of two rat monoclonal antibodies against the extracellular part of the leptin receptor (α-LR) and subsequently immunoblotted (IB) with anti-flag or anti-HA. (D) TLR2ic interaction profile. Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-TLR2ic, various TLR adaptor prey constructs, a Mal expression vector (or empty vector) and the rPAPI-luci reporter. Experimental setup was as in Table 2.

preparation, Kagan and Medzhitov showed that Mal contains a PIP2 binding domain that mediates Mal recruitment to membranes and that the primary function of Mal in TLR signalling is to facilitate MyD88 delivery to TLR4 via PIP2 binding [27]. These observations are also consistent with the phenotype of Mal-deficient mice, which is analogous to MyD88-deficient mice in terms of TLR2 and 4 signalling [7,10]. Similar findings were obtained supporting a TLR2/Mal/MyD88 bridging complex (Fig. 3D) in line with the need for Mal in TLR2 signalling. Of note, our findings are in contrast with a recent report [30], demonstrating direct TLR2/MyD88 binding with a yeast two-hybrid approach. The reason for this contradictory finding is unclear at present.

We also evaluated the interactions between the TIR domains of additional TLRs (TLR5, TLR7 and TLR 9) and MyD88 (data not shown). Most interestingly, in none of these cases could we observe any signal. Although we cannot rule out at present that our strategy fails to detect these interactions, we would like to point out that a control using a JAK2-binding-prey was clearly positive, indicating that the attached TIR do-

mains did not interfere with the MAPPIT read out. Moreover, the yeast two-hybrid method also failed to detect direct interaction between TLR9 and MyD88 [30]. In addition, TLR7 and TLR9 signal from acidic endosomes. How MyD88 is recruited to those distinct cellular compartments remains unknown, and the use of accessory molecules, next to Mal and Tram, therefore cannot be excluded.

We next investigated the dimerisation properties of Mal and MyD88 in more detail. MyD88 and Mal both are two-domain proteins containing a C-terminal TIR domain preceded by a Death Domain and a N-terminal domain, respectively. Mutation of a single proline to histidine in the so called "BB-loop" of the TIR domain blocks TLR signalling. Various models of these TIR domains have been described, but the precise role of this conserved proline remains elusive [32,33]. Strikingly, mutation of the TIR domain of Mal (MalP125H) in either prey or bait, contrary to GST pull-down experiments [32], completely abolishes homo-dimerisation (Fig. 2A). These data support the model in which the BB loop of the TIR domain plays a crucial role

in TIR-TIR interactions [33] and provide a biochemical explanation for the drastic effect of this mutation on signalling. Moreover, in contrast with wild type Mal-bait, the MalP125H-bait is no longer capable of activating NF-κB signalling (Fig. 1C). This again reflects the importance of this proline residue in TLR signalling. In addition, we observed that the contribution of each interaction domain of Mal and MyD88 differs from homo- and hetero-dimerisation. For hetero-dimerisation, the isolated TIR domain of Mal or MyD88 is sufficient (Fig. 2). Mal homodimerisation on the other hand is dependent on the N-terminal domain of Mal, which is necessary to stabilise homodimer formation (Fig. 2A). This contrasts to MyD88 homodimerisation (Fig. 2B), where the isolated TIR domain is sufficient for interaction. Mutation of the conserved proline residue in the isolated TIR domain again abolished interaction. In contrast, full-size bait and prey homodimerisation is not affected by this P200H mutation nor does it eliminate NF-κB signalling (Fig. 1C). This implies that the MyD88 Death Domain not only interacts with downstream signalling molecules, as shown in several studies [17], but also can participate in MyD88 homodimer formation.

In brief, we examined the recruitment and dimerisation properties of the TLR adaptors Mal and MyD88 and provide a biochemical basis for the bridging function of Mal between an activated TLR and the TLR adaptor MyD88.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.01.026.

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CHAPTER 3: The C-terminus of CIS defines its interaction pattern

Members of the SOCS proteins are rapidly induced upon cytokine receptor and TLR activation and function as negative regulators of the aforementioned signalling pathways. These SOCS proteins are composed of a pre-SH2, a SH2 and a SOCS-box domain. In this study we evaluated the interaction profile of CIS, SOCS1, SOCS2 and SOCS3. By means of MAPPIT analysis, we could demonstrate that, besides the SH2-domain, the SOCS box of CIS is also essential for leptin receptor binding. Mutagenesis revealed that the conserved tyrosine Y253 in the C-terminus of the CIS SOCS-box is indispensable for receptor association. This C-terminal tyrosine is also critical for interaction with the Epo receptor, as demonstrated by both MAPPIT as well as peptide affinity chromatography experiments. Moreover, mutation of this tyrosine abolished the inhibitory function of CIS in Epo receptor signalling.

Conversely, the interaction of CIS and the universal TLR adaptor MyD88 was not dependent on this conserved tyrosine. Further MAPPIT and coimmunoprecipitation experiments revealed that CIS interacts with the N-terminal death domain of MyD88.

Finally, we could demonstrate that the SOCS-boxes of SOCS1, SOCS2 and SOCS3 are not essential for cytokine receptor association.

The C-terminus of CIS defines its interaction pattern

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Proteins of the SOCS (suppressors of cytokine signalling) family are characterized by a conserved modular structure with pre-SH2 (Src homology 2), SH2 and SOCS-box domains. Several members, including CIS (cytokine-inducible SH2 protein), SOCS1 and SOCS3, are induced rapidly upon cytokine receptor activation and function in a negative-feedback loop, attenuating signalling at the receptor level. We used a recently developed mammalian two-hybrid system [MAPPIT (mammalian protein–protein interaction trap)] to analyse SOCS protein-interaction patterns in intact cells, allowing direct comparison with biological function. We find that, besides the SH2 domain, the C-terminal part of the CIS SOCS-box is required for functional interaction with the cytokine receptor

motifs examined, but not with the N-terminal death domain of the TLR (Toll-like receptor) adaptor MyD88. Mutagenesis revealed that one single tyrosine residue at position 253 is a critical binding determinant. In contrast, substrate binding by the highly related SOCS2 protein, and also by SOCS1 and SOCS3, does not require their SOCS-box.

Key words: cytokine-inducible Src homology 2 protein (CIS), cytokine receptor, mammalian protein–protein interaction trap (MAPPIT), myeloid differentiation marker 88 (MyD88), signal transduction, suppressor of cytokine signalling box (SOCS-box).

INTRODUCTION

A wide spectrum of α-helical bundle cytokines contributes to growth, differentiation and survival of haemopoietic cells. Examples include the CSFs (colony-stimulating factors), Epo (erythropoietin) and several ILs (interleukins) such as IL-5. More recently, leptin, a hormone-like member of this family, was also shown to promote proliferation of haemopoietic progenitors [1–3]. All of these cytokines activate the highly conserved JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway upon receptor binding. Signalling via these receptors is under tight control, including negative feedback by rapidly induced SOCS (suppressor of cytokine signalling) proteins. CIS [cytokine-inducible SH2 (Src homology 2) protein] was the founding member of the SOCS protein family that consists of eight members: SOCS1–SOCS7 and CIS [4,5]. All SOCS proteins comprise an SH2-domain responsible for association with phosphotyrosine motifs, an N-terminal pre-SH2 domain and a C-terminal SOCS-box [5]. They can modulate receptor activation and signalling via at least three distinct mechanisms.

CIS can inhibit Epo and GH (growth hormone) signalling by competition for STAT5-docking sites at the receptor level [6–8]. Consistent with this, CIS suppresses Epo-induced cell proliferation and promotes apoptosis of erythroid progenitor cells [9,10]. Phenotypes of CIS-transgenic mice and of STAT5a- and/or STAT5b-knockout mice show clear similarities, lending further support for CIS as a specific negative-feedback regulator of STAT5-mediated cytokine signalling [4,6,7,11]. Direct interference with STAT5 recruitment has also been suggested for SOCS2-mediated inhibition of GH action [12,13]. Interestingly, SOCS2

shows a dual effect on GH signalling. Mice lacking SOCS2 and SOCS2-transgenic mice both exhibit increased growth [12,14,15]. This may be explained through direct binding and functional interference between SOCS proteins [16,17].

CIS-dependent receptor degradation was reported for the EpoR (Epo receptor) and GHR (GH receptor) [6,18]. The SOCS-box of SOCS proteins can interact with the Elongins B and C [19], which form a complex with proteins of the Cullin and Rbx families. This assembly is an E3 ubiquitin ligase complex that is responsible for specific targeting of associated proteins for polyubiquitination [20]. This way, several SOCS proteins can inhibit signalling by marking associated signalling components for proteasomal degradation.

SOCS1 and SOCS3 carry a KIR (kinase inhibitory region) domain in their N-terminal region that acts as a pseudo-substrate for direct inhibition of JAK activity. SOCS1 interacts directly with the phosphorylated activation loop of JAK2 via its SH2 domain [21], whereas SOCS3 shows only weak affinity for JAK2 and is thought to bind to the receptor in close proximity to the kinase [22]. This is exemplified for SOCS3, which was identified recently as a potent inhibitor of LR (leptin receptor) signalling involved in regulation of energy balance: SOCS3 haplo-insufficient mice or neural-cell-specific SOCS3-knockout mice show augmented leptin sensitivity in the hypothalamus associated with a remarkable attenuation of diet-induced obesity, suggesting a key role for SOCS3 in leptin resistance [23,24].

SOCS proteins are also involved in regulating JAK/STAT-independent pathways such as insulin and TNF-α (tumour necrosis factor α) signalling [25–27]. Also, triggering of TLRs (Toll-like receptors), which are key players in innate immunity, leads to the

induction of CIS, SOCS1 and SOCS3 [28–30]. Evidence linking SOCS proteins to TLR signalling arose from the analysis of SOCS1-deficient mice that show enhanced sensitivity to LPS (lipopolysaccharide)-induced sepsis [31,32] and from SOCS1-deficient mice that lack endotoxin tolerance. Moreover, macrophages lacking SOCS1 produce increased levels of nitric oxide and pro-inflammatory cytokines in response to LPS. Conversely, SOCS1 overexpression in macrophages suppresses LPS-induced NF-κB (nuclear factor κB) activation. On the basis of these data, SOCS1 was considered to be a negative regulator of TLR signalling, although no direct target of SOCS1 could be identified. Recently, two groups have reported that SOCS1 has an indirect inhibitory effect on TLR signalling, targeting the secondary type I interferon signalling pathway and not the main NF-κB pathway [30,33].

In the present study, we examined the binding modalities of SOCS proteins in more detail. We demonstrate that the SOCS-box of CIS, and more particularly its C-terminal tyrosine residue, is essential for interaction with recruitment motifs in the EpoR and LR, and for its inhibitory role on STAT5 activation. In contrast, the SOCS-box is not required for SOCS2 receptor interaction or for signalling inhibition by SOCS1 and SOCS3. Furthermore, we identified the universal TLR adaptor MyD88 (myeloid differentiation marker 88) as a target for CIS. This interaction is SOCS-box-independent, indicating a different binding modus compared with that of the cytokine receptors.

EXPERIMENTAL

Constructs

Generation of the mutant murine LRs by mutagenesis and cloning in the pMET7 expression vector has been published elsewhere [34]. EpoR-bait constructs containing two extra leucine residues in the transmembrane region were described previously [35]. The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was used as described previously [36]. The pGL3-β-casein-luciferase reporter consisting of five repeats of the STAT5-responsive motif of the β-casein promoter was a gift from Dr Ivo Touw. Generation of the prey constructs pMG2-CIS and pMG2-SOCS2 was described in [35].

CISd5, CISdbox (amino acids 1–221), CISY249F and CISY253F prey constructs were generated by site-directed mutagenesis on pMG1-CIS using the following primer pairs. CISd5: 5′-GACTACCTCCGACAGTGATATCTCCAACTCTGATCTA-G-3′ and 5′-CTAGATCAGAGTTGGAGATATCACTGTCGGA-GGTAGTC-3′. CISdbox: 5′-GTGCGCAGGAGCAGTTGATAT-CGCTTACAACATCTGTG-3′ and 5′-CACAGATGTTGTAAG-CGATATCAACTGCTCCTGCGCAC-3′. CISY249F: 5′-GGCG-TATGGCCGACTTCTTAAGACAGTACCCCTTCC-3′ and 5′-GGAAGGGGTACTGTCTTAAGAAGTCGGCCATACGCC-3′. CISY253F: 5′-GACTACCTCCGACAGTTCCCCTTCCAGCT-GTGATCTAGAGAAAAAACCTCC-3′ and 5′-GGAGGTTTT-TTCTCTAGATCACAGCTGGAAGGGGAACTGTCGGAGGT-AGTC-3′.

The CIS mutants were then transferred to the pMG2 vector and the pMET7-FLAG expression vector by EcoRI/XbaI cloning. SOCS2Y194F was generated using the previously described pMG1-SOCS2 as template [36] and the primer set 5′-GCAG-AATTCACCCTGCGGTGCCTGGAGCC-3′ and 5′-CGCTGCG-GCCGCTTATACCTGGAATTTGAATTCTTCCAAGTAATC-3′, and was first cloned in the pMG1 vector as an EcoRI/NotI fragment, and then transferred to the pMG2 vector using EcoRI/XbaI. SOCS2Y190F and SOCS2dbox (amino acids 1–159) were

amplified from the pEF-FLAG-I/mSOCS2 construct (a gift from Dr Robyn Starr) using the 5′-GCAGAATTCACCCTGCGGTGC-CTGGAGCC-3′ and the 5′-GGTCGTCTAGAGCGGCCGCTTA-TACCTGGAATTTATATTCTTCCAAGAAATCTTTTAGTCTT-GTTG-3′ and 5′-GCTGGGCGGCCGCTTATGATGTATACAG-AGGTTTGG-3′ primers respectively, and were also cloned into the pMG2 vector. SOCS2 was transferred from the pMG2 vector to the pMET7-FLAG expression vector as an EcoRI/XbaI or EcoRI/NotI fragment.

The pMET7-FLAG-SOCS3 expression vector was described previously [37]. SOCS3dbox was amplified from the pMET7-FLAG-SOCS3 template using primers 5′-GCGAGATCTCAG-AATTCGTCACCCACAGCAAGTTTCC-3′ and 5′-CGCTTCT-AGATTAGTTGGAGGAGAGAGGTCGG-3′ allowing EcoRI/XbaI-based cloning in the pMet7-FLAG vector.

The pMet7-FLAG/mSOCS1 and pMet7-FLAG/mSOCS1dbox constructs were generated by amplifying SOCS1 and SOCS1dbox from the pEF-FLAG-I/mSOCS1 construct (gift from Dr Robyn Starr) with the 5′-CCAGCGAATTCATGGCGCGCGCCAGGAC-TACAAGGAC-3′ and 5′-GGTCGTCTAGATCAGATCTGGAA-GGGGAAGGAAC-3′ or 5′-GGTCGTCTAGATCAGCGGCGC-TGGCGCAGCGGGGCCCCCAAC-3′ primer sets respectively and EcoRI/XbaI cloning in the pMet7-FLAG vector.

EpoR cDNA was amplified from TF1-derived using primers 5′-CGGGGTACCATGGACCACCTCGGGGCGTCC-3′ and 5′-CGCTCTAGACTAAGAGCAAGCCACATAGC-3′ and was cloned in the pSVsport vector by KpnI/XbaI cloning. The pECE-STAT5B expression vector was a gift from Dr Walter Becker.

Mouse full-length MyD88 was amplified using the 5′-GCG-CGAGCTCAATGTCTGCGGGGAGACCCCCGCG-3′ and 5′-GC-GTGCGGCCGCTCAGGGCAGGGACAAAGCC-3′ primer pair on a pCAGGSE-mMyD88 expression vector (a gift from Dr Rudy Beyaert). After SacI/NotI digestion, the fragment was cloned in the pCEL(2L) vector, which was described previously [35]. This resulted in the mMyD88 bait construct. The mMyD88(N) and the mMyD88TIR bait vectors were made in an analogous manner using primer pairs 5′-GCGCGAGCTCAATGTCTGC-GGGGAGACCCCCG-3′/5′-GCTCGCGGCCGCTTACGTTTGT-CCTAGGGGGTC-3′ and 5′-GCGCGAGCTCAATGCCGGAA-CTTTTCGATGCC-3′/5′-GCTCGCGGCCGCTCAGGGCAGG-GACAAAGCC-3′ respectively.

The pCAGGSE-mMyD88(N) and pCAGGSE-mMyD88 DD (death domain) expression vectors were generated by amplification using oligonucleotide pairs 5′-GGCAAAGAATTGAATT-CCACCATGGGTGCGC-3′ and 5′-GCGCCTCGAGTCAAAG-TTCCGGCGTTTGTCCTAGGGGGTC-3′, and 5′-GGCAAAG-AATTGAATTCCACCATGGGTGCGC-3′ and 5′-GCGCCT-CGAGTCAGACGACGCGCCAGAGCGCCCCTGCC-3′ respectively on pCAGGSE-mMyD88, followed by a EcoRI/XhoI digestion and ligation into the pCAGGSE vector.

Generation of the pMET7TAP2 construct was described in [17]. CIS, CISdbox, CISY249F and CISY253F were introduced in a TAP2 (tandem affinity purification 2) construct via EcoRI/KpnI cloning from the respective pMG2 constructs.

An overview of the bait constructs used in this study is given in Table 1.

Cell culture, transfection and reporter assays

Cell culture conditions, transfection procedures and luciferase assays for HEK-293T (human embryonic kidney) cells were described previously [37]. For a typical luciferase experiment, 4×10^5 cells were seeded in six-well plates 24 h before overnight transfection with the desired constructs together with the luciferase reporter gene. Cells were left untreated (negative control) or were stimulated overnight with 100 ng/ml leptin or

Table 1   Overview of the bait constructs used in the present study

(a) Extracellular EpoR–intracellular LRF3 (Y985F/Y1077F/Y1138F) bait constructs

Protein	Construction
Mock	No bait
EpoR Tyr^{402}	Tyr^{402} motif of the EpoR
EpoR Tyr^{430}/Tyr^{432}	Tyr^{430}/Tyr^{402} motif of the EpoR
mMyD88	MyD88
mMyD88(N)	N-terminal part of MyD88 (DD and intermediate domain)
mMyD88(TIR)	C-terminal TIR domain of MyD88

(b) Extracellular LR–intracellular LR bait constructs

Protein	Construction
LR(F3)	LR (Y985F/Y1077F/Y1138F)
LR(YYF)	LR (Y1138F)
LR(YFF)	LR (Y1077F/Y1138F)
LR(FYF)	LR (Y985F/Y1138F)

(c) Non-MAPPIT receptor constructs

Protein	Construction
EpoR	WT EpoR
LR(FFY)	LR (Y985F/Y1077F)
LR(YFY)	LR (Y1077F)

3.3 ng/ml Epo, and luciferase activity of the transfected cells was measured by chemiluminescence.

Co-immunoprecipitation

Approx. 2×10^6 HEK-293T cells were transfected with different combinations of mMyD88-E, mMyD88(N), mMyD88 DD, mCIS–FLAG, mCISY253F and mCISdbox. Cleared lysates [modified RIPA lysis buffer: 200 mM NaCl, 50 mM Tris/HCl, pH 8, 0.05 % SDS, 2 mM EDTA, 1 % Nonidet P40, 0.5 % sodium deoxycholate and Complete™ Protease Inhibitor Cocktail (Roche)] were incubated with 4 μg/ml anti-FLAG mouse monoclonal antibody (Sigma) and Protein G–Sepharose (Amersham Biosciences). After immunoprecipitation, SDS/PAGE and Western blotting, interactions were detected using anti-E-Tag antibody (Amersham Biosciences) and horseradish-peroxidase-conjugated anti-(mouse IgG) antibody (Amersham Biosciences).

Phosphopeptide affinity chromatography

The phosphopeptide affinity chromatography procedure was as described previously [37].

EMSA (electrophoretic mobility-shift assay)

HEK-293T cells transiently transfected with the desired constructs were starved for 4 h in serum-free medium and were subsequently stimulated with 5 ng/ml Epo for 15 min or were left untreated. Protein concentrations of the nuclear extracts were measured with the Bio-Rad protein assay. Double-stranded oligonucleotides based on the β-casein promoter (sense: 5′-CAGATTTCTAGGAATTC-3′; antisense: 5′-GGATTTGAA-TTCCTAGAAATC-3′) were labelled by filling in 5′ protruding ends with Klenow enzyme using [α-^{32}P]dATP (3000 Ci/mmol; 10 mCi/ml). This probe binds STAT5 homodimers. Nuclear extracts (5 μg of protein) were incubated with approx. 10 fmol (20000 c.p.m.) of probe in gel-shift incubation buffer [10 mM Hepes, pH 7.8, 1 mM EDTA, 5 mM MgCl$_2$, 5 % glycerol, 5 mM dithiothreitol, 2 mM Pefabloc® SC, 1 mg/ml BSA and 0.1 mg/ml

poly(dI-dC) · (dI-dC)] for 10 min at room temperature (25 °C). The supershifting anti-STAT5 antibodies were incubated with the nuclear extracts for 10 min at room temperature before addition of the radiolabelled β-casein probe. The protein–DNA complexes were separated on a 4.5% (w/v) polyacrylamide gel containing 7.5 % glycerol in 0.5-fold TBE (Tris/borate/EDTA) at 20 V/cm for 90 min. Gels were fixed in water/methanol/ethanoic (acetic) acid (80:10:10, by vol.) for 30 min, dried and autoradiographed.

TAP2 purification and MS

HEK-293T cells were transfected with the appropriate TAP2 constructs. The TAP2 purification procedure was followed as described previously [17]. Proteins were visualized on a polyacrylamide gel by silver staining, or for MS analysis with Sypro Ruby protein gel stain (Molecular Probes) according to the manufacturer's instructions. Proteins of interest were excised, prepared for MS and applied for nano-LC-MS/MS analysis on an UltiMate™ system (Dionex) connected inline to an Esquire HCT (high-capacity trap) ion trap (Bruker Daltonics).

Modelling method

Molecular models were built for the CIS–Elongin B–Elongin C complex, using the crystal structure of the SOCS2–Elongin B–Elongin C complex as a template [38]. The sequences of CIS and SOCS2 were aligned automatically using the sequence-alignment editor of MOE (Molecular Operating Environment; Chemical Computing Group). Using this alignment, 150 models were built for the CIS–Elongin B–Elongin C complex in MODELLER version 8.1 [39], and ten models with the best DOPE (discrete optimized protein energy) and molpdf (molecular probability density function) scores were selected and evaluated.

RESULTS

Design of MAPPIT (mammalian protein–protein interaction trap) experiments

We previously reported a new two-hybrid method to study protein–protein interactions in intact mammalian cells, termed MAPPIT [36]. Briefly, a bait protein is C-terminally linked to a chimaeric EpoR/LR that is deficient in STAT3-recruitment sites, while a prey protein is attached to the string of four functional STAT3-recruitment sites of gp130 (glycoprotein 130). Association of bait and prey leads to STAT3 activation and subsequent activation of the STAT3-responsive rPAPI promoter–luciferase reporter. To examine interactions with the EpoR, we used its intracellular receptor tyrosine motifs as baits. We also analysed interactions with the LR itself by mutating the STAT3-recruiting Tyr^{1138} to phenylalanine. One or both of the two membrane-proximal tyrosine residues at positions 985 and 1077 were mutated to phenylalanine to examine tyrosine-specific interactions. These LR mutants were termed LR(YFF) and LR(FYF). MAPPIT configurations used in this manuscript are shown in Figure 1(A).

The C-terminus of CIS but not that of SOCS2 is required for receptor binding

We recently showed interaction of CIS and SOCS2 with the EpoR and the LR [17,35]. CIS and SOCS2 both interact with Tyr^{402}, and to a lesser extent also with Tyr^{344} and the double Tyr^{430}/Tyr^{432} motifs of the EpoR, and with Tyr^{1077} in the LR. Although both are highly related, only CIS associated with Tyr^{985} of the LR and only SOCS2 with the pTyr^{480} motif of the EpoR [17,35]. In the present study, we examined the binding modus of CIS and SOCS2 in more
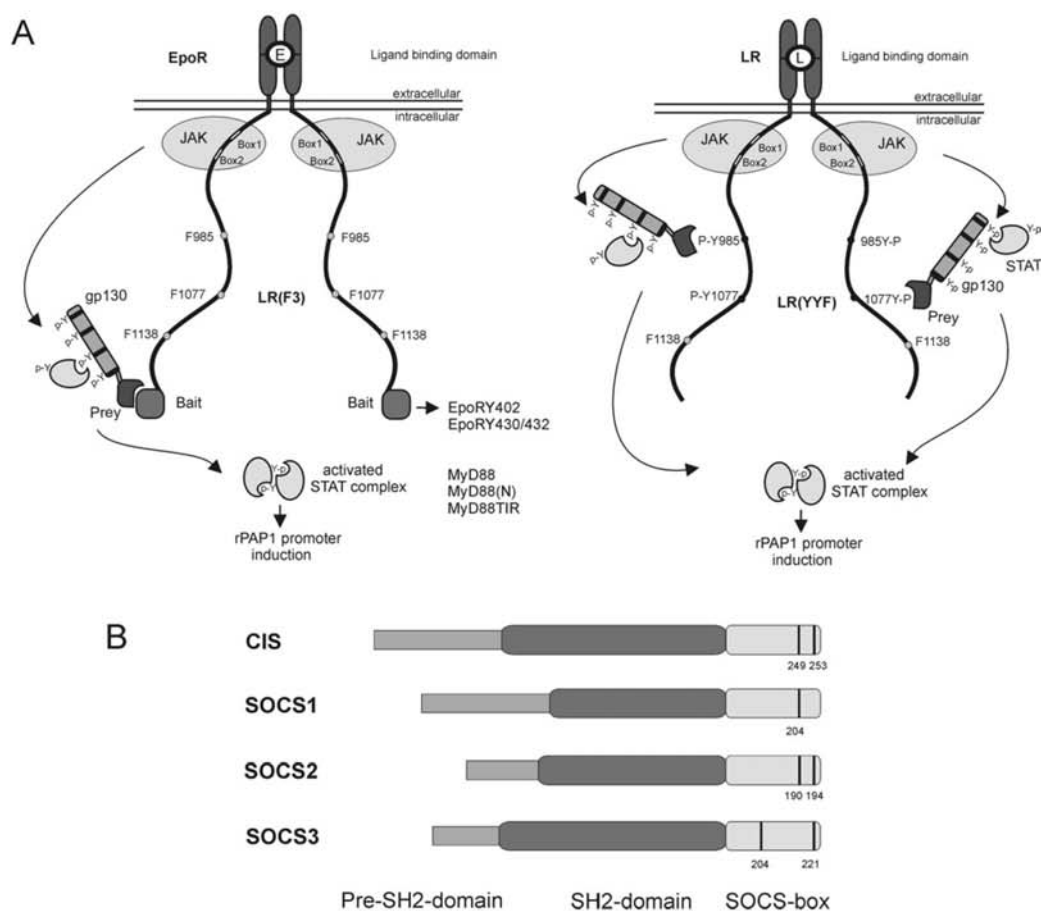
Figure 1   MAPPIT

(**A**) MAPPIT, a cytokine receptor-based two-hybrid method, is displayed in the left-hand panel, with the various receptor motifs used in the present study. The right-hand panel shows a variant of the MAPPIT technique using the STAT3 signalling-deficient LR as bait. Both MAPPIT methods are described in more detail in the Results section. (**B**) Schematic structure of SOCS proteins. Conserved tyrosine residues in the SOCS-box are indicated with a black line.

detail. It is well established that interaction of SOCS proteins with their receptor targets depends on their SH2 domains [40,41]. Figure 1(B) shows a diagrammatic representation of the structure of SOCS proteins. For MAPPIT use, CIS and SOCS2 preys were generated by N-terminal fusion with part of gp130. Figure 2(A) shows the critical requirement of the CIS SOCS-box for CISprey binding to the EpoR Tyr[402] motif in a MAPPIT experiment. C-terminal deletion of the entire SOCS-box resulted in complete loss of MAPPIT signalling. Detailed mapping showed that deletion of the five C-terminal amino acids and even a single Y253F mutation leads to impaired MAPPIT signalling (Figure 2A). In contrast, no effect was observed upon replacing the second conserved tyrosine residue at position 294 with phenylalanine. We next performed a similar analysis for SOCS2. Here, deletion of the entire SOCS-box, or tyrosine-to-phenylalanine mutation of both conserved tyrosine residues did not significantly affect signalling (Figure 2B). Very similar observations were obtained in MAPPIT experiments for the EpoR Tyr[430]/Tyr[432] motif (Figure 2C). In Figure 2(D), we expand this dataset further to the LR Tyr[985] and

Tyr[1077] positions, demonstrating that these findings are not limited to the EpoR system.

CIS binding to the EpoR pTyr[402] motif was also evaluated by phosphopeptide affinity chromatography. Figure 2(E) clearly shows loss of CIS binding by deletion of the SOCS-box or by introduction of the single Y253F mutation. In contrast to the MAPPIT dataset, complete loss of binding is also observed with the Y249F mutant. This may be explained by a lowered binding affinity so that the interaction with the EpoR pTyr[402] motif is still detected with MAPPIT, but not with peptide affinity chromatography. In line with such an assumption, MAPPIT detects interactions without the need for any purification step.

No role for the SOCS1 and SOCS3 SOCS-box for receptor binding

We also examined the role of the SOCS-box for the inhibitory function of SOCS1 and SOCS3. In this assay system, with clear inhibition of EpoR and LR signal transduction by co-expression of SOCS1 and SOCS3, deletion of the complete SOCS-box of
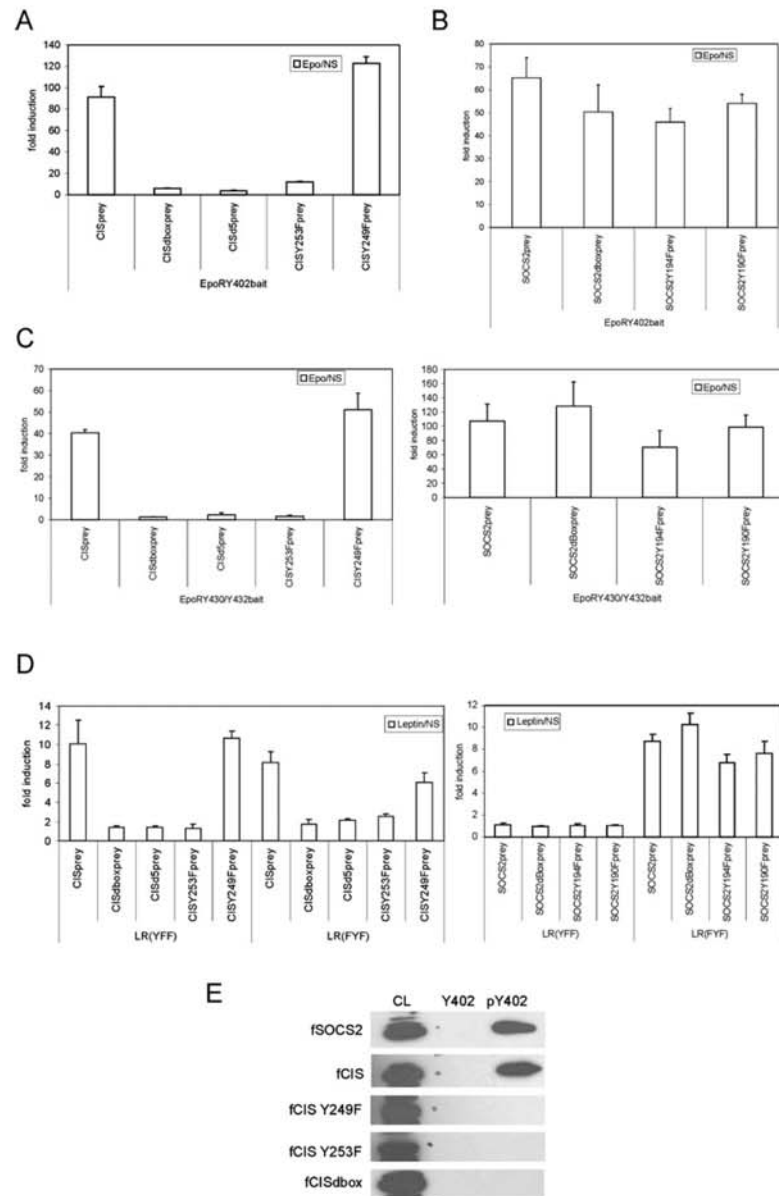
Figure 2   The CIS C-terminus is critical for interaction with EpoR and LR

(A) Interaction of CISprey mutants with EpoR Tyr[402]. HEK-293T cells were transiently co-transfected with plasmids encoding the EpoR Tyr[402] bait, various mutants of the pMG2-CIS prey construct and with the pXP2d2-rPAPI-luciferase reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 h. Luciferase activities were measured in triplicate. All preys were also tested for interaction with a 'mock bait' lacking an EpoR tyrosine motif, and consistently showed absence of any signalling (results not shown). Data are expressed as the stimulated/NS ratio + S.D. for triplicate measurements. (B) Interaction of SOCS2 prey mutants with EpoR Tyr[402]. As in (A), except HEK-293T cells were transiently co-transfected with plasmids encoding the EpoR Tyr[402] bait, various mutants of the pMG2-SOCS2 prey construct and with the pXP2d2-rPAPI-luciferase reporter. (C) Interaction of CISprey or SOCS2prey mutants with EpoR Tyr[430]/Tyr[432]. As in (A), except HEK-293T cells were transiently co-transfected with plasmids encoding the EpoR Tyr[430]/Tyr[432] bait, various mutants of the pMG2-CIS and pMG2-SOCS2 prey constructs and with the pXP2d2-rPAPI-luciferase reporter. (D) Interaction of CIS/SOCS2 prey mutants with LR(YFF) and LR(FYF). HEK-293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants, various mutants of the pMG2-CIS or pMG2-SOCS2 prey constructs and with the pXP2d2-rPAPI-luciferase reporter. The transfected cells were either stimulated for 24 h with leptin or left untreated (NS). Luciferase measurements were performed in triplicate. All preys were also tested for interaction with the LR lacking intracellular tyrosine residues and consistently showed absence of any signaling (results not shown). Data are expressed as the stimulated/NS ratio + S.D. (E) Peptide-affinity chromatography. HEK-293T cells were transfected with various mutants of CIS or SOCS2. The lysates were incubated with the (phospho-)tyrosine peptides corresponding to the Tyr[402] motif of the EpoR. Specific protein binding was revealed by SDS/PAGE and immunoblotting using the anti-FLAG antibody.

A

B
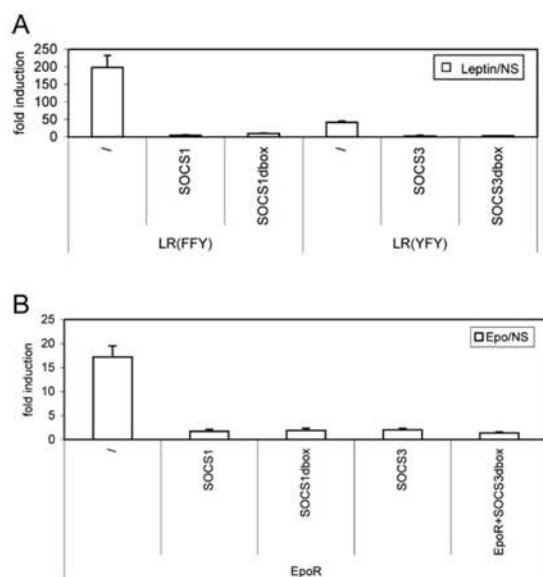
**Figure 3   The C-terminus of SOCS1 and SOCS3 is not essential for signalling inhibition**

Inhibition of EpoR (**A**) and LR (**B**) signalling by SOCS1/SOCS3 mutants. HEK-293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants or EpoR, various mutants of SOCS1 and SOCS3 and with the pXP2d2-rPAPI-luciferase reporter. The LR tyrosine mutants were used to minimize interference of other inhibitors. The transfected cells were either stimulated for 24 h with ligand or left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as the stimulated/NS ratio + S.D.

SOCS1 or SOCS3 did not significantly alter the inhibitory effect (Figure 3). We conclude that the SOCS-box of SOCS1 and SOCS3, in analogy with SOCS2, does not contain critical determinants involved in substrate binding. This highlights the unique new property of the CIS SOCS-box, which we next evaluated in more detail.

Critical role of Tyr^{253} in CIS function

We first investigated the functional implications of the CIS SOCS-box mutations. CIS operates in a classical negative-feedback loop on EpoR signalling: it is rapidly and strongly induced by activated STAT5 upon EpoR activation, subsequently binds to the Tyr^{402} site in the EpoR, and there inhibits STAT5 activation. We first looked at the effect of CIS mutations on STAT5-dependent β-casein promoter-reporter activity using the EpoR Tyr^{402} bait. Wild-type CIS clearly abrogated reporter induction as expected. In contrast, co-expression of mutant proteins with a C-terminal deletion of the SOCS-box, or with the Y253F mutation was unable to impair reporter induction. The CISY249F mutant inhibited signalling to a similar extent as did wild-type CIS protein (Figure 4A). Expression of all CIS variants was verified via Western blot (results not shown).

Confirmation was obtained using EMSAs. A ^{32}P-labelled probe corresponding to a β-casein STAT5-binding site was used to visualize bound STAT5 complexes. Whereas wild-type CIS clearly suppressed the formation of nuclear STAT5–DNA complexes, deletion of the complete SOCS-box, as well as the Y253F mutant, resulted in loss of inhibition (Figure 4B). Again, the Y249F mutant behaved as wild-type CIS. Supershift with

A

B

C
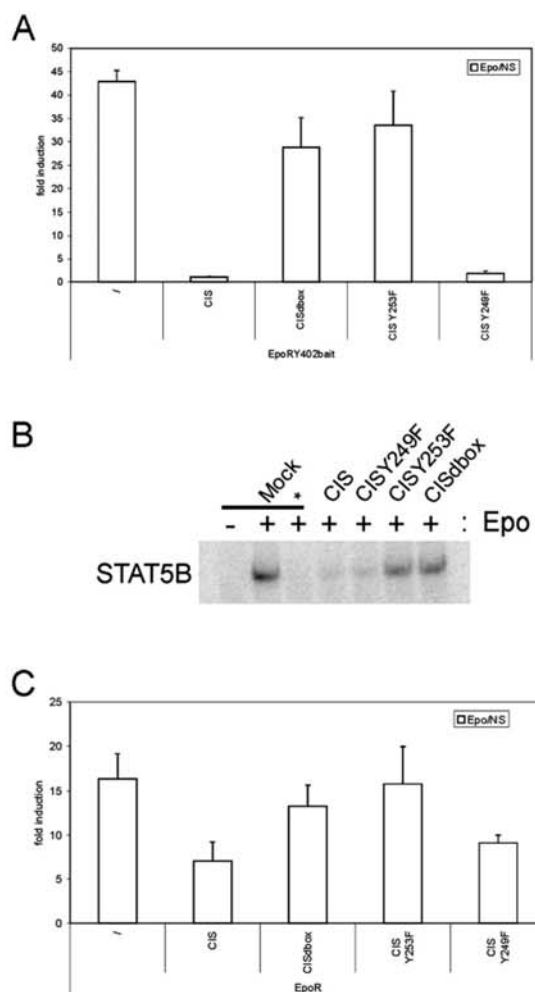
Figure 4   Critical role of CIS C-terminus in blocking STAT5 activation

(**A**) STAT5 reporter assay using the EpoR Tyr^{402} motif. HEK-293T cells were transiently co-transfected with plasmids encoding the EpoR Tyr^{402} bait, various mutants of FLAG-tagged CIS and with the pGL3-β-casein-luciferase reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 h. Luciferase activities were measured in triplicate. Data are expressed as the stimulated/NS ratio + S.D. (**B**) EMSA using the EpoR Tyr^{402} motif. HEK-293 Flp-In cells were transiently co-transfected with plasmids encoding the EpoR Tyr^{402} bait, various FLAG-tagged CIS mutants and STAT5B. Nuclear lysates were incubated with ^{32}P-labelled probe corresponding to a β-casein STAT5-binding site to reveal active STAT5 complexes. The EpoRy402F bait (*) was used as a negative control. (**C**) STAT5 reporter assay using the wild-type EpoR. HEK-293T cells were transiently co-transfected with plasmids encoding the EpoR, various mutants of FLAG-tagged CIS and with the pGL3-β-casein-luciferase reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 h. Luciferase activities were measured in triplicate. Data are expressed as the stimulated/NS ratio + S.D.

anti-FLAG antibody confirmed the presence of STAT5B in the complexes (results not shown).

Reporter assays were also performed on the wild-type EpoR. Although the effects were less pronounced, the tendencies clearly corresponded to what we observed for the EpoR bait construct (Figure 4C). This weaker effect is most likely explained by the incomplete overlap of STAT5- and CIS-binding sites [6,35].
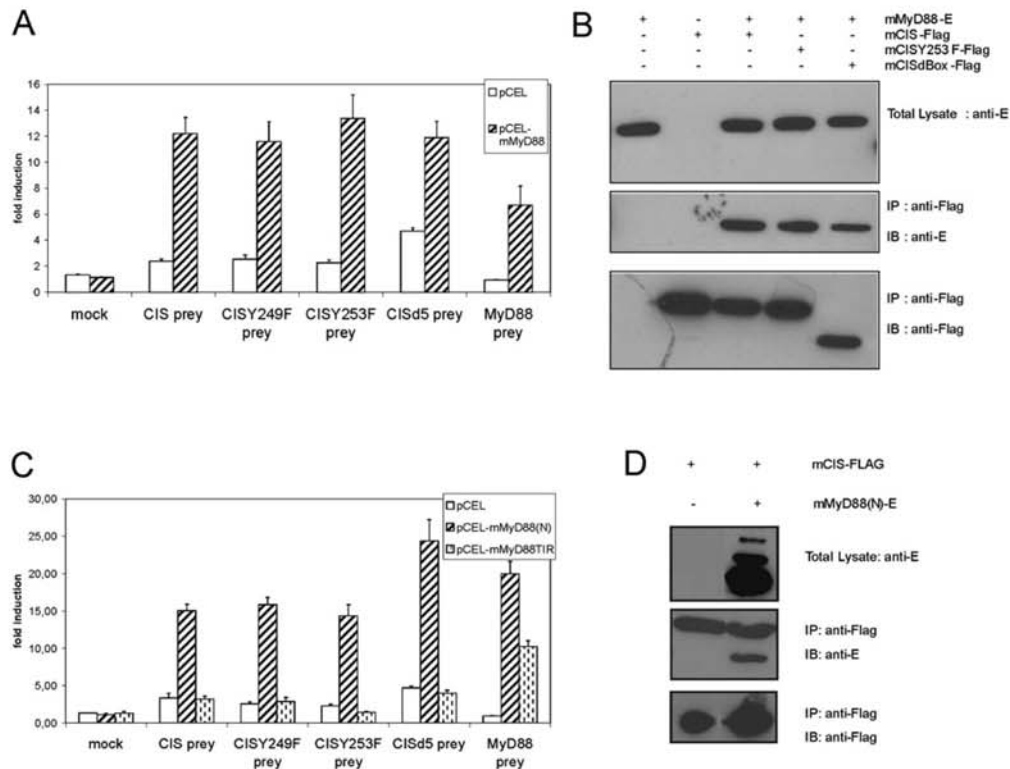
Figure 5   The SOCS-box of CIS is not critical for interaction with MyD88

(**A**) Interaction of MyD88 and CIS prey constructs. HEK-293T cells were transiently co-transfected with the MAPPIT mock bait or the mMyD88 bait plasmid (0.1 μg), various CIS or CIS-mutant prey (0.5 μg) constructs and with the pXP2d2-rPAPI-luciferase reporter (0.2 μg). MyD88 prey was used as a positive control. The transfected cells were either stimulated for 24 h with Epo or left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as the stimulated/NS ratio + S.D. (**B**) Co-immunoprecipitation analysis. HEK-293T cells were transiently co-transfected with combinations of mMyD88-E, mCIS–FLAG, mCISY253F and mCISdbox. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E. (**C**) Role for the N-terminal domain of MyD88 in CIS binding. HEK-293T cells were transiently co-transfected with the MAPPIT mock bait, the mMyD88(N) bait or the mMyD88TIR bait vector (0.1 μg), various CIS or CIS-mutant prey (0.5 μg) constructs and with the pXP2d2-rPAPI-luciferase reporter (0.2 μg). The MyD88 prey construct was used as a positive control. The transfected cells were either stimulated for 24 h with Epo or left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as the stimulated/NS ratio + S.D. (**D**) Co-immunoprecipitation analysis. HEK-293T cells were transiently co-transfected with combinations of mMyD88(N)-E and mCIS–FLAG. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E.

CIS interaction with MyD88 does not depend on its SOCS-box

SOCS proteins are also rapidly induced after TLR stimulation. However, no interaction partner of the SOCS proteins in TLR signalling has been described so far. A possible target of SOCS proteins in TLR signalling is the adaptor protein MyD88, which is used by most TLRs. To investigate this possibility, we performed MAPPIT analysis using MyD88 as bait and the CISprey. As shown in Figure 5(A), we observed clear induction of luciferase activity implying an interaction between CIS and MyD88. To investigate the role of the SOCS-box of CIS in this interaction, we next analysed the effect of the above mentioned CIS mutants. Much in contrast with association of CIS with the EpoR and LR, interaction between MyD88 and CIS was not affected by any CIS mutation, including deletion of its entire SOCS-box. These data were confirmed by co-immunoprecipitation (Figure 5B). Here, we transiently co-expressed E-tagged MyD88 (MyD88-E) and FLAG-tagged CIS (CIS–FLAG), CISY253F (CISY253F–FLAG) or CIS lacking its SOCS-box (CISdbox–FLAG). In every

case, MyD88-E was co-immunoprecipitated using an anti-FLAG antibody.

MyD88 consists of two interaction domains: a C-terminal 'TIR' (Toll/IL-1 receptor) domain and a N-terminal DD, linked by a short intermediate domain. To examine the role of either domain in CIS binding, we created MAPPIT baits containing the N-terminal part of MyD88 encompassing the DD and the intermediate domain, or the C-terminal TIR domain. MAPPIT analysis showed clearly that only the N-terminal part of MyD88 interacted with CIS (Figure 5C). Again, co-immunoprecipitation studies confirmed these findings (Figure 5D). Taken together, we clearly document a role for the MyD88 DD in CIS recruitment, and show that this interaction depends solely on the CIS SH2 domain.

C-terminal mutations in CIS do not affect Elongin B/C and Cullin 5 recruitment

We used a variant, called TAP2, of the tandem affinity purification method developed by Puig et al. [42] (S. Eyckerman, unpublished
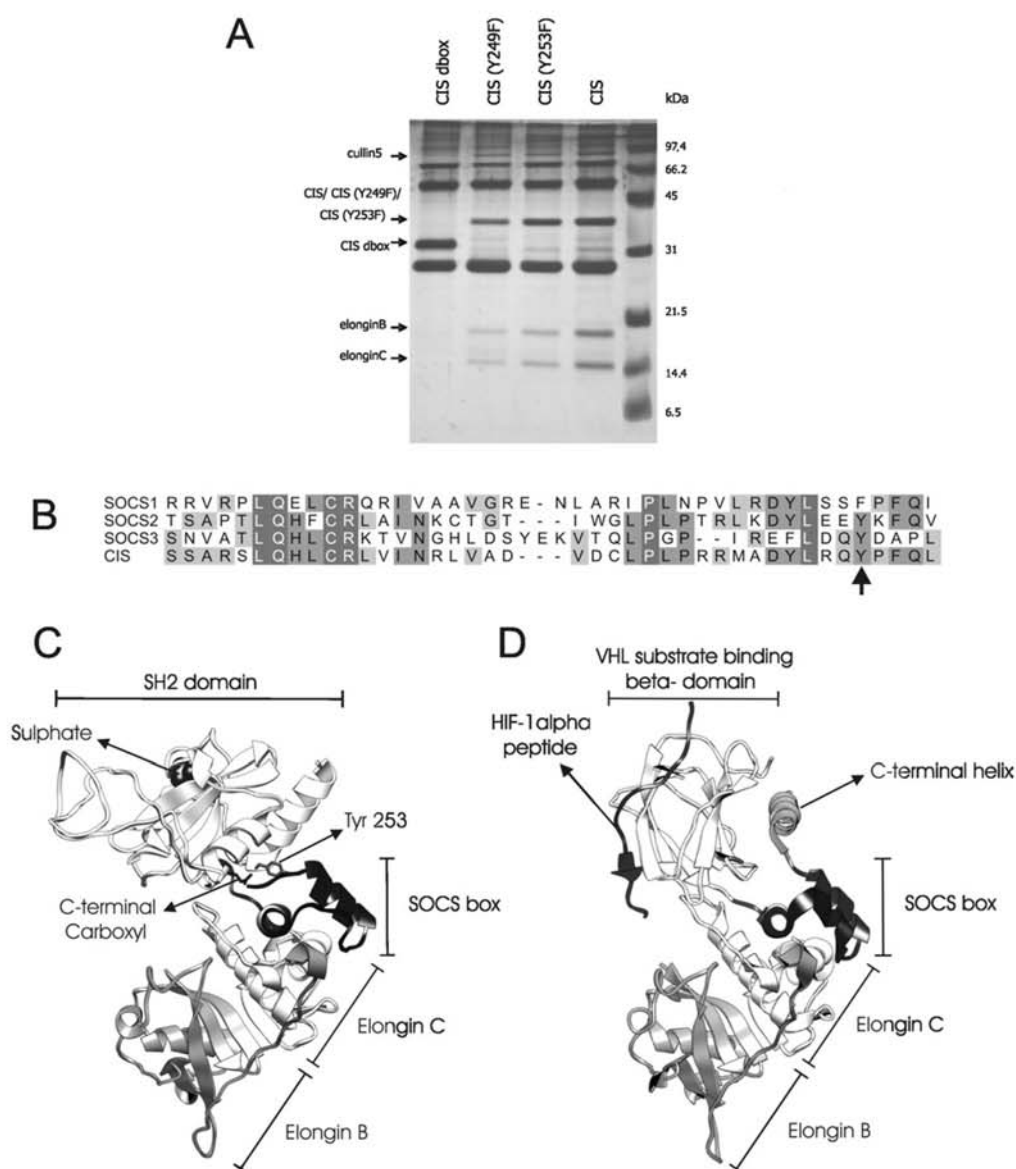
Figure 6 Role of the CIS C-terminus in Elongin B/C association

(**A**) TAP2 purification using CIS mutants. HEK-293T cells were transiently transfected with various mutants of the pMet7TAP2-CIS construct. Cell lysates were purified by the TAP2 method and were then loaded on a 14% polyacrylamide gel. After silver-staining, the protein bands indicated with an arrow were analysed by MS and identified as the annotated protein. (**B**) Sequence alignment of SOCS-boxes. SOCS-box sequences of murine SOCS1, SOCS2, SOCS3 and CIS were aligned using the t_coffee algorithm. An arrow indicates Tyr[253]. Increasing grey shading corresponds with increasing percentage identity. (**C**) Homology model of mouse CIS, in complex with Elongin C and B. The CIS SOCS-box is shown in black. The position of the phosphopeptide substrate in the model is indicated by a sulfate ion, copied from the SOCS2 template structure, that mimics the phosphate group of the phosphopeptide substrate. Tyr[253] hydrogen bonds with the C-terminal carboxy group. (**D**) Crystal structure of VHL protein (PDB code 1LQB), bound to its hydroxylated HIF-1α substrate (black) and to Elongins C and B. The pVHL SOCS-box and the hydroxyproline-binding domain are in black. The extra C-terminal helix (dark grey) is indicated.

work) to analyse protein complexes of CIS and its mutants. Cullin 5 and Elongins B and C were identified as interacting partners of CIS. While deletion of the complete SOCS-box of CIS abrogates their association, mutation of the tyrosine residues at position 249 or 253 to phenylalanine did not significantly influence Cullin 5 or Elongin B and C binding (Figure 6A).

Modelling of the SOCS-box of CIS

We used the crystal structure of the SOCS2–Elongin C–Elongin B complex [38] to build a molecular model for CIS bound to Elongin B and C (Figure 6C). This model, together with sequence alignment with other SOCS proteins (Figure 6B), showed that residues involved in Elongin C binding are highly conserved. As for SOCS2, the C-terminal residues of CIS mutated in this work are not part of its actual Elongin C-binding site and mutation of the C-terminus of CIS is thus not predicted to affect Elongin C binding directly. As in SOCS2, the C-terminus of CIS is buried in the interface between the SH2 domain and he SOCS-box domain. The hydroxy group of the completely buried Tyr[253] hydrogen bonds to the buried C-terminal carboxy group. The C-terminus of CIS or SOCS2 is not able to make direct contact with a phosphopeptide substrate bound to the SH2 domain (Figure 6C).

DISCUSSION

SOCS proteins typically consist of a phosphotyrosine-binding SH2 domain, a C-terminal SOCS-box involved in proteasome recruitment and a pre-SH2 domain that only in the case of SOCS1 and SOCS3 contains a JAK-blocking KIR domain. Association of SOCS proteins with their target substrates is believed to occur solely via their SH2 domain. In the present study, we took a closer look at the binding modus of SOCS proteins using the MAPPIT approach, combined with biochemical and functional analyses.

A key finding is that the SOCS-box of CIS is essential for association with recruitment motifs in cytokine receptors, including the EpoR and LR. Deletion of the entire SOCS-box abrogated binding completely, and more detailed mutagenesis analysis revealed the critical role of the single C-terminal Tyr[253]. These findings were confirmed by peptide-affinity chromatography using the phosphorylated or non-phosphorylated EpoR Tyr[402] motif. Furthermore, reporter assays and EMSAs extended these findings to functional activation of STAT5. Indirect effects of the mutations on the structural integrity of CIS could be ruled out, since clear SOCS-box-independent interaction was observed with the unrelated MyD88 protein as bait. Association of the CISY249F mutant with the EpoR pTyr[402] motif could not be demonstrated by peptide-affinity chromatography, suggesting that Tyr[249] might serve a similar role as Tyr[253]. However, MAPPIT experiments showed indisputable association of this CIS mutant with the same EpoR Tyr[402] motif. Only this latter interaction was functionally confirmed by the clear inhibitory effect seen in EpoR Tyr[402]-dependent STAT5 recruitment and activation. The Y249F mutant thus only modestly reduced binding affinity compared with wild-type CIS. This reduced binding affinity of CISY249F completely abolished binding with the EpoR pTyr[402] motif in a peptide-affinity chromatography experiment, much in contrast with MAPPIT. The MAPPIT technique therefore reveals itself as a sensitive tool for the identification of weaker, but functionally highly relevant, protein interactions.

In line with our findings that the SOCS-box of CIS is essential for EpoR association, Ketteler et al. [10] reported previously that the SOCS-box of CIS is essential for the apoptotic effect of CIS on erythroid progenitor cells. Seemingly contradictory to our observations, they also found that the SOCS-box of CIS was not required for inhibition of EpoR-induced proliferative responses [10]. However, this anti-proliferative effect may be due to CIS interference with intermediate signalling molecules coupling to the cell cycle. CIS can indeed associate with downstream effector molecules in a SOCS-box-independent modus as we showed for MyD88.

This critical role of the CIS SOCS-box in substrate binding may be a unique feature of CIS, and was not seen for the highly related SOCS2 protein, or for SOCS1 and SOCS3. The corresponding mutation of the conserved tyrosine residue in the SOCS-box of SOCS2 or even deletion of its entire SOCS-box did not show any significant effect on receptor association. Likewise, the inhibitory effect of SOCS1 and SOCS3 on cytokine receptor signalling was hardly affected by removal of the SOCS-box. Previously, the SOCS-box of SOCS1 was also reported to be dispensable for LIF, IL-6 and GHR signalling inhibition but not for G-CSF (granulocyte CSF) signal transduction [40,41,43,44]. *In vivo* deletion of the entire SOCS-box of SOCS1, however, leads to partial loss of SOCS1 function [45], most likely reflecting its role in Elongin B/C binding, thus establishing an E3 ubiquitin ligation complex leading to proteasomal degradation of associated receptor complexes.

Using MAPPIT, we could also demonstrate the association of CIS with the universal TLR adaptor MyD88. This interaction was confirmed by co-immunoprecipitation. In contrast with the data described above, mutation of the conserved C-terminal Tyr[253] to phenylalanine or deletion of the complete SOCS-box of CIS had no effect at all on MyD88 binding. Further analysis of this association revealed a critical role for the DD of MyD88 in CIS binding. More studies are required to elucidate the functional consequences of this interaction. We also observed interaction of other members of the SOCS protein family with MyD88 and its splicing variant lacking the intermediate domain (P. Ulrichts, unpublished work), and structural and functional analyses of these interactions are ongoing. Interestingly, our results imply differential modulation by CIS of signalling via cytokine receptors and TLRs.

A crystal structure for SOCS2 in a complex with Elongin C and B was determined recently [38]. A molecular model was built for the CIS–Elongin C–Elongin B complex in order to get structural insight into the role of the CIS C-terminus on substrate recognition. In the SOCS2 structure and the CIS model, the C-terminus is buried in the interface between the SH2 domain and the SOCS-box, excluding the possibility that this C-terminus could make direct contact with a phosphopeptide substrate bound to the SH2 domain (Figure 6C). In both CIS and SOCS2, the hydroxy group of the last tyrosine residue hydrogen bonds to the buried C-terminal carboxy group. The Y253F mutation in CIS can thus be expected to influence the protein structure or folding: removing the tyrosine hydroxy group may render burial of the C-terminus energetically unfavourable. Tyr[253] of CIS may therefore play a structural role. One can speculate that this affects stability of the packing between SOCS-box and SH2 domain, but how this might affect binding to a phosphopeptide substrate remains unclear. Allosteric effects on the substrate-binding pocket cannot be excluded. However, the direct environment of the C-termini in the CIS model and SOCS structure are very similar, hinting that the same phenomenon would be expected for the Y194F mutation in SOCS2, whereas this mutation has no effect on interaction with its phosphopeptide substrates.

Tyrosine phosphorylation of SOCS proteins has been reported previously: Cacalano and co-workers [46,47] showed Epo-induced phosphorylation of the two conserved tyrosine residues in the SOCS-box of SOCS3, including Tyr[221] that corresponds to Tyr[253] in CIS. Interestingly, the C-terminal pTyr[221] allowed binding and functional coupling to the Ras signalling pathway on the one hand, while both phosphorylated tyrosine residues, situated centrally and C-terminally in the SOCS-box, were involved in abrogation of Elongin C interaction [46,47]. Intriguingly, as in CIS and SOCS2, Tyr[221] in SOCS3 is also predicted (results not shown) to be buried in the interface between the SH2 domain

and the SOCS-box, and its hydroxy group hydrogen bonds to the buried C-terminal carboxy group. It is therefore likely that phosphorylation of Tyr^{221} in SOCS3, and possibly Tyr^{253} in CIS, requires changes in the conformation of the C-terminus. One possibility is that burial of the C-terminus as seen in the SOCS2 crystal structure depends on binding of the Elongin complex. In the absence of Elongin binding, the C-terminal tyrosine motifs may be accessible for phosphorylation. In this structural modus, phosphorylation-dependent interactions may occur with signalling molecules or with accessory proteins that facilitate interactions with (a subset of) substrates. Mutating Tyr^{253} in CIS may then prevent the phosphorylation-driven structural change required for downstream interactions. Deletion of the four C-terminal amino acids in the CISprey, eliminating the putative phosphorylation context, leads to impaired MAPPIT signalling, adding evidence to this phosphorylation hypothesis (results not shown). Of note, it seems unlikely that disturbed Elongin binding causes the substrate-binding defects in the CIS mutants: TAP2 purification of wild-type CIS and of its C-terminal tyrosine mutants showed clear association with Elongins B and C and with Cullin 5, which was lost completely when the entire SOCS-box of CIS was deleted. Thus Elongin C binding does not involve the C-terminus of CIS and has no role in the effect of mutating Tyr^{253}.

The role of the C-terminus of CIS in substrate binding is remarkably similar to the role of the C-terminal helix of the VHL (von Hippel–Lindau) tumour-suppressor protein. The VHL protein is part of a VHL–E3 ligase complex involved in ubiquitination of transcription factors such as HIF (hypoxia inducible factor)-1α, targeting them for degradation. The VHL–E3 ligase complex binds and ubiquitinates two oxygen-dependent degradation domains (HIF-ODDD). The crystal structure of VHL protein, bound to a hydroxylated HIF-1α peptide, and to the Elongins C and B has been determined (Figure 6D) [48]. This revealed that the SOCS-box of VHL is followed by a C-terminal helix, which is not directly involved in binding to the hydroxylated HIF-1α peptide. Like the C-terminus of CIS, this helix interacts tightly with the SH2 domain. Lewis et al. [49] reported that this C-terminal helix is critical for ubiquitination of HIF-1α. Deletion of this C-terminal peptide impaired VHL binding and ubiquitination of the C-terminal HIF-ODDD, while ubiquitination and degradation of the N-terminal HIF-ODDD is hardly affected [49]. The role of the C-terminal peptide in VHL thus shows some striking parallels with the CIS C-terminus. As for CIS, deletion of the VHL C-terminus specifically affects certain functions/interactions, leaving other functions unaltered. It was suggested that deletion of the C-terminus might affect VHL substrate binding by secondary folding effects.

In brief, we have shown that the SOCS-box of CIS is essential for interaction with target cytokine receptors but not with the universal TLR adaptor MyD88. It appears that the biological role of the SOCS-box is more complex than simple recruitment of a ubiquitin–ligation complex, and is also involved in (regulated) substrate binding. Depending on the type of SOCS protein, this may include receptor recruitment motifs, alternative signalling pathways and other SOCS proteins [16,17]. The precise underlying controls that are involved in these diverse functions of the SOCS-box remain to be clarified.

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PART 3: Conclusions

Interactions between proteins are essential in almost all cellular processes. Proteins can form stable structures including the proteasome complex, ribosomes and the cytoskeleton. However, protein interactions are also involved in the regulation of cellular responses. As these processes are context-dependent, the protein complexes involved are often of a temporary nature. Identification of interactors of a protein in a given cellular context, can contribute considerably to its functional definition.

As discussed in chapter 4, a wide spectrum of biochemical and genetic techniques have been developed to study these protein-protein interactions. Biochemical techniques have the advantage that signalling complexes can be analysed in whole. However, the lysis step involved in these methods can lead to a loss of weak interactions. Moreover, cell lysis disrupts the normal cellular environment, which can result in false positive interactions between proteins normally residing in separate cellular compartments. Genetic methods rely on hybrid bait and prey molecules, of which interaction lead to detectable signals. The frequently used yeast two-hybrid method is based on functional complementation of a transcription factor (Fields and Song, 1989). However, this technique suffers from some intrinsic drawbacks. Bait and prey interaction need to occur in the nucleus, thus excluding the analysis membrane-bound proteins. Moreover, interactions between mammalian proteins often require post-translational modifications, which are hard to reproduce in a yeast context.

To circumvent these restrictions, MAPPIT was developed in our laboratory (Eyckerman et al., 2001). The MAPPIT system relies on the fundaments of type I cytokine signalling, exploiting the cardinal JAK/STAT signalling pathway. Interaction of bait- and prey-hybrids leads to the functional complementation of a STAT3 recruitment-deficient leptin receptor by STAT3 recruitment sites on the gp130 chain. Next to the advantages intrinsic to its mammalian context, MAPPIT disposes of some additional assets. Since signal readout is mediated by STAT molecules, commuting between cytosol and nucleus, nuclear translocation of bait and prey molecules is avoided. Moreover, these bait and prey proteins cannot directly interfere with signal readout. The versatile MAPPIT system appeared to be a valuable technique for the analysis of various signalling pathways including erythropoietin (Montoye et al., 2005), leptin (Lavens et al., 2006); Wauman *et al.*, submitted) and growth hormone receptor signalling (Lavens et al., 2006; Uyttendaele et al., 2007). The flexibility of the

MAPPIT system is also reflected by a number of reports where the technique has been applied in haematopoietic (Montoye et al., 2006) and neuronal (Wauman *et al.*, submitted) cell lines. Other cell types can be used, on the condition that these cells endogenously express STAT3. Moreover, STAT5 expressing cells can also be used through the application of the β_{common}-MAPPIT variant (Montoye et al., 2006).

The MAPPIT system can also be used to screen for new interactors (Lievens et al., 2004). Therefore the luciferase reporter is changed by a surface tag, which is used as a selectable marker gene. Cells expressing a bait-interacting prey are enriched through a series of selection steps using magnetic-activated cell sorting and next selected by fluorescence-activated cell sorting (FACS). Ultimately, these positive cells are deposited as single cells, allowing easy identification of the bait-interacting proteins.

Throughout this thesis, the MAPPIT technology was extensively used to study TLR signalling events. In that way we generated a TLR-adaptor interaction map. Both well-documented and new interactions could be detected. The strength of the MAPPIT technology was underscored by the observation that Mal acts as a bridging molecule, linking MyD88 to the activated TLR4, thereby demonstrating the potency of MAPPIT to detect indirect interactions. Our data are in line with a recent report, describing the essential role of a PIP2 binding domain in the N-terminus of Mal in TLR4 signalling (Kagan and Medzhitov, 2006). This domain ensures Mal recruitment to PIP2-rich areas in the plasma membrane and was shown to be crucial in facilitating MyD88 delivery to an activated TLR4. Quite surprisingly, we could not detect direct interaction between MyD88 and other TLRs including TLR5, TLR7 and TLR9 using MAPPIT. Consistently, yeast-two hybrid methods also fail to demonstrate interaction between MyD88 and TLR9 (Brown et al., 2006). Moreover, MyD88 should be recruited to different subcellular compartments, as it is used by both endosomal as membrane expressed TLRs. The mechanisms involved in this differential translocation remain to be unravelled. Although we cannot exclude that the MAPPIT system fails to detect these interactions, these findings suggest that TLR5/7/9 use accessory molecules, with an analogous role as Tram and Mal in TLR4 signalling.

In addition, we evaluated the homo-and heterodimerisation properties of Mal and MyD88. Moreover, analogous mutations as the TLR4P712H mutation in the BB

loop of the TLR4 TIR domain were made for Mal and MyD88, a mutation which is known to abrogate TLR4 signalling (Poltorak et al., 1998). These mutated adaptors failed to homo- and heterodimerise and also TLR interaction was abrogated. In that way we provided biochemical evidence for the observed signalling defects. These initial data prompt us to further characterise the TIR-TIR interaction surface using MAPPIT, through the evaluation of additional TIR domain mutations.

TLR signalling balances on a tight rope, balancing between a potent immune response and excessive inflammation. Therefore, the TLR pathway is strictly controlled by various means. Inhibition of TLR signalling is achieved either by inhibiting ligand binding, by modulating TLR expression or by interfering in the intracellular signalling pathways. One group of intracellular TLR regulators is the SOCS protein family. The inhibitory function of SOCS1 in TLR4 signalling is well established, targeting the adaptor protein Mal for proteasomal degradation (Kinjyo et al., 2002; Nakagawa et al., 2002; Mansell et al., 2006). Using MAPPIT, we could demonstrate interaction between another member of the SOCS family, CIS, and the universal TLR adaptor MyD88. These finding suggest a regulatory role for CIS analogous to SOCS-1. However, functional data supporting this hypothesis remain to be found. This interaction was, much in contrast with interaction of CIS with the epo and leptin receptor, not dependent on a conserved tyrosine in the SOCS box domain of CIS. These findings suggest a differential interaction modus of CIS between receptor binding and recruitment of MyD88. SOCS proteins are subjected to tyrosine phosphorylation (Cacalano et al., 2001) and therefore, it is tempting to speculate that the critical tyrosine in the CIS SOCS-box needs to be phosphorylated to allow receptor interaction. Moreover, the corresponding tyrosine in the SOCS-box of SOCS3 was shown to be phosphorylated and this event was crucial for elongin C recruitment (Haan et al., 2003). Metabolic labelling experiments indicated that CIS can be phosphorylated. Moreover, an altered distribution of the tyrosine mutated CIS compared with the wild type was observed using 2D gel electrophoresis. However, future effort will need to clarify the nature and location of these modifications, which is essential to provide mechanistic insight of CIS interactions.

The dramatic consequences of several autoimmune diseases, chronic inflammation and bacterial sepsis can be conferred to uncontrolled TLR signalling.

Because of their specific role in the signalling pathway, TLR adaptor molecules are considered as ideal therapeutical targets. Another MAPPIT variant, reverse MAPPIT, allows relatively easy discovery and analysis of disruptor molecules (Eyckerman et al., 2005). Using reverse MAPPIT in a TLR context, could lead to the identification of therapeutics disrupting the TIR-TIR interaction and in that way alleviating the harmful effects of inappropriate TLR signalling. Moreover, mutational studies using MAPPIT could provide a better insight in the nature of the TIR-TIR interaction surface, which will considerably facilitate therapeutic development.

Taken together, our data prove the functionality of the MAPPIT system in a TLR framework. The MAPPIT toolbox can contribute substantially to the characterisation of TLR signalling pathways both by the analytical use of MAPPIT as well as by the FACS-based screening method. Moreover, the reverse MAPPIT methology appears to be a valuable tool for the screening of drugs inhibiting uncontrolled and harmful TLR signalling.

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