Intracellular regulation of TLR signalling

Basic mechanisms and importance for intestinal inflammation

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Sweden, 2011

"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after"

JRR Tolkien

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Abstract

Toll-like receptors (TLRs) recognize conserved structures on/in microorganisms. The intracellular signalling pathways of TLRs are shared with IL-1R and IL-18R and their activation leads to transcription of pro-inflammatory cytokines and type-I interferons. Signalling downstream of these receptors is strictly regulated via diverse mechanisms including downregulation of proteins important for signalling transduction and upregulation of proteins that negatively regulates signalling transduction. The intestinal lumen is populated with an enormous number of bacteria separated from the immune system with only a single layer of intestinal epithelial cells (IECs). Interestingly, IECs and immune cells in the lamina propria (LP) have a restricted expression of TLRs and an increased expression of negative regulators contributing to intestinal homeostasis. Mutations in several TLRs have been associated with inflammatory bowel disease (IBD) whereas less is known about the importance of intracellular signalling components. The aim with this thesis was to investigate the regulation of TLR signalling during homeostasis and intestinal inflammation.

First, we tried to identify serum markers for early detection of intestinal inflammation in Gαi2$^{-/-}$ mice that spontaneously develop intestinal inflammation 12-25 weeks after birth. Serum concentrations of IL-18 was upregulated in ongoing colitis whereas IL-1Ra was upregulated in ongoing and in early coilits. Furthermore, splenocytes from Gαi2$^{-/-}$ mice had increased production of pro-inflammatory cytokines in response to TLR stimulation and Gαi2$^{-/-}$ peritoneal macrophages had an intact TLR cross-tolerance.

To investigate the mechanisms involved in TLR signalling and cross-tolerance, IRAK-1$^{-/-}$ peritoneal macrophages were stimulated with LPS and/or LTA. IRAK-1$^{-/-}$ peritoneal macrophages had a reduced production of TNF and IL-10 in response to low concentrations of LTA whereas high concentrations of LPS resulted in decreased IL-10, but not TNF, production. Interestingly, increased concentration of LTA restored TNF production and reduced concentrations of LPS impaired TNF production from IRAK-1$^{-/-}$ peritoneal macrophages. With regard to TNF production, cross-tolerance was intact in IRAK-1$^{-/-}$ peritoneal macrophages after pre-stimulation with LPS followed by LTA stimulation whereas pre-stimulation with LTA followed by LPS stimulation induced a hyporesponsive trend. With regard to IL-10 production, cross-tolerance was not induced in IRAK-1$^{-/-}$ peritoneal macrophages after pre-stimulation with LPS followed by LTA stimulation whereas pre-stimulation with LTA followed by LPS stimulation, unexpectedly, resulted in increased IL-10 production.

Next, we investigated the importance of IRAK-1 for intestinal inflammation by treating IRAK-1$^{-/-}$ mice with dextran sulfate sodium (DSS). IRAK-1$^{-/-}$ mice had reduced body- and spleen weight at sacrifice. However, only male IRAK-1$^{-/-}$ mice were protected from intestinal inflammation as judged by colon inflammation score and thymic weights, indicating that the importance of IRAK-1 might be gender dependent.

IRAK-M is a negative regulator that inhibits IRAK-1 signalling transduction in response to TLR stimulation. DSS treatment of IRAK-M$^{-/-}$ mice resulted in increased intestinal inflammation and reduced body- and thymic weight. Furthermore, mRNA expression of pro-inflammatory cytokines was up-regulated in distal colon tissue and in plasma. These results suggest that IRAK-M has an important role in intestinal homeostasis.

In conclusion, results presented in this thesis highlight the delicate regulation of TLR/IL-1R signalling involved in homeostasis and intestinal inflammation. We identify IL-1Ra as a candidate serum marker for early detection of colitis in Gαi2$^{-/-}$ mice, we demonstrate that IRAK-1 is of importance for TLR2 and TLR4 signalling regulation and that IRAK-1 and IRAK-M regulates the immune response during intestinal inflammation induced in mice.

Keywords: TLR/IL-1R signalling, Gαi2$^{-/-}$ mice, IRAK, peritoneal macrophages, cross-tolerance, dextran sulfate sodium, IBD
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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. **Serum interleukin-1 receptor antagonist is an early indicator of colitis onset in Gαi2-deficient mice.**
OH Hultgren, M Berglund, M Bjursten, E Hultgren Hörnquist.
World J Gastroenterol. 2006 Jan 28;12(4):621-4.

II. **Toll-like Receptor Cross-hyporesponsiveness is Functional in Interleukin 1-receptor-associated Kinase-1 (IRAK-1)-deficient Macrophages: Differential Role Played by IRAK-1 in Regulation of Tumour Necrosis Factor and Interleukin-10 Production.**
M. Berglund, J. A. Thomas, E. H. Hörnquist, O. H. Hultgren.
Scandinavian J Immunol. 2008 May; 67(5):473-9.

III. **Gender dependent importance of IRAK-1 in dextran sulfate sodium induced colitis.**
Berglund M, Thomas JA, Fredin MF, Melgar S, Hörnquist EH, Hultgren OH.
Cell Immunol. 2009 May; 259(1):27-32.

IV. **IL-1 Receptor-associated Kinase M Downregulates DSS-induced Colitis.**
Berglund M, Melgar S, Kobayashi KS, Flavell RA, Hörnquist EH, Hultgren OH.
Inflamm Bowel Dis. 2010 Oct; 16(10):1778-86.

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Abbrevations

5-ASA	5-aminoacylate
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
ASCA	Anti-saccharomyces cereviseae antibodies
BAFF	B lymphocyte activating factor
CARD	Caspase recruitment domain
CBA	Cytometric bead array
CD	Crohn's disease
CP	Cryptopatches
CRP	C-reactive protein
CT	Computer tomography
CYLD	Cylindromatosis
DC	Dendritic cell
dsRNA	Double-stranded RNA
DSS	Dextran sulfate sodium
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAE	Follicular associated epithelium
GPCR	G-protein-coupled receptor
H&E	Hematoxylin and eosin
HPRT	Hypoxanthine guanine phosphoribosyltransferase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
KC	Keratinocyte chemoattractant
LGP	Laboratory of genetics and physiology
LP	Lamina propria
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MAL	MyD88 adapter-like protein
MAP	Mitogen-activated protein
MBL	Mannan-binding lectin
MDA	Melanoma differentiation associated gene
MLN	Mesenteric lymph node
MRI	Magnetic resonance imaging
MUC	Mucin
MyD88	Myeloid differentiation factor-88
NALP	Nacht leucine-rich repeat and pyrin domain containing protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptors
NLRC	NLR family CARD domain-containing protein
NOD	Nucleotide-binding oligomerization domain

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PP	Peyers patches
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern regognition receptor
RAG	Recombination activation gene
RIG	Retinoic acid-inducible gene
RLR	RIG-I-like receptor
RT-PCR	Reverse transcriptase polymerase chain reaction
SARM	Sterile α- and armadillo-motif-containing protein
SCID	Sever combined immunodeficiency
SED	Subepithelial dome
SIGIRR	Single immunoglobulin IL-1R-related molecule
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
TAB	TAK binding protein
TAK	TGF-β activated kinase
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
Tollip	Toll interacting protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
WT	Wild-type

Introduction

The immune system and pathogen recognition

The immune system has developed in parallel with pathogenic organisms during evolution. It is specialized for invoking immune responses to bacteria, virus and parasites and at the same time allowing the presence of commensal bacteria and absorption of food antigens. These criteria are fulfilled by a complex interplay and a strict regulation of numerous immune cells spread throughout our bodies. Defectively regulated immune responses can result in inflammatory disease and cancer. The immune system is traditionally divided into innate and adaptive immunity, but the two branches are intimately linked as the innate immune system is necessary for the induction of the adaptive immune system [1].

The innate immune system is evolutionarily primitive and variants of it exist in all multicellular organisms. Innate immune detection of microbes is mediated by a low number of germ-line encoded pattern recognition receptors (PRRs) recognizing conserved structures, also known as pathogen associated molecular patterns (PAMPs), present on a wide range of microorganisms [2-4]. Metabolism of bacteria, fungi and protozoa is clearly distinguished from eukaryotic metabolism and the expression of e.g. glycolipids, peptidoglycan and lipopeptides can be detected by PRRs. Viruses hijack the host metabolism and can therefore not be detected in this way. Instead, PRRs recognize viral RNA, DNA and replication intermediates obligate for survival [5]. The innate immune system consists of e.g. macrophages, granulocytes, dendritic cells (DCs) and mast cells that are specialized in detection and uptake of pathogens followed by internal lysis and production of inflammatory mediators attracting other cells to sites of infection. In addition, uptake of antigen by macrophages and DCs, also known as antigen presenting cells (APCs), results in their activation, migration to lymph nodes and subsequent presentation of antigen fragments on major histocompatibility (MHC) complex to T cells of the adaptive immune system.

Activation of the adaptive immune system in the absence of activated APCs results in clonal inactivation whereas antigen recognition in presence of activated APCs results in clonal expansion [6,7]. The adaptive immune system is only present in vertebrates and consists of different subsets of T- and B lymphocytes with somatically generated receptors able to distinguish and respond to an enormous number of antigens (Table 1). T lymphocytes can be divided into many subgroups including T helper 1 (Th1), Th2, Th17, T regulatory (Treg) and cytotoxic T cells depending on cytokine profile and effector function [8,9] whereas activated B lymphocytes can develop into antibody secreting plasma cells [10]. In contrast to cells of the innate immune system, primary antigen encounter by the adaptive immune system results in the generation of memory T- and B lymphocytes that elicits a more rapid response towards a second encounter to the same antigen [7,11].

Pattern recognition receptors

Pathogenic microbes are recognized by multiple PRRs that can be divided into secreted, endocytic and signalling classes. Secreted PRRs such as C-reactive protein (CRP) and mannan-binding lectin (MBL) bind to structures on microbial surfaces and activate the classical- and the lectin pathway, respectively, of the complement system resulting in opsonisation, inflammation and target cell lysis. Furthermore, opsonisation by secreted PRRs induce phagocytosis of microbes by innate immune cells [12-15]. Endocytic PRRs, such as the macrophage mannose receptor and scavenger receptors, are present in cell membranes of phagocytic cells and bind to bacterial cell walls and mediates uptake of pathogens into lyzosomal compartments where they are destroyed [16,17]. Fragments of the lysed pathogen can then be presented at the cell surface on MHC complexes. Signalling PRRs, such as the Toll-like recep-

tors (TLRs), are present both in the cell membrane and intracellularly where they recognize microbial structures and induce signalling cascades resulting in gene expression [2].

Toll-like receptors

The Toll protein was first discovered in 1985 in Drosophila as a protein involved in the establishment of the dorso-ventral polarity during embryogenesis [18]. It shares a highly conserved region with the interleukin-1 (IL-1) receptor, now known as the Toll/IL-1 receptor (TIR) domain, and it was, therefore, proposed that the Toll-pathway was a part of the immune system [19]. The proposition was confirmed in 1996 when mutant flies deficient in various proteins of the Toll-pathway were found to be highly sensitive to fungal infection [20]. In 1997 the first mammalian homologue to Drosophila Toll was identified [21] and, subsequently, a family of structurally related receptors has been identified; the Toll-like receptors (TLRs).

TLRs are transmembrane type I receptors characterized by a leucin-rich domain responsible for ligand binding and an intracellular TIR domain important for signalling initiation [22]. Recently, the crystal structures of a few TLRs have been solved and reveal an extracellular horseshoe-shaped structure anchored to the cell membrane with only a short intracellular region (Fig. 1). Upon ligand binding TLRs form dimers, bringing two TIR domains into close contact resulting in the initiation of an intracellular signalling cascade [23-26]. Importantly, certain TLRs are dependent on accessory proteins facilitating extracellular ligand binding to the receptor [27-29]. TLRs can form homodimers as well as heterodimers and today there are 10 and 12 functional TLRs discovered in humans and mice, respectively, recognizing evolutionarily conserved PAMPs from bacteria, virus and fungi [30] (Table 1).

TLRs can be divided in two groups based on their cellular localisation. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed at the cell surface and recognize structures on the surface of pathogens whereas TLR3, TLR7, TLR8 and TLR9 are located intracellularly in endosomal compartments and recognize nucleic acids from bacteria and viruses [31]. Interestingly, new studies have proposed that TLR4 initially signals from the cell membrane and, subsequently after internalisation, from endosomal compartments [32]. Whether this is true also for other TLRs is not known.

Receptor	Microbial ligand	Expression
TLR 1/6	Tri-acyl lipopeptides	Mouse/Human
TLR2/6	Di-acyl lipopeptides	Mouse/Human
TLR3	dsRNA	Mouse/Human
TLR4	LPS	Mouse/Human
TLR5	Flagellin	Mouse/Human
TLR7	ssRNA	Mouse/Human
TLR8	ssRNA	Mouse/Human
TLR9	CpG DNA	Mouse/Human
TLR10	Unknown	Human
TLR11	Profilin	Mouse
TLR12	Unknown	Mouse
TLR13	Unknown	Mouse

Table 1. TLRs in humans and mice b[33-43].

Toll-like receptor signalling

Ligand binding to TLRs induces an intracellular signalling cascade starting by the recruitment of TIR domain-containing adaptor molecules to the TLR TIR domain. Myeloid differentiation factor-88 (MyD88) can bind to the TIR-domain of all TLRs, except TLR3 [36,44]. Interestingly, also IL-1R and IL-18R use MyD88 as adaptor molecule and, thereby, share intracellular signalling pathways with most TLRs [45]. TLR1, TLR2, TLR4 and TLR6 use MyD88 adapter-like protein (MAL) as a bridging adaptor between the TLR TIR domain and MyD88 [46,47]. Activation of TLR3 and TLR4 recruits TIR domain-containing adaptor protein induc-

ing IFN-β (TRIF) [48]. TLR4 use TRIF-related adaptor molecule (TRAM) as a bridging adaptor between the TLR TIR domain and TRIF [49]. Recently, a fifth TIR domain-containing adaptor molecule was discovered; sterile α- and armadillo-motif-containing protein (SARM). However, SARM has no signalling property and acts as a negative regulator of TRIF after TLR stimulation [50].

Signalling downstream of TLRs can be divided into MyD88-dependent and MyD88-independent based on the adaptor molecule used for signalling initiation. In MyD88-dependent signalling several members of the interleukin-1 receptor-associated kinase (IRAK) family are recruited to MyD88 forming an intracellular complex [51-54]. The IRAK proteins activate TNF receptor-associated factor 6 (TRAF6) [52,53,55] resulting in the subsequent activation of the TGF-β activated kinase-1 (TAK1)/TAK binding protein-2/3 (TAB2/3) complex. TAK1 stimulates mitogen-activated protein (MAP)-kinases and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which leads to induction of pro-inflammatory cytokines [56]. MyD88-independent signalling, initiated by TRIF, also signals via TRAF6 and induces NF-κB activation [57]. Furthermore, MyD88-independent signalling can activate TRAF3 and interferon regulatory factor 3 (IRF3) leading to interferon-β (IFN-β) transcription [57,58] (Fig. 1).

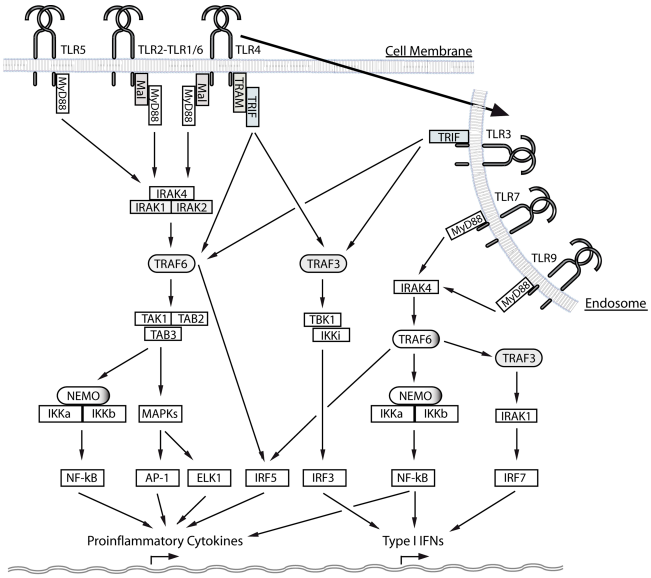

Figure 1. Schematic picture of the TLR signalling network [36,44,46-49,51-53,55,57-59].

Cytosolic pattern recognition receptors – NOD-like receptors and RIG-I-like receptors

TLRs can recognize intracellular bacteria and viruses present in endosomal compartments. However, many bacteria and viruses replicate within the cytoplasm and, therefore, cytosolic detectors are required. NOD-like receptors (NLRs) are a large family of cytosolic signalling proteins containing a variable C-terminal domain, a central nucleotide-binding oligomerization domain (NOD) and a leucine-rich repeat N-terminal domain. NLRs recognize degradation products of peptidoglycan, noninfectious crystal particles and other microbial products [60]. NOD1 and NOD2 are two examples of NLRs recognizing parts of the peptidoglycan molecule resulting in expression of inflammatory cytokines [61,62] whereas nacht leucine-rich repeat and pyrin domain containing protein 1 (NALP1), NALP3 and nucleotide binding domain-leucine rich repeat protein (NLRC4) respond to diverse stimuli and induce formation of multi-protein complexes, known as the inflammasomes [63-66]. An important function of inflammasomes is the activation of caspase-1 and subsequent conversion of pro-IL-1β and pro-IL-18 into their biologically active forms IL-1β and IL-18 [67,68].

The retinoic acid-inducible gene-1 (RIG-I)-like receptors (RLRs) consists of RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) involved in cytosolic detection of viral RNA [69-71]. Structurally, RLRs have an N-terminal tandem caspase recruitment domain (CARD)-like region, a central helicase domain containing ATP-binding motifs and a C-terminal domain that binds RNA. Activation of RLRs induces expression of type I interferons and pro-inflammatory cytokines [72].

The interleukin-1 receptor-associated kinase family

The IRAK proteins are a family of serine-threonine kinases recruited to MyD88 upon ligand binding to TLRs. Today there are four IRAKs discovered; IRAK-1, IRAK-2, IRAK-M and IRAK-4. Initially, all IRAKs were thought to play redundant roles in TLR signalling but recent studies have revealed that each IRAK member has distinct functions. Structurally, the IRAK family members have an N-terminal death domain, a central kinase domain and a C-terminal domain important for TRAF6 activation (not present in IRAK-4) [73].

IRAK-1 was the first protein in the IRAK family identified [74]. It is expressed in various cell types and tissues and studies have demonstrated that mice deficient in IRAK-1 are resistant to lipopolysaccharide (LPS)-induced shock and infection with gram-negative bacteria [75]. Furthermore, mouse macrophages deficient in IRAK-1 have reduced NF-κB activation and production of pro-inflammatory cytokines in response to e.g. LPS and IL-1β [76] . However, these cells still respond to TLR stimulation implying that other IRAK members can compensate for IRAK-1 function. Interestingly, IRAK-1 is critical for the induction of type I interferons after TLR9- and TLR7-mediated stimulation of mouse plasmacytoid DCs (pDCs) [77]. In addition to the importance of IRAK-1 for NF-κB activation, sumoylated IRAK-1 can enter the nucleus and activate signal transducer and activator of transcription 3 (STAT3) [78]. In humans, IRAK-1 exists in two additional splice forms; IRAK-1b and IRAK-1c which are found in most cell types. However, IRAK-1b is only found in minute amounts and has an unknown function whereas the expression of IRAK-1c is more pronounced and negatively regulates induction of MAP-kinases after TLR/IL-1R stimulation [79,80]. Mutations in the IRAK-1 gene in humans are associated with both adult- and childhood-onset systemic lupus erythematosus (SLE) [81].

Similar to IRAK-1, IRAK-2 is important for TLR signalling transduction [52]. Mice deficient in IRAK-2 have a reduced pro-inflammatory cytokine production in response to TLR stimulation and IRAK-2 is critical for sustaining NF-κB activation after TLR2 stimulation. Furthermore, mice deficient in IRAK-2 are resistant to TLR4 induced shock. Interestingly, macrophages deficient in both IRAK-1 and IRAK-2 have a substantially reduced production of pro-inflammatory cytokines indicating that IRAK-1 and IRAK-2 act redundantly in TLR signalling [82]. IRAK-2 has also been reported important for signalling downstream of TLR3 [83]. So far, there are no clinical studies investigating the role of IRAK-2 in humans.

IRAK-M has no kinase activity and it is preferentially expressed in monocytes/macrophages [84]. Studies have revealed that IRAK-M deficient mice have a hyperactivated phenotype. Bone marrow-derived macrophages (BMDM) from IRAK-M deficient mice produce more inflammatory cytokines in response to TLR stimulation, are more sensitive to bacterial infection and have an impaired endotoxin tolerance [85]. Furthermore, IRAK-M deficient mice have increased survival and lower bacterial load in blood during sepsis and an elevated activation of osteoclasts [86,87]. Elevated concentrations of IRAK-M correlate with mortality in human sepsis patients and variations of the IRAK-M gene are associated with early-onset persistent asthma [88,89].

IRAK-4 deficient mice are completely resistant to LPS-induced shock and have severely impaired pro-inflammatory cytokine production in response to bacterial and viral challenge [53,90]. IRAK-4 deficiency in humans is associated with non-responsiveness to ligands for all

TLRs, except TLR3, and life threatening recurrent infections of extracellular pyogenic bacteria such as *Streptococcus pneumoniae* during childhood [91-93]. Interestingly, patients with a deficiency in the upstream adaptor molecule MyD88 have similar cellular phenotype and are susceptible to pyogenic bacteria [94,95]. IRAK-4 is also important for proliferation of T lymphocytes in mice after activation of the T-cell receptor (TCR) *in vivo* indicating that IRAK proteins may be involved in adaptive immune responses [96].

Kinetics of interleukin-1 receptor-associated kinase signalling

TLR4 is often used as example to describe TLR signalling and it will be used to describe the kinetics of IRAK signalling below. Upon TLR4 stimulation, the adaptor molecules MAL and MyD88 bind to the TIR domain of the intracellular part of TLR4 [44,46,47]. This is followed by binding of IRAK-4 to MyD88 [53] (Fig. 2a) and the subsequent recruitment of IRAK-1 and IRAK-2 to IRAK-4 (Fig. 2b) [51,52]. IRAK-4 phosphorylates IRAK-1 and IRAK-2 [82,97] (Fig. 2c) resulting in their dissociation from the receptor complex (Fig. 2d) and subsequent polyubiquitination of TRAF6 and further downstream signalling [83] (Fig. 2e). Interestingly, a recent publication suggests that several MyD88, IRAK-4, IRAK-2 and IRAK-1 molecules form an oligomer, called the Myddosome, at the intracellular part of the TLR [54]. IRAK-M binds to and prevents the dissociation of IRAK-1 from MyD88 and, thereby, the downstream signalling [85]. Additionally, new data speculates that IRAK-M can inhibit dissociation of IRAK-2 from MyD88 [54].

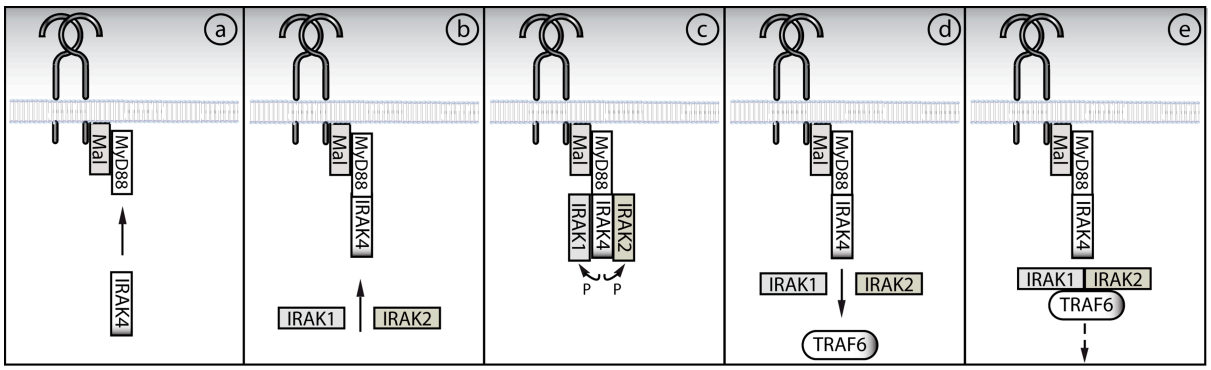

Figure 2. Simplified figure of IRAK signalling kinetics downstream of MyD88 [51-54,82,83,97].

Negative regulation of Toll-like receptor signalling

TLRs are specialized in inducing immune responses towards microbial structures, resulting in production of pro-inflammatory cytokines and type 1 interferons. However, an exaggerated inflammatory reaction can cause harmful tissue damage. Therefore, evolution has favoured a rapid burst of TLR signalling followed by a strictly controlled regulation at multiple cellular locations. Extracellular, soluble TLR decoy receptors bind antigens and make them inaccessible to membrane bound receptors. Soluble TLR2 (sTLR2) binds extracellular lipopeptides and whole Gram-positive bacteria and, thereby, prevents them from binding membrane TLRs and eliciting immune responses [98]. Some studies have suggested that TLR stimulation of both human and mouse macrophages can induce TLR downregulation, per se, [99,100] whereas other studies suggest that TLR expression is unchanged or even increased [100-102]. Intracellular regulatory proteins including IRAK-M, single immunoglobulin IL-1R-related molecule (SIGIRR), Toll interacting protein (Tollip) and suppressor of cytokine signalling-1 (SOCS-1), bind to and interfere with the signalling cascade at different cytoplasmic levels resulting in decreased cytokine production (Fig. 3) [85,103,104]. Additionally, cytoplasmic signalling proteins, such as IRAK-1, are downregulated after the primary signalling cascade resulting in decreased responsiveness to a secondary stimulation [105]. Nuclear receptors,

such as peroxisome proliferator-activated receptors (PPARs), downregulate diverse components of the inflammatory response including pro-inflammatory cytokine production [106]. Recent studies have shown that intranuclear gene-specific chromatin modifications can induce transient silencing of pro-inflammatory mediators and upregulation of antimicrobial peptides after TLR stimulation that further contributes to regulation of the TLR response [107]. Finally, microRNAs (miRNAs) have emerged as post-transcriptional regulators of multiple TLR signalling components functioning mainly by decreasing mRNA levels and, thus, the subsequent protein synthesis [108].

Repeated stimulation of human and mouse monocytes/macrophages by endotoxin results in downregulation of pro-inflammatory cytokine production, also known as endotoxin tolerance [109,110]. Furthermore, stimulation of other TLRs and subsequent stimulation with different TLRs and the IL-1R also results in inflammatory cytokine hyporesponses, known as cross-tolerance [110-114]. The exact molecular mechanism responsible for TLR/IL-1R induced tolerance is at present unknown but it is thought to involve upregulation of negative regulators acting on multiple levels of TLR/IL-R signalling and downregulation of signalling components [85,103-105,115-118] (Fig. 3).

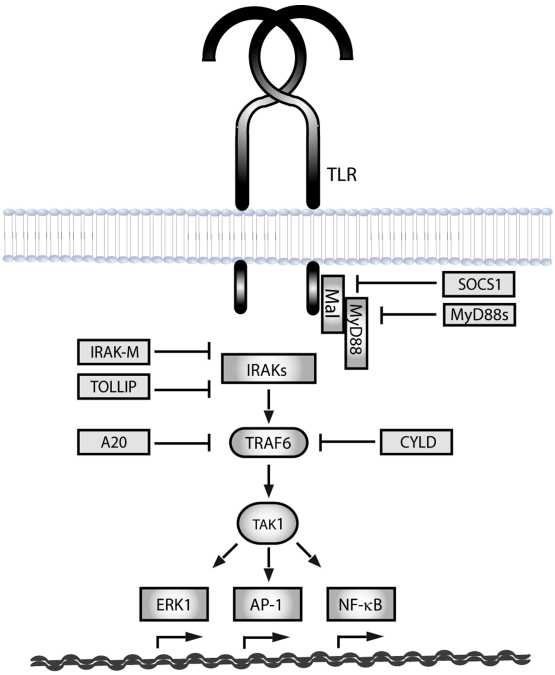

Figure 3. Negative regulators and their target proteins in the MyD88-dependent signalling pathway [85,103,104,116-118].

The intestinal epithelial barrier

The human colon is colonized by approximately 10^{13} to10^{14} bacteria [119] separated from the immune system by a single layer of intestinal epithelial cells (IECs) forming a selectively permeable barrier where transport of water and nutrients is supported and the translocation of pathogenic microbes into intestinal tissues is prevented. IECs have a polarized phenotype with an apical surface facing the intestinal lumen and a basolateral surface facing the underlying lamina propria (LP) [120]. The apical surface of IECs is covered by mucins secreted by specialized epithelial cells, such as intestinal goblet cells. The mucins form an inner firm layer that e.g. prevents bacteria from gaining access to the epithelial surface and a loose outer layer populated by bacteria [121,122]. The importance of the mucus layer for intestinal homeostasis has been demonstrated in mouse studies in which mice deficient in mucin 2 (MUC2) spontaneously develop colitis [123]. In addition to the regulated transcellular transport through

IECs, the paracellular space between IECs is sealed by protein complexes known as tight junctions and subadjacent adherent junctions minimizing translocation of luminal content to the underlying LP [124]. Pro-inflammatory cytokines are known to increase paracellular permeability by regulating the expression of junctional proteins [125,126]. Steady state pro-inflammatory cytokine production is thought to facilitate low-rate antigen translocation contributing to the induction of immune tolerance, whereas high concentrations have been associated with pathological conditions, such as inflammatory bowel disease (IBD) [127,128].

Commensal bacteria and intestinal homeostasis

Bacteria have existed on earth for 3.4 billion years [129] and is today colonizing diverse niches including the human gastrointestinal tract. Bacterial colonisation of mucosal surfaces is important for host metabolism [130-133] but also confers protective effects from pathogenic microbes by physically competing for space and by limiting the availability of dietary nutrients. In addition, intestinal bacteria influence human IECs by promoting epithelial cell maturation, lymphocyte development, cell-to-cell integrity, epithelial repair and immune tolerance [133-136]. Studies suggests that healing of IEC damage requires bacterial signalling through TLRs since mice deficient in MyD88 and TLR2 are unable to heal epithelial damage caused by ingestion of dextran sulfate sodium (DSS) [133,137-140]. Commensal bacteria induce intracellular proteins in IECs that interfere with pro-inflammatory signalling and, thereby, contribute to a hyporesponsive IEC phenotype [141,142]. Furthermore, the nuclear receptor PPAR-γ is induced by TLR4 stimulation of IECs and diverts nuclear NF-κB into the cytoplasm, resulting in blunted pro-inflammatory cytokine production (Fig. 4a) [143,144]. IECs, such as Paneth cells, actively affect the intestinal milieu by secreting antimicrobial peptides into the lumen, resulting in protection from pathogenic microbes and manipulation of commensal bacteria composition [145].

Intestinal bacteria also influence IECs to secrete anti-inflammatory mediators, such as thymic stromal lymphopoietin (TSLP) and transforming growth factor-β (TGFβ), into the underlying LP, that promotes tolerogenic DC phenotypes important for induction of tolerance towards food antigens and commensal bacteria [146]. Bacteria-derived products penetrating IECs can bind DCs that, subsequently, induce Treg cells [147]. LP Treg cells suppress the immune response by e.g. production of anti-inflammatory cytokines such as IL-10 and TGF-β, cytolysis and metabolic disruption [148-152]. LP macrophages are highly phagocytic but have a tolerogenic phenotype with regard to production of pro-inflammatory cytokines due to e.g. decreased TLR expression and inactivation of intracellular signalling pathways, possibly reflecting a conditioning effect from intestinal bacteria (Fig. 4b) [153,154]. Tolerogenic LP DCs and macrophages produce anti-inflammatory cytokines in response to TLR stimulation further contributing to intestinal tolerance [155,156]. Luminal bacteria can also stimulate adaptive immune cells important for intestinal homeostasis. Stimulation of IEC TLRs induce production and secretion of a proliferation inducing ligand (APRIL) and B lymphocyte activating factor (BAFF) that stimulate LP plasma cell survival and class switching into IgA [135]. IgA is actively transported over the IEC layer into the intestinal lumen were it bind to and neutralize bacteria (Fig. 4b) [157].

The importance of crosstalk between IECs and intestinal bacteria is exemplified by that endotoxin exposure of IECs from neonatal mice, naturally occurring during vaginal delivery, results in a rapid termination of IEC activation, as judged by NF-κB activation, associated with a transient downregulation of IRAK-1. Interestingly, termination of IEC activation does not occur in IECs from neonatal mice delivered by caesarean section [158]. In humans, caesarean delivery is associated with allergic disorders such as asthma and celiac disease in children [159,160]. Thus, a hyporesponsive IEC layer in neonates might be necessary for the establishment of a stable host-microbe homeostasis preventing allergic diseases later in life.

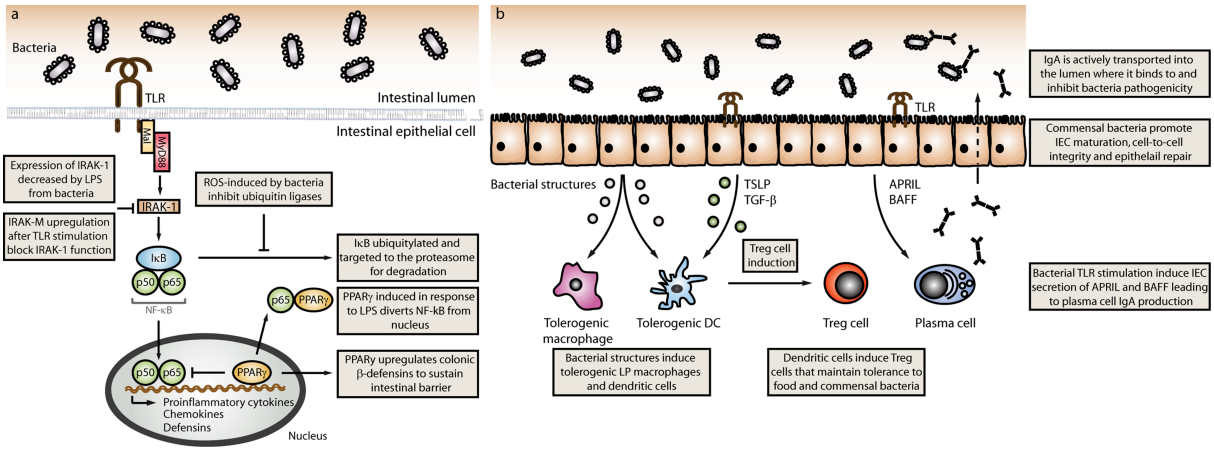

Figure 4. The tolerizing effects of intestinal bacteria on IECs (a) and the underlying LP (b). Reprinted and adapted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology, The immune system and the gut microbiota: friends or foes?, Cerf-Bensussan et al. 2010 Oct;10(10):735-44, copyright 2011.

Toll-like receptor expression on intestinal epithelial cells

IECs express several TLRs including TLR2, TLR3, TLR4, TLR5 and TLR9 without inducing inflammation under normal conditions, suggesting that TLR expression and signalling is strictly regulated [161-164]. Indeed, TLR2 and TLR4 are expressed in low concentrations in IECs from human adults whereas TLR2 and TLR4 expression of ileal crypt enterocytes from human foetuses is restricted to the basolateral surface [162,165]. A study using a small intestinal epithelial cell line from mice demonstrates that TLR4 is located intracellular and that luminal LPS needs to be actively internalized into the Golgi apparatus of IECs to stimulate TLR4 [166]. TLR3 is expressed constitutively, both in human colonic and small intestinal IECs [162]. TLR9 is expressed both on the apical and basolateral surface of polarized human IECs. Interestingly, basolateral TLR9 stimulation of polarized human IECs induces activation of NF-κB and secretion of pro-inflammatory cytokines, whereas apical TLR9 stimulation leads to inhibition of NF-κB. Moreover, apical TLR9 stimulation inhibits the production of pro-inflammatory cytokines after a subsequent basolateral stimulation of TLR9 as well as TLR2, TLR3 and TLR5, indicating a state of cross-tolerance [164]. TLR5 expression in the human intestine is restricted to the basolateral surface of, predominantly, colon IECs and studies have shown that luminal flagellin (a TLR5 ligand) can only stimulate IECs after epithelial injury [163].

Local cytokine production regulates the expression of TLR expression in IECs. Pro-inflammatory Th1 cytokines, such as IFN-γ, induce expression of TLR4 and its co-receptor MD2 whereas Th2 cytokines, such as IL-4 and IL-13, decrease the responsiveness of IECs to LPS stimulation [167-170].

Intestinal structure and function

The basement membrane separates IECs from the underlying LP populated by a mixture of immune cells such as macrophages, DCs, neutrophils, mastcells and T- and B lymphocytes. A thin muscle layer, muscularis mucosa, separates the mucosa from the submucosa. External to the submucosa are circular and longitudinal muscle layers responsible for peristaltic movement. Finally, the muscle layers are surrounded by the serosa, or connective tissue (Fig. 5).

The small intestine has important functions in absorbing nutrients and antigens from the gut lumen, while colon is the main location for uptake of salt and water. Anatomically, the small intestine is composed of crypts and villi whereas the colon is composed of a large number of crypts [171]. Peyer's Patches (PP), present only in the small intestine, are the most important route for uptake of antigen in the intestine and are composed of B lymphocyte follicles

together with areas of T lymphocytes [172]. A follicular-associated epithelium (FAE) with antigen sampling microfold (M) cells overlays the PP and regulates the transport of antigens from the lumen into the subepithelial dome (SED) were antigens are taken up and processed by DCs [173,174]. In addition to PP, LP is constituted with organised cellular structures such as cryptopatches (CP) composed of clusters of lymphocytes and DCs located at the base of intestinal crypts [175], and isolated lymphoid follicles with a structure resembling follicles in PP [173]. The mesenteric lymph nodes (MLNs) are the largest lymph nodes in the body and are thought to be the draining site for antigen presenting DCs in the intestine and a major site for T lymphocyte activation [176].

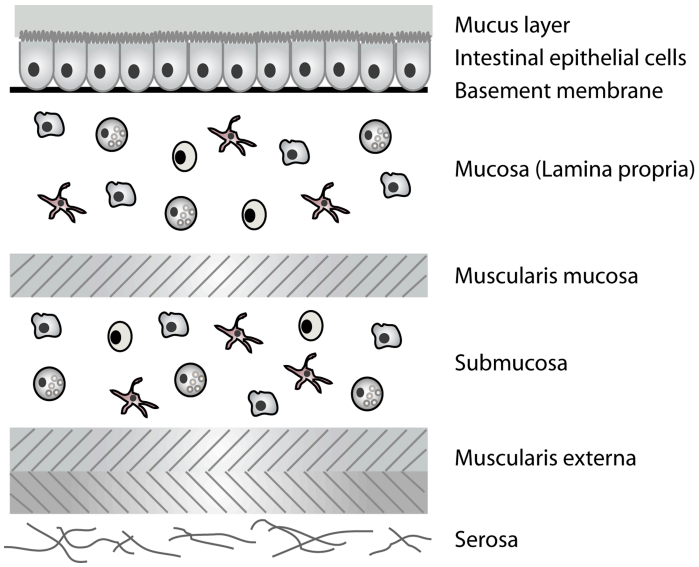

Figure 5. Intestine in cross-section. Based on figure 2B from Maria Fritsch Fredin thesis "Dynamic changes in T cell compartments and new approaches in evaluating DSS induced and Gαi2 deficient colitis". Used with permission.

Pathogen detection in the lamina propria

In addition to commensal bacteria, the intestine contains pathogenic organisms that can infect host tissue. Consequently, the intestinal epithelium and the underlying LP is crowded with cells specialized in pathogen detection and clearance. The IEC layer in both small intestine and colon is populated by a subpopulation of T cells known as intraepithelial lymphocytes (IELs) [177]. The exact functions of IELs remain unknown but they are thought to contribute to mucosal homeostasis by monitoring the epithelium for pathogenic microbes [178]. Additionally, studies have shown that IELs have protective functions during experimental colitis in mice [179-181].

Although IECs form a physical and chemical barrier, luminal microbial antigens do penetrate into the LP, partially by active uptake in PP and partially by pathogenic translocation [173,174]. Within the LP, antigens are initially recognized by a wide range of immune cells. In a non-pathogenic state large numbers of macrophages are present just below the IECs monitoring the LP for pathogens via PRRs. Intestinal macrophages are highly phagocytic and can engulf whole bacteria and kill them within phagolysosomes by lysis [154]. Mast cells have important functions for pathogen recognition during early inflammatory events and microbial recognition by mast cells induces production of pro-inflammatory cytokines, chemokines and vasodilatory substances, such as histamine, leading to recruitment of large amounts of neutrophils and other immune cells [182]. DCs are the main APC in the LP and contribute to pathogen surveillance by uptake of pathogens crossing the epithelial layer and studies also suggest that DCs can protrude dendrites between IECs and sampling the lumen for bacteria [183,184]. TLR activation and phagocytosis of bacteria induce DC maturation,

microbial lysis in lysosomal compartments, loading of peptides on MHC-molecules and migration to lymph nodes where DCs activate naïve T cells [185-187]. Subsequently, activated T lymphocytes can migrate to LP where they stimulate B-lymphocytes to produce antibodies and activate macrophages [188]. Alternatively, activated T lymphocytes can become memory T lymphocytes that reside in the body for many years.

Inflammatory bowel disease

The immune system acts to respond to invading pathogens in the intestinal tract. However, exaggerated immune responses can lead to inflammatory disorders, such as IBD, resulting in tissue damage. IBD is a group of chronic intestinal inflammatory disorders traditionally divided into ulcerative colitis (UC) and Crohn's disease (CD) based on their clinical, pathological, endoscopic and radiological features. The prevalence of IBD is steadily increasing and today approximately 1.4 million people in the United States and 2.2 million people in Europe are affected by the disease [189]. The most common age of IBD onset is between 15 and 30 years of age with a second peak of onset occuring at 50 to 70 years of age. About 10 percent of the patients are under 18 years of age [190].

UC is characterized by superficial and continuous mucosal ulcerations of the colon extending proximally from the rectum, accompanied by increased number of neutrophils in the LP and the crypts and, commonly, depletion of goblet cells and mucin content. In contrast, CD can affect the whole gastrointestinal tract and is characterized by aggregation macrophages that can form granulomas. CD can be patchy and segmental and the inflammation is normally transmural [191]. In serious cases of UC and CD surgical removal of the inflamed tissue might be necessary. Furthermore, both UC and colonic CD are associated with an increased risk of colorectal cancer [192].

The cause of IBD is unknown, but both UC and CD result from an inability to control immune responses in the gastrointestinal tract. IBD has a higher prevalence in the western world compared to developing countries, but in recent years the incidence has increased in developing countries adopting a westernized lifestyle identifying this as a risk factor for IBD [193]. Examples of other environmental risk factors for IBD are smoking (only for CD) [194] and stress [195]. IBD is more aggregated in certain families and twins have an increased disease concordance, especially for CD, highlighting the impact of genetic factors [196]. A number of susceptibility loci for both UC and CD have been identified including genes for several TLRs and NOD2 [197-201]. Families with IBD have increased epithelial permeability and decreased levels of tight junction proteins [202-204]. Furthermore, mouse studies indicate that deficiency in goblet cells and mucus production are contributing factors for developing IBD [123,205].

IBD is characterized by periods of relapse with mild to severe disease followed by periods of remission where symptoms can decrease or totally disappear. Patients with IBD commonly suffer from multiple symptoms such as bloody diarrhoea, abdominal pain, fever, nausea and vomiting that can result in loss of appetite, weight reduction and growth retardation. Furthermore, in rare cases also extra-gastrointestinal complications including arthritis, spondylitis, iritis and osteoporosis can be seen in IBD patients [206].

The first step in diagnosing IBD is an abdominal examination and to take a blood and faecal sample. Increased levels of the acute phase protein CRP in blood is commonly detected in patients with CD whereas it is less common in patients with UC [207]. Furthermore, specific serum antibodies can be detected in CD and UC patients (including anti-saccharomyces cereviseae antibodies (ASCA) and anti-neutrophil cytoplasmic antibody (ANCA), respectively) and are, therefore, used to distinguish between the two IBD subgroups [208]. Faecal samples are analyzed for the concentration of inflammatory markers, such as the neutrophil-derived protein calprotectin, to detect both CD and UC [209]. Based on the results from the

initial tests, endoscopic evaluation with multiple biopsies can be used to further diagnose the ongoing inflammation. Magnetic resonance imaging (MRI), computer tomography (CT) and ultrasound are imaging techniques used as non-invasive alternatives/complements to discomforting endoscopic evaluation and can detect e.g. intestinal thickening, luminal narrowing and fibrofatty proliferation features commonly exhibited in IBD patients [210].

Since the underlying mechanisms remains unknown there is no universal treatment for IBD patients. 5-Aminosalicylates (5-ASA) are used in the first line therapy for UC and CD (most effective in colon) and function by reducing e.g. the production of leukotrienes, inhibiting inflammatory cytokine production and macrophage chemotaxis and inducing T cell apoptosis [211-214]. How 5-ASA mediates these diverse effects is presently unknown but studies have indicated that both inactivation of NF-κB and activation of PPAR-γ are possible mechanisms of action [215,216]. Corticosteroids have diverse immunosuppressive effects and are used when 5-ASA treatment is inadequate. Although treatment with corticosteroids is often effective it is also associated with frequent and serious side effects [217]. Cytotoxins such as azathioprine and its metabolite mercaptopurine are used as complement to corticosteroids and is thought to induce apoptosis in activated T lymphocytes. However, toxicity and a slow onset of benefit limit its clinical use [218,219]. To reduce the side effects seen with broad spectrum drugs, a group of more specific immune inhibitors have been developed and are today used for IBD treatment; the tumor necrosis factor (TNF) inhibitors. There are today four approved therapeutic monoclonal antibodies that all bind to and neutralize the pro-inflammatory effects of TNF; infliximab, adalimumab, golimumab and certolizumab pegol [220-223]. Additionally, the soluble TNF receptor, etanercept, is used to prevent binding of TNF to its biologically active receptors [224]. Since the anti-TNF constructs are structurally distinct they have different effects on different inflammatory diseases. Studies have shown that infliximab, adalimumab and certolizumab pegol have effect on IBD. Interestingly, although all constructs binds to and block both soluble and membrane bound TNF, only infliximab and adalimumab induce T cell and macrophage apoptosis [225]. Manipulation of luminal bacteria with antibiotics has been successful for treatment of CD and there are studies indicating that probiotics can ameliorate IBD [226,227]. Finally, in patients with severe IBD where anti-inflammatory treatment is insufficient surgery can be used to remove the inflamed tissue.

Toll-like receptor signalling and inflammatory bowel disease

Patients with IBD have increased expression of TLR2, TLR4 and TLR8 whereas the expression of TLR3, TLR5 and TLR9 is unchanged or lower than control individuals [162,201,228,229]. Furthermore, the expression of CD14, a TLR4 accessory protein needed for LPS binding, is upregulated in patients with IBD [230]. However, it is difficult to interpret whether the increased/decreased TLR expression observed in IBD patients is an inducer or is secondary to the inflammation. Mutations in human TLR1, TLR2, TLR4, TLR6 and TLR9 genes have all been associated with an increased risk for IBD demonstrating that TLR signalling is critical for intestinal immune homeostasis [197-199]. The importance of TLRs for IBD pathogenesis is further supported by indirect evidence, for example that alteration in the commensal flora is seen in IBD patients [231-233]. However, if the alterations in commensal flora of IBD patients are the cause or a result from the ongoing inflammation is still unclear. Less is known about the contribution of intracellular TLR signalling components to human IBD.

Animal models for inflammatory bowel disease

Murine models of mucosal inflammation generally do not fully reflect the complexity of human IBD, but they are an important instrument for analysis of distinct disease aspects that are impossible to study in human patients and today there are over 60 different animal models

used for IBD research [234]. A category of chemically induced models including the DSS and the trinitrobenzene sulfonic acid (TNBS) models, are widely used both to study basic pathology of disease and for evaluation of therapeutics [235,236]. Many murine models of IBD are spontaneously induced by the genetic removal of immunological signalling components, such as IL-2, IL-10 and TLR5 [237-239]. In other animal models, colitis is induced via the transfer of T lymphocytes from wild-type (WT) mice into mice deficient in T- and B lymphocytes (i.e. SCID or RAG1/2$^{-/-}$ mice), resulting in intestinal inflammation [240,241]. Interestingly, although the genetic defects represent different parts of the immune system, they converge in the common end result of mucosal inflammation. So why is the intestinal mucosa particularly sensitive to immunological defects? An obvious explanation is the proximity and large burden of intestinal bacteria present in the colon. Indeed, murine studies have shown that, in most models, mucosal inflammation fails to develop in a milieu devoid of bacteria [242]. The involvement of bacteria in IBD is further supported by chemically induced murine colitis models and by models using mice deficient in various barrier proteins where the intestinal epithelial barrier function is compromised, resulting in bacterial translocation and intestinal inflammation [243,244]. In this thesis I have used the spontaneous Gαi2$^{-/-}$ and the induced DSS mouse model of colitis.

The Gαi2 deficient mouse model of colitis

Guanine nucleotide-binding proteins (G proteins) are signalling transducers attached to the cell membrane that connect G protein-coupled receptors (GPCRs) to intracellular signalling pathways important for e.g. transcription, motility, contractility and secretion [245]. G proteins are composed of three types of subunits; α, β, and γ, each with numerous subgroups that could be associated into different combinations [246]. Mice deficient in Gαi2 spontaneously develop intestinal inflammation 12-25 weeks after birth characterized by weight loss, mucus filled diarrhoea, shortening and thickening of the colon, crypt loss, goblet cell depletion, ulceration and adenocarcinomas [247]. The inflammation is confined to the colonic mucosa and is characterized by infiltration of neutrophils and T- and B lymphocytes and increased concentrations of IL-1β, TNF, IL-6, IL-12p40, IL-17 and IFN-γ [248-251]. What causes colitis in Gαi2 deficient mice is incompletely understood. GPCRs are involved in signal transduction in response to chemokines and LP lymphocytes from Gαi2 deficient mice have an impaired chemotactic migration [245,252]. Furthermore, studies have demonstrated that signalling via GPCRs leads to induction of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway that negatively regulates TLR signalling in certain cell types [253]. Interestingly, Gαi2 is also involved in development and maintenance of the epithelial barrier tight junctions [254]. Bone marrow derived cells are important for disease induction since irradiated WT mice reconstituted with Gαi2-deficient bone marrow develop colitis and irradiated Gαi2 deficient mice reconstituted with WT bone marrow have substantially increased lifespan. Furthermore, the importance of adaptive immune cells for induction of colitis in Gαi2 deficient mice has been demonstrated as transfer of splenic Gαi2 deficient CD3^{+} T cells into immunodeficient mice results in severe colitis [255]. Since Gαi2-deficient mice are born healthy the Gαi2 deficient model is suitable for investigation of early and late inflammatory events and treatment studies.

DSS model of colitis

Mice treated with 3-10% DSS in drinking water develop weight loss, bloody diarrhoea, shortening of the colon, epithelial loss, fibrosis, crypt loss, goblet cell depletion and focal ulceration. The first cells to infiltrate the colonic mucosa and submucosa after DSS treatment are neutrophils and macrophages, followed by T- and B lymphocytes [235,256-258]. DSS treat-

ment induces high concentrations of pro-inflammatory cytokines such as IL-1α/β, IL-6, IL-12p40, IL-17, IL-18, TNF and IFN-γ in colonic tissue [259,260]. The mechanism for DSS induced colitis is incompletely understood, but it has been suggested that DSS acts as a toxic agent on IECs resulting in a compromised epithelial barrier and increased translocation of intestinal bacteria [235,261]. Studies have demonstrated that DSS treatment makes the colon layer more permeable and that DSS treated mice have decreased expression of IEC tight junction proteins demonstrating that barrier function is compromised in DSS colitis [122,262]. The DSS molecules are not restricted to intestinal tissues of treated mice as macrophages can engulf DSS and migrate to the mesenteric lymph node and the liver [263]. Interestingly, mice devoid of bacteria (i.e. germ-free) develop severe rectal bleeding without any clinical signs of colitis in response to short-term high dose of DSS whereas prolonged low dose DSS treatment induces colitis [264]. These results indicate that germ-free mice, with an under-developed immune system, are more susceptible to non-bacterial-associated effects of DSS. Adaptive immune cells seem dispensable for induction of DSS colitis since SCID mice, without T- and B lymphocytes, develop colitis similar to WT control mice [265]. In comparison to Gαi2 deficient model of colitis, the DSS model can be used on a wide range of genetically engineered mice making it suitable for mechanistic studies of ongoing colitis.

The role of individual TLR signalling components in IBD has been extensively studied using the DSS model of colitis as the majority of immunodeficient mice do not spontaneously develop colitis. Mice deficient in the adapter protein MyD88 develop severe intestinal inflammation in response to DSS whereas TRIF deficient mice are protected from colitis compared to both WT control mice and MyD88 deficient mice [138,266]. Mice deficient in the intracellular negative TLR regulators SIGIRR or SOCS-1 (heterozygous) display increased colonic inflammations after DSS treatment [267,268]. DSS treatment of TLR2 deficient mice induces a more severe colitis compared to control mice [269]. This finding echoes the important function of TLR2 in maintaining barrier integrity [270]. In another DSS study, however, treatment of TLR2 deficient mice resulted in less colon inflammation compared to WT control mice [271].

Aims

The overall aim of this thesis was to study the importance of TLR signalling and regulation during homeostasis and intestinal inflammation.

Specific aims for the papers:

Paper I — To study IL-1 and IL-18 production, two endogenous IL-1/TLR signalling cytokines, in induction and progression of colitis.

Paper II — To investigate the importance of IRAK-1 for TLR2 and TLR4 signalling and TLR cross-tolerance upon repeated stimulation.

Paper III — To investigate the importance of IRAK-1 for the induction and progression of colitis.

Paper IV — To investigate the importance of IRAK-M for the induction and progression of colits.

Methodological considerations

Mice

Gαi2$^{-/-}$ mice on a C57BL/6X129SvEv backcrossed four generations into 129SvEv and then intercrossed, IRAK-1$^{-/-}$ mice on a 129SvEv x C57BL/6 background backcrossed more than 9 generations into C57BL/6, IRAK-M$^{-/-}$ mice backcrossed 10 generations into C57BL/6, MyD88$^{-/-}$ mice on a 129SvEv x C57BL/6 background backcrossed more than 9 generations into C57BL/6, MyD88$^{-/-}$ mice on a 129SvEv x C57BL/6 background backcrossed more than 9 generations into C57BL/6 as well as C57BL/6 mice were kept and bred at the animal facility of the Department of Experimental Biomedicine, University of Gothenburg, Sweden. All animals were specific pathogen free and were maintained in microisolator racks under standard conditions of temperature and light and fed with standard laboratory chow and water *ad libitum*. All studies were approved by the Local Animal Ethical Committee at University of Gothenburg.

Serum study of Gαi2 deficient mice

Gαi2$^{-/-}$ and Gαi2$^{+/-}$ mice were sacrificed at 6, 12 and 24 weeks of age, when they were considered healthy, pre-colitic and colitic, respectively. Blood was collected from the tail vein and spleens were weighed. The colons were opened longitudinally, rinsed in phosphate-buffered saline (PBS) and macroscopically scored for colitis based on the following criteria: 0 = normal, 1 = mild colitis, 2 = moderate colitis and 3 = severe colitis.

Isolation and stimulation of peritoneal macrophages

Isolation of mouse peritoneal macrophages was performed by injecting cold PBS into the peritoneal cavity followed by one minute of abdominal massage. Subsequently, the peritoneal fluid was collected and diluted to 1 x 10^6 cells/ml in Iscove's medium containing foetal calf serum, gentamicine, L-glutamine and mercaptoethanol. Diluted cells were transferred to 96-well plates and incubated for 24h at 37^{o}C, 5% CO_2. On day 2 the cells were carefully washed in warm PBS, to remove non-adherent non-monocytic cells. TLR-tolerance was induced in adherent cells by incubation with 1 μg/ml phenol extracted LPS, 1 μg/ml lipoteichoic acid (LTA) or medium (control) for 20h. After additional washes with warm PBS the cells were incubated with 3 μg/ml LPS or 10 μg/ml LTA for 6h where after the supernatants were collected and analyzed for TNF and IL-10 with enzyme-linked immunosorbent assays (ELISA). The kinetic study was performed by stimulating adherent cells (day 2) with 100 ng/ml or 1 ng/ml LPS where after supernatants were sampled at 1, 3, 5 12 and 24 hours post stimulation.

Isolation and stimulation of splenocytes

Whole spleens were passed through a nylon mesh and erythrocytes were depleted by Tris-buffered ammonium chloride. Sterile PBS was added to the splenocyte suspension which was followed by centrifugation (5 min at 1500 rpm). This procedure was repeated and thereafter, the splenocytes was diluted to 2.2 x 10^6 cells/ml in Iscove's medium containing foetal calf serum, gentamicine, L-glutamine and mercaptoethanol. Diluted cells were transferred to 24-well plates and incubated together with 1 μg/ml LPS, 1 μg/ml LTA or medium.

In vivo treatment of Gαi2 deficient mice

Gαi2$^{-/-}$ mice were treated with intraperitoneal injections of 100 μg/ml LTA or PBS three times per week starting at diarrhoea onset. The mice were weighed and observed at the time of injections. After 2.5 weeks the mice where anesthetized with isofluorane and sacrificed with cervical dislocation. The colons were opened longitudinally, rinsed in PBS and macro-

scopically scored for colitis based on the following criteria: 0 = normal, 1 = mild colitis, 2 = moderate colitis and 3 = severe colitis. Colon tissue was collected for histopathology and complete spleen was removed and weighed. Blood samples were taken before the first injection and at sarcrifice and were analyzed for the expression of IL-6, IL-12p40, keratinocyte chemoattractant (KC) and haptoglobin.

DSS-induced colitis

6-9 week old age-matched mice weighing 17-24 g were treated with 3% DSS diluted in drinking water for 5 days followed by 2 days with regular drinking water. Fresh DSS was prepared on a daily basis and the mice were weighed daily. The consumption of DSS/water and total body weight at treatment start did not differ between groups. At day 7 the mice were anesthetized with isofluorane and blood was collected by retro-orbital puncture. Subsequently, the mice were sacrificed by cervical dislocation and the colons were opened longitudinally, rinsed in PBS and macroscopically scored for colitis. Colon tissue was collected for RT-PCR analysis and histopathology. Additionally, complete spleen and thymus was removed and weighed.

Scoring of DSS-induced colitis

The entire colon was macroscopically scored based on the following parameters; thickness (0-4), stiffness (0-2), oedema (0-3) and visible ulcerations (0-1) (Table 2a) [272]. Tissue from the distal and proximal colon was fixed as swiss-rolls in formaldehyde containing zinc and stained with hematoxylin/eosin (H&E). Cross-sectioning of colon swiss-rolls was performed by Histocenter-Skandinaviskt Centrum för Histoteknik AB. Histopathological scoring was performed in a blinded fashion and was based on the number of inflammatory cells, epithelial degeneration and ulceration (Table 2b) [273].

a

Parameter	Score
thickness	0-4
stiffness	0-2
oedema	0-3
visible ulceration	0-1
Total	10

b

Charateristics	Score
no signs of damage	0
few inflammatory cells, no signs of epithelial degeneration	1
mild inflammation, few signs of epithelial degeneration	2
moderate inflammation, few epithelial ulcerations	3
moderate to severe inflammation, ulcerations in more than 25% of tissue section	4
moderate to severe inflammation, ulcerations in more than 50% of tissue section	5
severe inflammation and ulcerations of more than 75% of tissue section	6

Table 2. Criteria for macroscopical (a) and histopathological (b) scoring of DSS-induced colitis [272,273].

ELISA

ELISA was used to detect TNF and IL-10 in culture supernatants. A quantitative sandwich ELISA was used in which a polyclonal antibody, specific for the cytokine of interest, was pre-coated (only IL-10 as the TNF-ELISA was pre-coated by the supplier) onto a microplate followed by the blockage of non-specific binding sites and, subsequently, addition of sample, standard and controls. If the investigated cytokine was present in the samples it would bind to the pre-coated polyclonal antibodies. Unbound substances were washed away and an enzyme-linked second polyclonal antibody, specific for the cytokine of interest, was added to the microplate. Following a wash to remove unbound enzyme-antibody complexes a substrate was added to the wells resulting in a blue product that turns yellow when a stop solution was added. The colour in the wells was proportional to the amount of cytokine of interest in the well and the exact concentrations were calculated from the standard curve by a computer measuring the absorbance at 450 nm.

Cytometric bead array

Cytometric bead array (CBA) was used to detect concentration of IL-2, IL-4, IL-6, IL-10, IL-17, TNF and IFN-γ in plasma from DSS treated mice collected at sacrifice. The CBA technique is based on capture beads of different sizes conjugated to specific antibodies that are used to detect soluble antigens. An advantage using the CBA technique is that you can detect multiple cytokines simultaneously from the same sample. The beads coated with antibodies are mixed together with sample and standard. The antibody binds to its corresponding cytokine in the mixture whereafter the antibody/bead is coupled to a secondary antibody conjugated to a fluorescent dye that can be detected by flow cytometry. The fluorescence is proportional to the amount of cytokine in the sample and the concentration can be calculated from the standard curve included in each experiment.

Real-time RT-PCR

Real time reverse transcriptase PCR (RT-PCR) was used to detect mRNA levels of IL-1β, IL-6, IL-10, IL-12p40, IL-17A, IL-23p19, TNF, IFN-γ, CCL5, CXCL10, T-bet, GATA3, RoRγt, FoxP3 and IRAK-M in mid (Paper III) or distal (Paper IV) colon tissue from DSS-treated mice at sacrifice. Total RNA was isolated with an RNeasy mini kit where RNA binds to a silica-based membrane and unbound contaminants are washed away. Subsequently, the RNA was reverse transcribed into cDNA in the presence of random hexamers. To amplify the mRNA of interest primers were designed using the Roche Probe Library (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). In contrast to genomic DNA, cDNA contains no introns and, therefore, primers are designed to span an exon-exon boundary to avoid contamination.

The PCR-procedure starts by denaturation of the cDNA chain at high temperature by disrupting the hydrogen bonds between complementary bases resulting in a single stranded DNA chain. Subsequently, the temperature is lowered allowing annealing of primers to the complementary section of the single stranded DNA and binding of DNA polymerase. Finally, DNA polymerase synthesizes a new strand complementary to the DNA template strand at temperatures suitable for the DNA polymerase used. For each PCR cycle the cDNA is duplicated resulting in exponential amplification. To visualize the amount of mRNA present in the samples RT-PCR was performed in the presence of SYBRgreen dye that binds dsDNA and can be detected by the RT-PCR instrument. Thus, the amount of SYBRgreen dye is proportional to the amount of mRNA of interest in the starting material. The characteristics of the obtained products was measured using melting curve analysis where each particular dsDNA has it own melting temperature based on the length and AT/CG content. At the melting temperature the two strands of DNA will separate and the fluorescence rapidly decreases. Since the SYBRgreen fluorescence increase with every cycle it will eventually rise above the background fluorescence and this time-point is called the crossing point (CP). To be able to quantify the amplified products a standard curve is prepared by serial dilutions of a positive calibrator for every primer pair. The CP of the amplified products was then compared to the standard curve. An identical positive calibrator can be included in every plate to enable comparison between different RT-PCR runs. The samples were assayed in duplicates and the expression levels were normalized to a housekeeping gene (Hypoxanthine Guanine Phosphoribosyltransferase (HPRT)) constitutively expressed at the same levels in all cells. Primers and their target genes used in this thesis are listed in Table 3. All the analysis was performed on a Roche LightCycler 480.

Gene target	Sequence	Gene target	Sequence
IL-1β For	5′-tgtaatgaaagacggcacacc-3′	CXCL10 For	5′-gctgccgtcattttctgc-3′
IL-1β Rev	5′-tcttctttgggtattgcttgg-3′	CXCL10 Rev	5′-tctcactggcccgtcatc-3′
IL-6 For	5′-gctaccaaactggatataatcagga-3′	T-bet For	5′-tcaaccagcaccagacagag-3′
IL-6 Rev	5′-ccaggtagctatggtactccagaa-3′	T-bet Rev	5′-aaacatcctgtaatggcttgtg-3′
IL-10 For	5′-cagagccacatgctcctaga-3′	GATA-3 For	5′-cttatcaagcccaagcgaag-3′
IL-10 Rev	5′-tgtccagctggtcctttgtt-3′	GATA-3 Rev	5′-cccattagcgttcctcctc-3′
IL-12p40 For	5′-agacagagacgccattccac-3′	RoRγt For	5′-tgaggatgagattgccctcta-3′
IL-12p40 Rev	5′-cctgaagtgtgaagcaccaa-3′	RoRγt Rev	5′-ttgcagatcttccactctcct-3′
IL-17A For	5′-tcttcattgcggtggagagt-3′	FoxP3 For	5′-agctggagctggaaaagga-3′
IL-17A Rev	5′-cccttggcgcaaaagtg-3′	FoxP3 Rev	5′-gctacgatgcagcaagagc-3′
IL-23p19 For	5′-caccagcgggacatatga-3′	IRAK-M N-term For	5′-tcgacagattacagtgcacaaa-3′
IL-23p10 Rev	5′-ccttgtgggtcacaaccat-3′	IRAK-M N-term Rev	5′-ggctattcctatcaatacgctga-3′
TNF For	5′-tgcctatgtctcagcctcttc-3′	IRAK-M C-term For	5′-tgtctacagcttcggaatcg-3′
TNF Rev	5′-gaggccatttgggaacttct-3′	IRAK-M C-term Rev	5′-catcagttccatgaggaggtc-3′
IFNγ For	5′-gccatcagcaacaacataagc-3′	HPRT For	5′-tcctcctcagaccgctttt-3′
IFNγ Rev	5′-tgagctcattgaatgcttgg-3′	HPRT Rev	5′-cctggttcatcatcgctaatc-3′
CCL5 For	5′-tgcagaggactctgagacagc-3′		
CCL5 Rev	5′-gagtggtgtccgagccata-3′		

Table 3. Primers for RT-PCR.

Statistical analysis

Predictive Analytic SoftWare (PASW, SPSS) was used for statistical analysis. Results were presented as mean ± standard error of the mean (SEM), unless otherwise stated, and evaluated using the Mann-Whitney *U*-test. Values were considered statistically significant if $p \leq 0.05$.

Results and discussion

Detection of serum markers for early and ongoing colitis in Gαi2 deficient mice

Presence of bacteria is considered as an important factor for the induction of intestinal inflammation in animal models and human patients [274,275]. Bacterial structures are recognized by PRRs, including TLRs, which lead to the induction of pro-inflammatory cytokines such as IL-1β and IL-18. IL-1R, IL-18R and TLRs share intracellular signalling pathways and since signalling downstream of these receptors is highly regulated IL-1β and IL-18 may have important functions during early stages of intestinal inflammation. Gαi2 deficient mice have increased spontaneous production of IL-1β as well as other pro-inflammatory cytokines from intestinal tissue [249,251]. To identify serum biomarkers for early detection of intestinal inflammation induced in Gαi2 deficient mice on a 129SvEv background that are born healthy and gradually develop colon inflammation at 12-25 weeks of age, the expression of IL-1β, IL-18 and IL-1Ra in serum from pre-colitic and colitic Gαi2 deficient mice was measured. Serum concentration of IL-18 was upregulated in colitic mice but not in pre-colitic mice whereas concentration of IL-1Ra was increased in both pre-colitic and colitic mice. Serum concentration of IL-1β was not upregulated at any time-point. Why can we detect increased IL-1Ra but not IL-1β concentrations in serum from Gαi2 deficient mice? One explanation might be that IL-1β quickly binds to its receptor locally in the intestine. Another explanation might be that Il-1Ra, in contrast to IL-1β, is produced by liver cells in response to e.g. IL-6 which might contribute to increased serum concentrations [276]. Future studies are necessary to address whether the kinetics of IL-1Ra, IL-1β and IL-18 production seen in Gαi2 deficient mice can be detected also in human IBD patients.

Increased production of TNF after TLR stimulation of Gαi2 deficient cells

Studies from our group and others have demonstrated that cells from Gαi2 deficient mice have an increased production of pro-inflammatory cytokines in response to bacterial stimulation [248,277]. In concordance with these data, we found that splenocytes (Fig. 6a) from Gαi2 deficient mice produced increased concentration of TNF in response to LPS and LTA stimulation. We could detect no significant increase in TNF production after stimulation of Gαi2 deficient peritoneal macrophages (Fig. 6b) as judged by the number of mice used in this experiment.

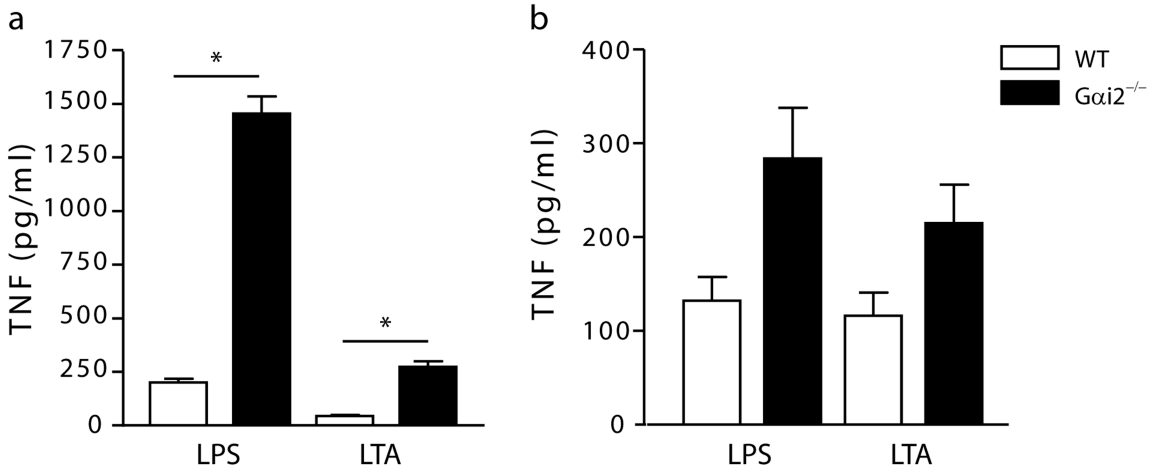

Figure 6. Increased TNF production from Gαi2 deficient cells after LPS and LTA stimulation. TNF concentrations in culture supernatants after stimulation with LPS (1 μg/ml) or LTA (1 μg/ml) of 6 week old Gαi2 deficient (n = 6) or WT (n = 6) splenocytes (a) and 6 week old Gαi2 deficient (n = 5) or WT (n = 5) peritoneal macrophages (b). Data values are presented as mean ± SEM. Data were analyzed using Mann-Whithey *U*-test. A value of $P \leq 0.05$ (*) was considered statistically significant.

Gαi2 deficient peritoneal macrophages have a functional TLR cross-tolerance *in vitro*

Gαi2 deficient mice had an increased production of IL-18 in serum and increased spontaneous production of IL-1β from intestinal tissue [249]. As IL-1β and IL-18 exerts their pro-inflammatory functions via TLR-associated signalling pathways, and IL-1R stimulation of macrophages induce hyporesponsiveness towards subsequent TLR/IL-1R stimulation [110], i.e. downregulation of pro-inflammatory cytokine production after repeated TLR/IL-1R stimulation, the ability of Gαi2 deficient cells to induce TLR-tolerance was investigated. Peritoneal macrophages from Gαi2 deficient mice had a reduced pro-inflammatory cytokine production after repeated stimulation with the same TLR ligand and after repeated stimulation with different TLR ligands (i.e. cross-tolerance) demonstrating that non-functional TLR-tolerance not was involved in the pathogenesis of Gαi2 deficient mice (unpublished data).

Earlier studies have shown that peritoneal injections of TLR ligands can induce TLR tolerance *in vivo* [111,278]. Therefore, Gαi2 deficient mice were treated with repeated injection of LTA into the peritoneal in order to alleviate colon inflammation. Treatment started at disease onset and continued with three injections per week for 2.5 weeks. However, no effects on colitis as judged by colitis score, pro-inflammatory cytokine concentrations in plasma, total body weight and spleen weight were detected (unpublished data). The choice of *in vivo* treating Gαi2 deficient mice with LTA, instead of LPS, was based on an assumed lower toxicity and positive results reported from other groups [111,278]. One could speculate that rectal administration might have more pronounced effects locally in the colon. Thus, it is possible that treatment with other TLR ligands and/or other routes of administration could result in alleviated colon inflammation in Gαi2 deficient mice.

Reduced production of cytokines from IRAK-1 deficient peritoneal macrophages

The downregulation of pro-inflammatory cytokines seen after repeated TLR-stimulation of Gαi2 deficient peritoneal macrophages revealed the effectiveness and biological relevance of TLR-tolerance and cross-tolerance for cellular homeostasis. Several cytosolic proteins and mechanisms have been linked to TLR tolerance/cross-tolerance, including downregulation of IRAK-1 [105,279-281]. However, no studies had addressed the importance of IRAK-1 for TLR tolerance/cross-tolerance using IRAK-1 deficient cells. Furthermore, IRAK-1 was initially described as a signalling kinase important for IL-1RI and TLR4 mediated production of pro-inflammatory cytokines [74,282]. However, the importance of IRAK-1 has been questioned in recent years as two additional IRAK-family members with partly redundant roles in TLR signalling have been discovered; IRAK-2 and IRAK-4 [52,53].

IRAK-1 deficient peritoneal macrophages have a reduced production of TNF and IL-10 in response to stimulation with low LTA concentration, whereas the absence of IRAK-1 resulted in decreased IL-10, but not TNF, production after high LPS stimulation. Interestingly, the role of IRAK-1 is of less importance for TNF production after stimulation with increased LTA concentration (i.e. after Med-LTA stimulation). Furthermore, kinetic studies demonstrated that IRAK-1 deficient peritoneal macrophages stimulated with low concentration of LPS (1 ng/ml) had a reduced production of TNF already 3 hours post stimulation (Fig. 7a). In contrast, the absence of IRAK-1 had no effect on TNF production after stimulation with increased concentration of LPS (100 ng/ml) at any time-point measured (Fig. 7b). These results corroborate earlier studies regarding TNF production after LPS stimulation of IRAK-1 deficient peritoneal macrophages and further showed that LTA stimulation follows the same concentration-dependent signalling pattern [75]. Interestingly, low concentrations of IL-1β induce reduced activation of NF-κB in IRAK-1 deficient mouse fibroblasts whereas no difference is seen at higher concentrations [283]. How can the importance of IRAK-1 for cytokine production be dependent on the concentration of the ligand? One possibility is the involvement of

redundant proteins, such as IRAK-2. Hypothetically, IRAK-1 could have a generally higher binding affinity for TRAF6, compared to IRAK-2, resulting in impaired signalling at low stimulation whereas increased stimulation activates sufficient IRAK-2 to fully activate TRAF6 in the absence of IRAK-1. This hypothesis is supported by molecular studies demonstrating that IRAK-1 has three TRAF6 interaction motifs whereas IRAK-2 only has two [284] which potentially could result in different abilities to activate TRAF6.

Studies from other groups confirm that IRAK-4 is critical for TLR signalling and that IRAK-1 together with IRAK-2 is important for TLR signalling during the first hours after TLR4 stimulation. However, whereas the kinase activity of IRAK-1 decreases within a few hours after TLR4 stimulation, IRAK-2 kinase activity is sustained for a longer time-period [82]. Collectively, these results suggest that IRAK-1 is important for the initial signalling events in response to TLR stimulation where after other signalling factors take over. The reason that we detected decreased TNF concentrations in supernatants from IRAK-1 deficient peritoneal macrophages up to 24h post stimulation with 1 ng/ml LPS may be explained by decreased ability to secrete TNF during early signalling events.

LPS is a stronger inducer of TNF production than LTA. Other studies confirm this finding and show that whereas LPS alone is a potent inducer of the immune system, gram-positive bacteria (i.e. with LTA-rich cell walls) may have to be phagocytised to fully activate the immune system [285,286]. Speculatively, this discrepancy may be partly explained by how the immune system fights bacteria of different structure. For example, in contrast to gram-negative bacteria, gram positive bacteria are more resistant to the membrane attack complex (MAC)-induced lysis by the complement system and might, therefore, need to be phagocytised [287]. Inside the cell, structures from gram-positive bacteria can be recognized by intracellular receptors, such as NOD2 and the NALP3 inflammasome, which facilitates full activation and bacterial killing [288,289].

LTA stimulation of peritoneal macrophages induces low production of IL-10. As TLR4, and not TLR2, can signal via the MyD88-independent pathway, peritoneal macrophages from MyD88 deficient mice were isolated to investigate whether the MyD88-independent pathway was responsible for IL-10 production seen after LPS stimulation. However, in the absence of MyD88, LPS stimulation induced very low concentration of IL-10 demonstrating that the MyD88-independent pathway was not responsible for IL-10 production after TLR4 stimulation at the time interval analysed. These data indicate that other signalling components are responsible for the differences in IL-10 production after LPS and LTA stimulation.

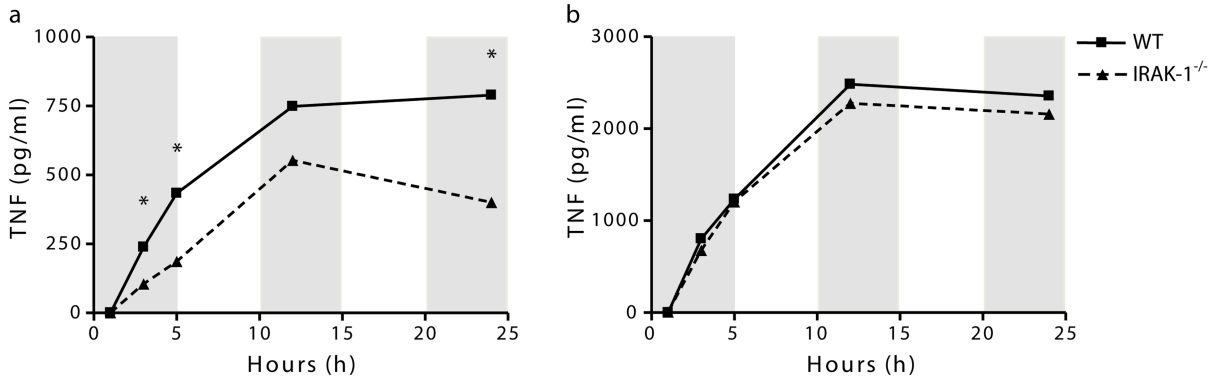

Figure 7. Decreased production of TNF from IRAK-1$^{-/-}$ peritoneal macrophages stimulated with low concentrations of LPS. TNF concentration in supernatants from IRAK-1$^{-/-}$ (n=4) and WT mice (n=5) at indicated time-points after stimulation with 1 ng/ml (a) or 100 ng/ml (b) LPS. Data values are presented as mean. Data were analyzed using Mann-Whithey *U*-test. A value of $P \leq 0.05$ (*) was considered statistically significant.

Altered cross-tolerance from IRAK-1 deficient peritoneal macrophages

As stimulation of IRAK-1 deficient peritoneal macrophages with high concentration of LPS and LTA resulted in substantial levels of TNF, the importance of IRAK-1 for TLR cross-tolerance was investigated. Cross-tolerance was intact in IRAK-1 deficient peritoneal macrophages after pre-stimulation with LPS, whereas pre-stimulation with LTA induced a hyporesponsive trend, as judged by TNF production. Thus, IRAK-1 was of more importance for cross-tolerance after stimulation with LTA, whereas cross-tolerance induced after LPS stimulation may be more dependent on other regulatory mechanisms. With regard to IL-10, decreased IL-10 concentrations were detected after pre-stimulation with LPS in WT, but not in IRAK-1 deficient peritoneal macrophages. Pre-stimulation of WT peritoneal macrophages with LTA resulted in a trend towards decreased IL-10 production whereas the absence of IRAK-1, interestingly, induced an increased IL-10 production after re-stimulation with LPS. The increased IL-10 production after repeated TLR stimulation have been observed by other groups which further supports this interesting finding (unpublished data, personal communication). Thus, presence of IRAK-1 downregulates IL-10 production after LPS and LTA pre-stimulation via an unknown mechanism.

The importance of IRAK-1 for cross-tolerance with regard to TNF production was more pronounced after pre-stimulation with LTA than after pre-stimulation with LPS. Since LPS is a potent stimulator of the immune system other/additional mechanisms to downregulate TLR4 induced responses may have evolved, whereas a less potent stimulation by LTA may be more efficiently regulated at the IRAK-1 level. As discussed earlier, the production of IL-10 in response to TLR2 and TLR4 stimulation was attenuated in the absence of IRAK-1. Based on these observations it was surprising that presence of IRAK-1 downregulated IL-10 production after a primary LTA stimulation. What is the biological relevance of reduced IL-10 production after repeated TLR stimulation as IL-10 is considered as an anti-inflammatory cytokine? Speculatively, decreased IL-10 production may facilitate pathogen clearance and studies on mice have demonstrated that inhibition of IL-10 can reduce the severity of certain infections [290-292]. Another possibility is that IL-10 serves other, yet unknown, functions in addition to its anti-inflammatory properties.

Decreased colitis in IRAK-1 deficient male mice after DSS treatment

As IRAK-1 was of importance for TLR/IL-1R signalling in response to bacterial ligands *in vitro* and IL-1/IL-18 is upregulated in colitis [293] the importance of IRAK-1 for inflammatory disease outcome in a bacteria rich milieu *in vivo* was investigated. Chemical induction of colitis with DSS treatment showed that both male and female IRAK-1 deficient mice were protected from DSS-colitis based on decreased body weight loss and spleen weight. Interestingly, only male IRAK-1 deficient mice were protected from colitis based on inflammatory cell infiltration, ulceration and oedema in colon tissue. Surprisingly, IRAK-1 deficient male mice had increased expression of IFN-γ in colon tissue whereas the expression of IFN-γ in female mice was unchanged. Earlier mouse studies have demonstrated that thymic atrophy correlates with increased colitis after DSS treatment [294]. Correspondingly, thymic weights of IRAK-1 deficient male mice were increased whereas thymic weights of IRAK-1 deficient female mice were unchanged.

DSS treatment results in erosion of the intestinal epithelial layer and increased bacterial translocation [235]. Since IRAK-1 was important for TLR2 and TLR4 signalling and IRAK-1 not is fundamental for TLR-tolerance it was not surprising to find that the absence of IRAK-1 resulted in decreased inflammation. However, it was unexpected that the presence of IRAK-1 was pro-inflammatory in male but not female mice, as judged by colon inflammation. Sex hormones are known to influence a wide range of inflammatory disorders and oestrogen treatment of human IBD patients has ameliorating effects on disease activity [295,296].

Therefore, the presence of oestrogen might explain the somewhat milder colitis seen in female mice after DSS treatment. It may be informative that the *Irak-1* gene is situated on the X-chromosome and that the cellular levels of functional IRAK-1 could differ between WT male and female mice. Therefore, cellular homeostasis of male and female mice may be differently affected by the removal of *Irak-1*.

Increased colitis in IRAK-M deficient mice after DSS treatment

Signalling of TLR/IL-1R via IRAK-1 is negatively regulated by IRAK-M and macrophages deficient in IRAK-M have increased production of pro-inflammatory cytokines after TLR stimulation and a deficient tolerance and cross-tolerance after repeated TLR stimulation [85,297,298]. Mutations in the *Irak-m* gene have been associated with human autoimmune diseases such as asthma [89]. However, the role of IRAK-M in intestinal inflammation was still unknown. IRAK-M deficient mice had reduced total body weight and increased colon inflammation characterised by oedema and influx of inflammatory cells after DSS treatment. Isolation and weighing of the complete thymus demonstrated that IRAK-M deficient mice had significantly reduced thymic weights. Surprisingly, IRAK-M deficient mice had lower spleen weights compared to both WT control mice and non-treated IRAK-M deficient mice, which may indicate increased apoptosis or efflux of spleen cells.

Expression of TNF, IL-1β, IL-6, IL-17A, IL-23p19, IL-10 and CCL5 mRNA were all significantly upregulated in distal colon from IRAK-M deficient mice. Furthermore, analysis of T lymphocyte hallmark transcription factors revealed that GATA3 (Th2) and FoxP3 (Treg cells) were significantly upregulated in IRAK-M deficient mice, indicating increased numbers of Th2 cells and Treg cells. The increased pro-inflammatory mRNA transcription was also detected in plasma collected at sacrifice and display increased concentrations of TNF and IL-6.

A recent study demonstrates that presence of commensal bacteria induce IRAK-M expression in colon tissue [142]. Furthermore, mice deficient in IL-10, which develops spontaneous intestinal inflammation, have increased colitis score and production of inflammatory cytokines when they are deficient also in IRAK-M. These data further supports a role of IRAK-M for colon homeostasis. What is the mechanistic explanation for the increased colon inflammation seen in IRAK-M deficient mice? Macrophages are known as producers of diverse cytokines in response to TLR stimulation by pathogens. However, intestinal macrophages normally have a tissue-restricted phenotype characterized by normal phagocytosis but decreased pro-inflammatory cytokine production [154]. Since IRAK-M is preferentially expressed in monocytes/macrophages, the observed phenotype in IRAK-M deficient mice may directly result from the inability to negatively regulate pro-inflammatory cytokine production after increased bacterial TLR stimulation, in turn caused by a deficient epithelial barrier.

Personal reflections and future perspective

Symbiotic relationships between animals and bacteria are advantageous and have, therefore, been preserved during evolution. However, symbiosis in the intestine requires a strict control of the immune system to avoid unwanted responses towards non-pathogenic bacteria that could result in inflammatory disease and tissue damage. The list of genetic risk factors involved in the pathogenesis of IBD have been steadily increasing since the discovery of NOD2 in 2001 to over 30 identified genes today demonstrating that intestinal inflammation is the end product of numerous genetic defects [200,299]. Interestingly, most of these defects do not result in inflammation anywhere else in the body which most probably is due to the high bacterial load in the intestinal tract. In addition, studies suggest that environmental factors, such as high-fat diet and compositional changes of the intestinal bacterial flora, can cause IBD by themselves [233,300-302]. However, it is hard to interpret whether these risk factors actually are inducers or if the inflammation is a secondary effect to genetic abnormalities. Based on these reflections it is possible to argue that chronic gastrointestinal inflammation results from a large group of host deficiencies and environmental risk factors, grouped together as IBD.

Why does absence of the αi2-subunit of G-proteins lead to increased production of pro-inflammatory cytokines and extensive colon inflammation in mice? The complete mechanism is not known but G_i-proteins are involved in many processes influencing the immune system including diapedesis, maintenance and development of tight junctions and signalling via chemokine receptors [252,254,303]. Interestingly, studies have shown that stimulation of Gi-protein-coupled receptors inhibits production of IL-12p70 after LPS and IFN-γ stimulation via activation of PI3K [253]. PI3K is known to inhibit extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation which leads to decreased concentrations of IL-10 and increased concentrations of IL-12 [304]. Thus, the absence of Gαi2 can potentially lead to multiple immunological defects resulting in increased pro-inflammatory cytokine production and inflammation.

Stimulation of isolated cells with bacterial ligands can reveal the importance of different signalling factors. This knowledge is fundamental for basic understanding but also potentiates studies on more complex interactions of the immune system. Soluble bacterial/viral ligands bind to and activate e.g. TLRs *in vivo* but the immune response is dependent on the sum of all immune activating/inactivating signals expressed by particular microbes acting on host cells. The end product is a tailor made arsenal of immune factors that collectively cooperate to eliminate the invading microbe. Studying the immune response to individual microbes could result in more pathogen specific treatment therapies with reduced side effects compared to broad spectrum drugs.

As front line sensors in the human body, TLRs are optimally positioned to detect and induce immune reactions in response to invading pathogens. However, this also confers that TLR signalling needs to be tightly controlled in order to induce immune responses without causing extensive tissue damage. The importance of regulating innate signalling is evident as stimulation of TLRs and IL-1β is followed by downregulation of pro-inflammatory gene expression [110]. On the other hand, a rapid termination of the signalling cascade or a prolonged hyporesponsive signalling phenotype could result in deficient clearance of the initial pathogen or an increased risk for subsequent opportunistic pathogens, respectively. This thesis further highlights the delicacy of TLR regulation as removal of single TLR signalling-associated proteins leads to abnormal immune responses towards bacterial stimulation.

Studies have shown that repeated stimulation with combinations of LPS and IL-1β induce a hyporesponsive phenotype, similar to cross-tolerance observed after repeated stimulation of different TLRs [110]. Since TLRs share intracellular signalling pathways with the IL-1R this result is not surprising. However, to my knowledge no studies have addressed whether

signalling via the IL-18R can induce tolerance towards further IL-18R simulation or cross-tolerance in response to TLR/IL-1R stimulation. Increased IL-18 concentrations are detected in patients with auto-immune diseases, such as CD and systemic onset juvenile idiopathic arthritis, which further motivates future studies regarding the importance of IL-18R tolerance and cross-tolerance [305,306].

New studies regarding TLR-tolerance have turned the scientific attention towards the nucleus and shows that genes induced after TLR4 stimulation falls into two groups dependent on their cellular function and regulatory requirements; pro-inflammatory gene expression is downregulated whereas antimicrobial factors remain inducible. This is thought as a mechanism to minimize tissue damage caused by excessive production of inflammatory cytokines while an antimicrobial milieu is maintained. Gene-specific regulation is caused by changes in the chromatin structure rendering certain genes accessible whereas other genes become inaccessible for transcription [107,307]. In addition, miRNAs have emerged as important regulators of TLR signalling that interferes with and decreases the levels of transcribed mRNA [108]. Chromatin remodelling and the actions of miRNAs can be considered as a fine tuning part of TLR signalling regulation acting at the level of individual genes and mRNAs, respectively, whereas proteins addressed in this thesis accounts for a broader control of TLR signalling. Collectively, regulation of TLR/IL-1R signalling occurs at multiple levels reaching from early interactions of proteins at the cell membrane to protein synthesis.

In the past decade the knowledge of intracellular regulation of TLR signalling has increased exponentially and we now know that multiple cytosolic proteins work in concert to optimize pathogen recognition. IRAK-1 has since its discovery in 1995 been considered as an important signalling protein for several TLRs but also IL-1R and IL-18R. The discovery of IRAK-2 and IRAK-4 have further contributed to the knowledge of IRAK-1 signalling as being cooperatively responsible for signalling transduction downstream certain TLRs and absolutely necessary downstream of other TLRs. In this thesis we conclude that IRAK-1 is of importance for TLR2 and TLR4 signalling and the induction of TLR-tolerance. However, since IRAK-1 act redundantly with IRAK-2 and IRAK-4 antibodies directed towards IRAK-1 might be beneficial for treatment of inflammatory disease without rendering the patient hypersensitive to opportunistic bacteria. We also demonstrate that IRAK-1 is important for induction of intestinal inflammation after DSS treatment in male, but not in female, mice. Whether these data is dependent on the properties of sex hormones, specific interactions of sex hormones with IRAK-1 or a difference in IRAK-1 signalling homeostasis between male and female mice is unclear and requires further investigation.

The presence of IRAK-M in humans has been reported in cells of monomyeloic origin [84]. However, more recent publications indicate that IRAK-M might be of importance also in non-monomyeloic cell types, such as biliary and gingival epithelial cells [308,309], which suggests that IRAK-M may be involved in signalling homeostasis in diverse tissues. Clearly, more studies are needed to clarify what cells and tissues express IRAK-M and if the regulatory properties of IRAK-M are universal.

Several studies have identified IRAK-M as an important factor for the pathogenesis of cancer. Initially, it was discovered that human macrophages cultured in the presence of cancer cells produced very low concentrations of pro-inflammatory cytokines and that the hyporesponsive phenotype was dependent on cancer-induced upregulation of IRAK-M [310]. Subsequent studies have revealed that IRAK-M deficient mice have increased defence against tumour growth and that IRAK-M expression correlates with lethality of lung cancer [311,312]. These data demonstrates the importance of innate immune responses towards cancer and pinpoint IRAK-M as a candidate molecule for cancer therapy.

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