Toll-like receptors in development, health and disease

Citation for published version (APA):

Wolfs, T. G. A. M. (2010). *Toll-like receptors in development, health and disease*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht. <https://doi.org/10.26481/dis.20100305tw>

Document status and date:

Published: 01/01/2010

DOI:

[10.26481/dis.20100305tw](https://doi.org/10.26481/dis.20100305tw)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Toll-like receptors in development, health and disease

© 2010 Tim Wolfs, Maastricht

Layout: Tiny Wouters

Production: Datawyse | Universitaire Pers Maastricht

ISBN: 978-90-5278-914-9

The study presented in this thesis was performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM)

Toll-like receptors in development, health and disease

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen in het openbaar te verdedigen
op vrijdag 5 maart 2010 om 12.00 uur

door

Tim Guillaume Anna Marie Wolfs

Promotor

Prof. dr. W.A. Buurman

Beoordelingscommissie

Prof. dr. JW Cohen Tervaert (voorzitter)

Prof. dr. N. Gassler (Aachen University, Institute of Pathology, Aachen, Germany)

Prof. dr. L.W.E. van Heurn

Prof. dr. A.A.M. Masclee

Prof. dr. L.J.I. Zimmermann

Voor mijn ouders

Contents

Chapter 1	Introduction	9
Chapter 2	In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN- γ and TNF- α mediated up-regulation during inflammation The Journal of Immunology 2002;168:1286-93	33
Chapter 3	Apoptotic cell-death is initiated during normothermic ischemia in human kidneys American Journal of Transplantation 2005;5:68-75	53
Chapter 4	Increased release of sMD-2 during human endotoxemia and sepsis; a role for endothelial cells Molecular Immunology 2008;45:3268-77	69
Chapter 5	The localization of the LPS-recognition complex in the human healthy and inflamed premature and adult gut Inflammatory Bowel Diseases 2010;16:68-75	91
Chapter 6	Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep PLoS One 2009;4:e5837	107
Chapter 7	Summary and discussion	127
	Samenvatting	137
	Dankwoord	143
	Publications	147
	Curriculum vitae	151
	Colour figures	155

Chapter 1

General introduction

Introduction

An inflammatory reaction by the host innate immune system can arise following infection, ischemia, trauma, tissue damage or a combination of such insults¹. Toll-like receptors are an integral part of the innate immune system and these receptors recognize conserved pathogen-associated molecular patterns (PAMPs). In addition, TLR are considered to be implicated in the response to ligands, released by damaged or dead host cells, the so called damage-associated molecular patterns (DAMPs)². Engagement of TLR with their ligands initiates a signaling cascade which ultimately results in an inflammatory response, aimed at elimination of the infecting microbe and recovery of the tissue. However, excessive or continuous activation of the immune system driven by TLR is considered to be involved in acute and chronic inflammatory syndromes including septic shock, ischemia reperfusion injury, necrotizing enterocolitis and inflammatory bowel disease. Understanding the biology of TLR will provide further insight into processes responsible for initiation and maintenance of inappropriate immune activation. Increased insight in the role of TLR in health and disease, might help to develop therapeutic strategies aimed at manipulating TLR signaling pathways to treat organ injury accompanying uncontrolled inflammation.

Innate immunity

The innate immune system is a universal and evolutionary ancient form of host defence against infectious organisms. The central function of this system is the recognition of microbial invasion and the initiation of responses that limit the spread of pathogenic microorganisms in host tissues³. The innate immune system comprises of passive and active components. The passive arm of the innate immune system includes anatomical barriers that prevent physical access of microorganisms⁴. Examples of anatomical barriers are the intestinal epithelium and the dry skin with their secretions, ciliated epithelial cells in the respiratory tract, and mucous membrane secretions. Secretory molecules include mucus, fatty acids, interferons, lactoferrin, trefoil factors and antimicrobial substances. Members of the latter group such as alpha-defensins and beta-defensins, lysozyme and cathelicidins can directly destroy microorganisms⁵⁻⁷.

When microorganisms breach the anatomical barrier, the active innate immune system comes into play. This part of the immune system relies on evolutionarily ancient germline-encoded receptors, the pattern-recognition receptors (PRR) and circulating pattern recognition molecules (e.g. complement system), which both recognize highly conserved microbial structures, known as PAMPs. These microbial patterns are not unique to pathogenic organisms, but also occur in nonpathogenic

commensal microorganisms. Therefore, these immuno stimulating molecules are also referred to as micro-organism associated molecular patterns (MAMPs).

Circulating pattern recognition molecules such as mannose binding lectin (MBL), Ficolin, and C1q function as initiators of the complement system. The complement system can be activated by three different pathways: i.e. the lectin pathway, the classical complement pathway and the alternative complement pathway. All pathways converge at the level of C3, which ultimately leads to the activation of C5 and the lytic pathway. The lectin pathway is activated after the recognition and binding of PAMPs by lectin proteins (MBL, ficolin H and ficolin L16). The classical complement pathway is activated by antigen-antibody complexes, resulting in activation of the complement factor C1q. Alternatively, C-reactive protein may activate the classical pathway of complement by binding C1q.

The alternative complement pathway is activated upon binding of C3b to microbial surfaces or antibody molecules. The alternative pathway is continuously activated at a low level^{8,9}. This spontaneous “C3 tick-over” leads to the deposition of C3b on biological particles in plasma or on cell surfaces¹⁰. In addition, the alternative pathway can act as an amplification loop to the other two pathways once C3b has been generated¹¹.

Gram-negative bacteria are prone to direct lysis by the complement system through the C5-C9 membrane attack complex (MAC)^{12,13}. Gram-positive organisms are not susceptible to this extracellular killing¹³. Alternatively, they are opsonized by C3b, which allows phagocytosis and subsequent release of the pro-inflammatory anaphylatoxins (C5a and C3a) that attract white blood cells and finally formation of the MAC complex, which forms a pore in the target membrane¹¹.

Besides circulating pattern recognition molecules, cell associated PRR such as the Nod-like receptors (NLR) are an important component of the active innate immune system functional in recognition and activation of host defense responses against PAMPs¹⁴. NLRs are confined to the cytosol where they detect their ligands¹⁵. This localization enables NLRs to recognize and sense bacteria or their toxins that have evaded detection by PRR through invasion of cells or escaped from endosomal compartments. In addition, NLRs have been shown to detect ligands that are delivered intracellularly by non invasive pathogens. For instance, *Helicobacter pylori* induces immune activation via NOD1 by peptidoglycan that is delivered into the cell via the so called bacterial type IV secretion system¹⁶.

More than 20 human and 30 murine NLR proteins have been identified. They do not appear to have known homologs in nonvertebrates^{15,17}. To date, the functions of Nod-1 and -2, NALP3, Ipaf and Naip have been studied most^{15,17}. Downstream signals include activation of the NF- κ B pathway, caspase-1-dependent inflammasome (a high-molecular-weight complex composed of multiple NLRs with the ability to cleave and activate pro-caspase-1, IL-1 β and pro-IL-18)^{18,19}, and ERK, JNK and p38 pathways²⁰. Certain NLR mutations are associated with genetic risk factors for chronic inflammatory disorders^{21,22} such as Crohn's disease. This is likely related to abnormal

intestinal microbe recognition, leading to an inflammatory response instigated by commensal and pathogenic bacteria.

Toll like receptors (TLRs)

In addition to the NLRs, the most studied PRR are the Toll-like receptors, which are able to detect danger elements both inside and outside the cell. TLR are type I integral membrane glycoproteins characterized by extracellular domains that contain varying numbers of leucine-rich-repeat (LRR) motifs, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) signaling domain. To date, at least 13 mammalian TLR have been identified^{23,24}. From these TLR, TLR10 is present in man but not in mice²⁵. In contrast, TLR 11, 12, and 13 are expressed in mice but absent in man^{24,26}. TLR expressed at the cellular surface including TLR 1, 2, 4, 5, 6 and 11 sense lipids, lipoproteins or peptidoglycans from bacteria and fungi (summarized in Table 1.1)^{23,24}. The other TLR (3, 7, 8 and 9) detect bacterial and viral nucleic acids (Table 1.1) and these nucleic-acid receptors are found almost exclusively in intracellular compartments such as endosomes^{23,24}. The ligands for TLR 10, 12 and 13 remain unidentified.

Table 1.1 TLR and their microbial ligands.

TLR	Microbial ligands	Microorganism
TLR1	Tri-acyl peptides	Mycobacteria, Neisseria, Borrelia
TLR2	Lipoprotein, PGN, Lipoteichoic acid (LTA), phenol-soluble modulins, Lipoarabinomannan Zymosan	Gram-positive bacteria e.g. <i>S. epidermidis</i> , Neisseria and Yeast
TLR3	dsRNA	Viruses
TLR4	LPS	Gram-negative bacteria
TLR5	Flagellin	Flagellated Gram-positive and -negative bacteria
TLR6	Lipoteichoic acid, Zymosan, Diacyl lipopeptides	Gram-positive bacteria, Mycoplasma and Yeast
TLR7	ssRNA	Viruses (e.g. influenza, VSV, HIV)
TLR8	ssRNA	Viruses
TLR9	Unmethylated CpG-containing DNA	Gram-positive and -negative bacteria and viral DNA (e.g. HSV 1 and 2; murine CMV)
TLR10	Unknown	Unknown
TLR11	Unknown	Uropathogenic <i>E. coli</i>
TLR12	Unknown	Unknown
TLR13	Unknown	Unknown

Different adaptor proteins are implicated in TLR signaling including MyD88, Mal (MyD88 adaptor-like) also termed TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain-containing adapter-inducing IFN- β) and TRAM (TRIF related adaptor

molecule) (Figure 1.1)²⁷. MyD88 is the universal adapter protein for signal transduction of the interleukin (IL)-1 receptor family and most TLR, with the exception of TLR3²⁷. TRIF has been demonstrated to be the crucial adaptor protein for TLR3. TRIF is also involved in a TLR4-mediated MyD88-independent pathway²⁸⁻³⁰. TRIF activation ultimately results in activation of IFN regulatory factor-3 (IRF-3) and induction of IFN- β ³¹⁻³⁴.

Signaling via the MyD88 dependent pathway, induces activation of the mitogen-activated protein kinases JNK, p38 and ERK and nuclear factor-kappa B (NF- κ B). The transcription factor NF- κ B plays a critical role in the coordination of both innate and adaptive immune responses by regulating gene expression of many cellular immune mediators³⁵.

Figure 1.1

TLR signaling pathways.

Upon engagement with their ligands, all TLR with the exception of TLR3, recruit MyD88 to their TIR domain. For the extracellular TLR, TIRAP and TRAM act as bridging adapters, involved in recruitment of MyD88. MyD88 recruits IRAK1 and IRAK4 and then TRAF6, finally resulting in activation of inflammatory transcription factors. Ligation of the intracellular TLR 7, 8 and 9 induces recruitment of IRAK1 and IRAK4, finally leading to dimerization and subsequent translocation of IRF7 to the nucleus, where it binds to the promoter of IFN- α genes. A TLR4-mediated MyD88-independent pathway and TLR3 dependent activation are both mediated by TRIF, ultimately resulting in translocation of IRF-3 or 7 to the nucleus. Control of TLR activation is mediated by negative regulators including IRAK-M, TOLLIP, SIGIRR and A20.

TLR activation is essential for the ability of the immune system to combat invading pathogens. A strict regulation is of major importance, since a lack of control leads to detrimental and inappropriate inflammatory responses that might result in allergic diseases and autoimmune/inflammatory diseases such as lupus, atherosclerosis, type I diabetes, arthritis, inflammatory bowel disease, psoriasis and dermatomyositis^{24,36}. Therefore, negative immune regulators of the TLR signaling pathway such as IRAK-M, TOLLIP, SIGIRR and A20 protect the host against excessive immune activation³⁷.

Besides an important corner stone of the innate immune system, TLR play a crucial role in bridging innate and adaptive immunity by modulating dendritic cell (DC) maturation and their migration to lymph nodes where they instruct the antigen specific T-lymphocytes³⁸. Additionally, it has been shown that generation of T-cell dependent antigen-specific antibody responses also requires activation of TLR in B cells³⁹. However, later studies suggest that TLR signaling in B cells amplifies, but is not required for antibody production or maintenance of memory⁴⁰. Microorganisms, via their PAMPs, may also contribute directly to the perpetuation and activation of long term T-cell memory as T-cells also express TLR⁴¹. Taken together, a large body of evidence emphasizes the importance of TLR in the generation of an optimal and orchestrated response to bacterial stimuli.

Crosstalk between PRR systems

Infectious agents contain multiple immunostimulatory molecules, capable to activate the innate immune system via different PRR. Evidence is increasing that the strongest response against different types of microbes, is achieved by different PRR combinations. In addition, multiple TLR and even different PRR systems become simultaneously or consecutively activated. The resulting PRR signaling proceeds synergistically or antagonistically as a result of crosstalk between different PRR systems^{42,43}. For example, several studies have reported a synergistic outcome on cytokine expression in different cells stimulated with a mixture of NLR and TLR agonists, whereas a single TLR or NLR activator showed little activity^{44,45}. In contrast, Nod2 activation appeared to inhibit TLR-2 mediated cytokine release in murine splenocytes⁴⁶. In addition, C5a was shown to negatively impact TLR4-induced synthesis of IL-12, IL-23 and IL-27 in murine macrophages⁴⁷. These recent findings highlight the importance of considering TLR activation in the context of other signaling pathways, as occurs *in vivo* during infection and inflammation.

Lipopolysaccharide recognition

Host mechanisms that recognize Gram-negative bacterial LPS are among the most sensitive and best studied. Endotoxin (lipopolysaccharide, LPS) constitutes the outer leaflet of the outer membrane of Gram-negative bacteria (Figure 1.2). LPS consists of three covalently linked regions; lipid A, core oligosaccharide, and an O side chain (Figure 1.2).

Figure 1.2 Schematic structure of lipopolysaccharide (LPS) in the bacterial cell wall. LPS has three domains: the lipid A moiety, the core oligosaccharide and the O-antigen or O-side chain. The lipid-A component anchors the LPS molecule in the outer membrane of the bacterial cell wall. Lipid A is composed of a phosphorylated diglucosamine carrying acyl residues. The core oligosaccharide is composed of short series of different sugars. It is connected with the O-antigen, a polymer of repeating sugar units.

Lipid A is responsible for the toxicity of LPS, and is composed of fatty acyl chains linked to two *N*-acetylglucosamine residues. Both glucosamine backbones of lipid A are frequently phosphorylated. This phosphorylation process is important for the biological activity of LPS. More precisely, diphosphorylated lipid A is 1000-times more active than monophosphorylated lipid A⁴⁸. In addition, the immunostimulatory potency of LPS is influenced to a large extent by the number and length of the fatty

acyl chains^{49,50}. The most immunologically active form of lipid A contains six fatty acyl groups (hexa-acyl) of 12–16 carbons in length and it is found in pathogenic bacteria such as *Escherichia coli* and *Salmonella* species^{51,52}. Lipid A structures containing four or five fatty acids, can inhibit the strong endotoxic response triggered by hexa-acylated LPS^{53,54}. This phenomenon is used in the search for pharmacological tools for treatment of endotoxin-mediated indications such as sepsis. Likewise, a synthetic tetra-acylated lipid A compound, designated Eritoran is a strong antagonist of hexa-acyl LPS, with therapeutic potential⁵⁵.

TLR4 is the central signaling receptor for LPS in mammals. TLR4-deficient and knockout mice are hyporesponsive to LPS^{56,57} and therefore highly susceptible to certain Gram-negative bacterial infections. TLR4 requires MD-2, a secreted glycoprotein of 160 amino acids, to interact functionally with LPS⁵⁸. The efficiency of LPS-MD-2 interactions is markedly increased by pretreatment of LPS with LBP and CD14 to produce a LPS:CD14 monomeric complex⁵⁹⁻⁶². MD-2 associates with the ectodomain of TLR4 and the TLR4–MD-2 heterodimer is considered to form the entire recognition complex for LPS. Recent studies have revealed the crystal structure of MD-2, bound to two different tetra-acylated lipid A antagonists, namely Eritoran and lipid IVa^{55,63}. These studies showed that human MD-2 contains a deep hydrophobic cavity, situated between two β sheets. Interestingly, both studies showed that the four lipid chains of Eritoran and lipid IVa occupy almost all of the available space in this hydrophobic pocket of MD-2. This suggests that the agonistic hexa-acyl LPS molecule attaches to MD-2 in a different manner than the antagonistic molecules. Kim *et al.* hypothesized that structural changes of the hydrophobic pocket are required to allow binding of agonistic hexa-acyl LPS. They speculated that such changes may induce an otherwise hidden binding site which favors interaction of MD-2 with the central or C-terminal domain of a second TLR4 protein⁵⁵. As a consequence, TLR4 homodimerizes and recruits two sets of adaptor molecules required for signaling. Future research is warranted to verify the described hypothesis.

Soluble MD-2 (sMD-2)

Interestingly, only a small portion of MD-2 binds TLR4 while the majority is released by the cell as soluble MD-2 (sMD-2)^{64,65}. The MD-2 protein contains seven conserved cysteine residues that enable formation of large disulfide-linked oligomers. Interestingly, only monomeric sMD-2 associates with TLR4^{60,66,67}. This finding was further extended by a report showing that only monomeric sMD-2 is able to bind LPS⁶⁸. Moreover, Kim *et al.* showed that one MD-2 molecule binds to one TLR4 molecule, confirming that only monomeric MD-2 interacts with TLR4 and mediates LPS signaling⁵⁵.

On the search for the function of sMD-2, studies suggested that sMD-2 modulates LPS signaling during Gram-negative infections by supplementing cells that express TLR4

but lack MD-2 such as pulmonary and intestinal epithelial cells^{69,70}. This suggestion was extended by Ohnishi *et al.* who showed that LPS induces formation of CD14-TLR4 complexes on the cell surface in the absence of mMD-2 while subsequent binding of sMD-2 is essential for activation⁷¹. Moreover, sMD-2 mediates opsonization of Gram-negative bacteria by TLR4-dependent phagocytosis^{72,73}. Although a direct bacteriocidal effect was not proven by these studies, opsonization by MD-2 was shown to enhance intracellular killing. Finally, sMD-2 has been found in biological fluids such as plasma, milk, tears and urine.

Taken together, both TLR4-bound MD-2 and sMD-2 play an important role in the host defense against Gram-negative infections, making MD-2 a potential pharmacological target for drug development in LPS-related conditions such as sepsis.

Innate immunity of the gut

Immediately after birth, organs connected with the external environment like the skin and intestine are colonized by microbiota. The most diverse and abundant bacterial load is found in the gastrointestinal tract. Intestinal microbiota provide important functions to the host, including the synthesis of essential nutrients, in part by metabolism of indigestible compounds⁷⁴. In addition, gut microbiota prevent colonization of many pathogenic bacteria, a process referred to as colonization resistance⁷⁵. Finally, the gut microbiota contribute to the development of the intestinal architecture and the mammalian immune system^{74,76,77}.

The enormous and diverse number of intestinal bacteria and their proximity to host tissues raises the question how the symbiotic relationship between microbiota and the host is maintained without inducing potentially injurious immune responses.

An undesirable inflammatory response towards intestinal residents is thought to be avoided by a well organized and complex interplay between microbiota, the intestinal barrier and the innate immune system. The intestinal barrier separates the lumen that contains the intestinal microbiota from the lamina propria thereby confining the microbiota to the lumen. The intestinal barrier is formed by a single layer of enterocytes that is renewed every 4-5 days (Figure 1.3). The epithelial cells are connected by intercellular tight junctions, in order to prevent paracellular transport of macromolecules from the lumen to the underlying lamina propria⁷⁸⁻⁸⁰. In the small and large intestines goblet cells (Figure 1.3) secrete large quantities of mucin, which is composed of highly glycosylated proteins that form a thick protective layer of mucus over the surface epithelium. Besides preventing bacterial translocation, this mucus layer neutralizes stomach acid and protects against shear stress and chemical damage⁸¹.

Segregation of microbiota in the intestinal lumen is also accomplished by immunoglobulin A (IgA) which is secreted by subepithelial plasma cells and transcytosed across the epithelium into the lumen. Luminal IgA provides a barrier by

binding bacteria and other antigens. Consistently, IgA deficiency leads to increased penetration of symbiotic bacteria into host tissues^{82,83}.

In addition, antimicrobial factors prevent translocation of microbiota through the epithelial barrier. These natural antibiotics are released by different intestinal epithelial cells. Enterocytes play an active role in defending epithelial surfaces by secreting β -defensins and cathelicidins^{84,85}. Members of both families display a broad-spectrum killing activity against a wide range of bacteria, fungi, and viruses⁸⁶. In the small bowel at the base of the crypts of Lieberkühn, specialized epithelial cells called Paneth cells (Figure 1.3) are equipped with secretory granules that contain several proteins involved in host defence, including α -defensins, lysozyme, and phospholipase A₂⁸⁷. Paneth cells secrete antimicrobial peptides constitutively in order to keep intestinal crypts sterile. Moreover, exposure of Paneth cells to bacterial products such as LPS and muramyl dipeptide induces increased levels of these antimicrobial peptides⁸⁸⁻⁹¹. Recently, Paneth cell intrinsic MyD88 signaling was shown to limit penetration of luminal bacteria to tissues, disclosing a role for epithelial MyD88 in maintaining intestinal homeostasis⁹². In addition, using a Paneth cell ablation model, the same group revealed that Paneth cells are essential for controlling intestinal barrier penetration by commensal and pathogenic bacteria⁹².

Next, strategic localization of different TLR might contribute to intestinal immune tolerance to commensal bacteria. First, apical epithelial cells are virtually unresponsive to LPS due to reduced or absent expression of TLR4 on their cell surface^{93,94}. In line, Ortega-Cava *et al.* confirmed the absence of TLR4 expression on apical epithelial cells facing the gut lumen and showed that TLR4 is exclusively expressed by epithelial cells in intestinal crypts⁹⁵. Second, expression of TLR5 has been shown to be limited to the basolateral surface of enterocytes, indicating that innate immune activation can only be accomplished by invaded bacteria⁹⁶. These findings suggest that under physiological conditions, epithelial PRR are not exposed to luminal symbiotic bacteria and their products. Hence, these receptors are strategically positioned to respond to bacteria, once they have breached the epithelial barrier.

Finally, active suppression of molecules responsible for intracellular PRR, is considered to be involved in the intestinal immune tolerance to commensal microbiota. In particular, expression of Toll-interacting protein (Tollip), an intracellular protein which inhibits IRAK activation⁹⁷ correlates directly with the luminal load of microbiota *in vivo*⁹⁸. Consistently, the highest Tollip expression in the gastrointestinal tract is seen in healthy colonic mucosa, which contains the highest numbers of commensal bacteria⁹⁸. Also decreased IL-1R-associated kinase (IRAK) expression is considered to limit hyperreactivity of the healthy intestine to luminal microbiota⁹⁸. However, it can also be argued that suppression of proinflammatory responses towards the microbiota creates a problem for the host, as it renders the innate immune system incapable to elicit an adequate response when challenged by pathogens. Additional research is warranted to unravel how the intestinal immune system can respond adequately to pathogens while remaining tolerant against commensals.

Figure 1.3 Cellular composition of the small bowel. Stem cells are located at the base of the crypts where they divide, migrate and differentiate into goblet cells, enterocytes, and enteroendocrine cells (not shown) or Paneth cells. Paneth cells secrete their antimicrobial peptides in the crypt lumen to keep it sterile.

Although intestinal microbiota have beneficial roles in health as discussed above, these commensals are considered to be key players in the pathophysiology of systemic and local inflammatory disorders such as sepsis, IBD and NEC, when they breach the intestinal barrier.

Penetration of the gut barrier by luminal bacteria can occur under several non physiological conditions. For instance, splanchnic hypoperfusion such as encountered during major surgery or trauma, has been postulated as a cause of disruption of the barrier integrity⁹⁹. Another non physiological condition which is associated with a compromised barrier is prematurity. In premature infants the intestine is underdeveloped resulting in a diminished barrier function^{100,101}. Moreover, many pathogens possess virulence factors that favour adherence to and penetration of the epithelial barrier^{102,103}. During invasion of the epithelial cells lining the gut lumen, certain pathogens can cause disruption of the intestinal barrier. Translocation of bacteria or their toxins from the gut lumen into underlying intestinal layers provoke activation of inflammatory cells located in the lamina propria, in order to eliminate intruders¹⁰⁴. Proinflammatory cytokines produced by these inflammatory cells such as TNF-alpha and IFN-gamma induce degradation of intestinal integrity by breakdown of intestinal tight junction proteins, such as zonula occludens proteins¹⁰⁵⁻¹⁰⁷, resulting in increased leakage of luminal material and ensuing greater immune activation. Regardless of the cause, disruption of the intestinal barrier predisposes to translocation of microbiota and/or their products¹⁰⁸⁻¹¹⁰. Translocation of luminal bacterial sometimes results in excessive activation of the immune system. Such an

inappropriate response directed against translocated commensal microbiota is often considered to be central to the development of IBD.

Several lines of evidence indicate a fundamental role for commensal bacteria in the onset and or progression of these diseases¹¹¹⁻¹¹⁵. In particular, spontaneous colitis does not occur in IL-2, IL-10 or T-cell receptor (TCR) α/β knockout mice when maintained in a germ-free environment, but colitis develops rapidly when these mice are colonized by commensal bacteria¹¹⁶⁻¹¹⁹. Moreover, rodents with chemically induced disruption of the intestinal barrier develop inflammation of the gut in a pathogen-free or conventional environment, but not in a germ-free environment¹²⁰. In line, treatment with antibiotics has been shown to reduce intestinal inflammation and inflammatory responses, not only in experimental models of colitis but also in a subset of patients with IBD^{121,122}.

Supporting evidence for a role of bacteria in the pathogenesis of NEC is found in the fact that the onset of the disease occurs at a time when the intestinal lumen is colonized by the gut microbiota^{123,124}, which usually occurs at 8–10 days after birth. In concordance with observations in IBD patients, administration of broad-spectrum antibiotic therapy reduces inflammatory responses in patients suffering NEC^{125,126}. Taken together, these data indicate that intestinal inflammatory disorders such as IBD and NEC result from a dysregulated intestinal immune response to the host microbiota, once these commensals pass the compromised gut barrier. Considering the crucial role of microbiota in the pathogenesis of IBD and NEC, it is tempting to speculate that the onset or exacerbation of exaggerated immune activation, that characterizes these diseases may be at least in part, mediated by uncontrolled TLR signaling.

Therefore, data concerning the regulation and cellular distribution of TLR family members in the healthy and inflamed gut are of interest. Different groups investigated TLR localization and regulation in patients with IBD and healthy controls. Constitutive TLR3 expression was significantly down-regulated in active Crohn's disease but not in ulcerative colitis^{127,128}. In contrast TLR2 and TLR4 which are barely or even undetectable in non-diseased mucosa are upregulated in both Crohn's disease (CD) and ulcerative colitis (UC)^{127,128}. In addition, IBD patients showed an inflammation dependent increase of TLR2 expression on lamina propria monocytes^{127,128}.

Reports on TLR4 localisation in the inflamed human gut are controversial. In UC and CD patients, increased TLR4 protein expression as assessed by immunohistochemistry was predominantly observed at epithelial cells lining the lumen and at some lamina propria-located inflammatory cells¹²⁷. Hausmann *et al.* confirmed the presence of TLR4-expressing macrophages in the lamina propria but failed to detect TLR4 expression by primary epithelial cells¹²⁸. Thus, the debate regarding TLR expression by epithelial cells during health and disease is still open.

Collectively, more insight into the complex interplay between the gut microbiota, the intestinal barrier and the innate immune system is warranted. Such insight might help to develop therapeutic interventions to dampen excessive immune activation while maintaining immunoprotection.

TLR and endogenous ligands

Besides their crucial role in recognition and sensing of microbial molecular structures¹²⁹, TLR are also considered to be involved in the response to ligands, released by dying or damaged cells^{130,131}. Although direct binding between TLR and these so called damage-associated molecular patterns (DAMPs) remains to be proven, there is accumulating evidence that TLR play a role in sensing of endogenous ligands. Examples of such host molecules associated with TLR mediated immune activation include heat-shock proteins (engaging with TLR2 and -4), the nonhistone chromatin-binding protein high-mobility group box 1 (HMGB1) (binding with TLR2 and -4), and extracellular matrix breakdown components such as hyaluronan (ligand for TLR2 and -4), fibronectin (engaging with TLR4) and heparan sulfate (engaging with TLR4)^{130,132-135}. Release of DAMPs is for example seen following hypoxia and ischemia. Consistently, an important role for TLR2 and TLR4 in the induction of a sterile inflammatory immune response in ischemic organs has been frequently reported¹³⁶⁻¹⁴⁰. The absence or inhibition of TLR2 or TLR4 has been shown to decrease tissue injury and improve organ function in response to ischemia in the liver, brain, heart, lung and kidney^{137,141-151}.

The demise of cells can occur by autophagy, necrosis, apoptosis or intermediate forms of the latter types. Cells undergoing death associated with autophagy are characterized by the presence of double membrane autophagic vacuoles. Numerous studies have described autophagy as a survival mechanism under poor nutritional or growth factor conditions. When cellular stress continues, cell death may proceed by autophagy alone. In addition, it often becomes associated with features of apoptotic or necrotic cell death¹⁵².

Necrotic cell death is accompanied by cell swelling and loss of plasma membrane integrity which results in the passive release of intracellular inflammatory mediators¹⁵³. Until recently, necrosis was considered as an unintended and unprogrammed form of cell death lacking underlying signaling events. This might be true for cell death resulting from severe physical damage or after exposure to high concentrations of detergents, oxidants or ionophores. However, increasing evidence support the existence of tightly regulated caspase-independent forms of cell death, leading to rupture of plasma membranes^{154,155}.

Apoptosis is an active process, executed by a family of cysteine proteases, called caspases¹⁵⁶. Two major pathways of caspase activation have been described, namely the extrinsic and intrinsic pathway¹⁵⁷. The extrinsic pathway activates caspase-8, while

the intrinsic pathway activates caspase-9. Each of these proteases can activate caspase-3, which is primarily responsible for cell disassembly by cleaving proteins that are needed for normal cell maintenance. Activation of the extrinsic pathway is mediated by binding of extracellular ligands to cell surface death receptors¹⁵⁸. The intrinsic pathway converges at the mitochondrial level^{159,160}, and this pathway is controlled by proteins of the B-cell lymphoma protein 2 (Bcl-2) family, that include pro-apoptotic members such as Bax and anti-apoptotic members including Bcl-2 and Bcl-XL^{161,162}.

Cells dying apoptotically are characterized by plasma membrane blebbing, nuclear and cytoplasmic condensation and formation of so called membrane-bound apoptotic bodies^{163,164}. In apoptotic cells, the membrane and cytoplasmic organelles remain intact, and cells are rapidly phagocytosed by macrophages or surrounding cells before lysis¹⁶⁵ in order to prevent leakage of cellular contents. In line, multiple studies reported that the release of DAMPs is triggered by necrotic but not by apoptotic cell death¹⁶⁶⁻¹⁷¹.

However, it should be noted that a number of recent publications showed that the release of intracellular endogenous ligands is not solely a passive process that only occurs once the cell membrane of necrotic cells becomes disrupted. In particular, work from Keller *et al.* provided evidence that the release of “danger” proteins can be mediated by a caspase-1 dependent, active process¹⁷². In line, with this hypothesis, stimulation of macrophages with LPS resulted in the secretion of HMGB1, apparently in the absence of necrosis¹⁷³. In addition, it has recently been demonstrated that accumulation of apoptotic cells can stimulate macrophages to secrete HMGB1¹⁷⁴. The latter protein appeared to have a role, downstream of apoptosis, in the development of organ damage in severe sepsis¹⁷⁴. Collectively, these studies challenge the paradigm that only necrosis is accompanied by an inflammatory response, whereas apoptotic cell death is immunologically and inflammatorily silent.

Consistent with these studies, work from our and other laboratories demonstrated that tubular cell apoptosis acts as a primary and major contributor to the pathophysiology of renal ischemia/reperfusion (I/R) and determines the outcome of renal damage¹⁷⁵⁻¹⁷⁷.

Aim of this thesis

As discussed above, the innate immune system is involved in infectious and sterile inflammation by mediating responses to both bacterial toxins and damaged tissues. Inappropriate immune activation has been associated with surgically relevant diseases including sepsis, IBD, NEC and ischemia-reperfusion injury. Although evidence is increasing that TLR are actively involved in the pathophysiology of these diseases, many questions including the regulation and cellular distribution of these TLR in health and disease remain unanswered. Therefore, the main purpose of this thesis is to provide insight into aspects of TLR biology in surgical patients. In the first part of this thesis, studies on sterile inflammation after ischemia reperfusion (I/R) injury are described (chapter 2 & 3). Renal I/R injury is seen during major surgery and transplantation surgery in particular. I/R plays an important role in acute end stage organ failure. As TLR are considered to be involved in mediating sterile inflammation in the course of I/R, the distribution and regulation of TLR2 and TLR4 was explored in the kidney following ischemia reperfusion (chapter 2). In chapter 3, the degree of ischemic injury in human kidneys was assessed by analyzing the regulation and processing of apoptotic proteins during normothermic ischemia.

In the second part of this thesis, studies on bacterial inflammation are presented. During surgery, reduced splanchnic blood flow is considered to promote translocation of luminal gut microbiota (mainly Gram-negative Enterobacteriaceae) across the intestinal epithelial barrier, thereby acting as a source of sepsis at distant sites of the gut. In this context, the molecular appearance, regulation and TLR4 costimulatory activity of native human sMD-2 in control and septic plasma was analyzed (chapter 4). In addition, the *in vivo* distribution of human MD-2 and the potential cellular sources of circulating sMD-2 during endotoxemia and sepsis were studied (chapter 4). Epidemiological observations and animal studies suggest an important role for Gram-negative organisms in the development of intestinal inflammatory pathology in preterms and adults. In this context, the localization and regulation of MD-2 was studied in the human healthy and inflamed adult and premature gut (chapter 5). In chapter 6, the functional consequences of luminal endotoxin exposure of the premature and mature gut were studied in an experimental animal model.

References

1. Nathan C. Points of control in inflammation. *Nature* 2002;420:846-852.
2. Kroemer G, Zitvogel L. Death, danger, and immunity: an infernal trio. *Immunol Rev* 2007;220:5-7.
3. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.
4. Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. *Nat Rev Genet* 2001;2:256-267.
5. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003;3:710-720.
6. Boyton RJ, Openshaw PJ. Pulmonary defences to acute respiratory infection. *Br Med Bull* 2002;61:1-12.
7. Hiemstra PS. The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res* 2007;33:537-542.
8. Pangburn MK, Muller-Eberhard HJ. Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. *J Exp Med* 1980;152:1102-1114.
9. Lachmann PJ, Hughes-Jones NC. Initiation of complement activation. *Springer Semin Immunopathol* 1984;7:143-162.
10. Law SK, Dodds AW. C3, C4 and C5: the thioester site. *Biochem Soc Trans* 1990;18:1155-1159.
11. Sjöberg AP, Trouw LA, Blom AM. Complement activation and inhibition: a delicate balance. *Trends Immunol* 2009;30:83-90.
12. Jack DL, Klein NJ, Turner MW. Mannose-binding lectin: targeting the microbial world for complement attack and opsonophagocytosis. *Immunol Rev* 2001;180:86-99.
13. Mold C. Role of complement in host defense against bacterial infection. *Microbes Infect* 1999;1:633-638.
14. Chen G, Shaw MH, Kim YG, Nunez G. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol* 2009;4:365-398.
15. Carneiro LA, Travassos LH, Girardin SE. Nod-like receptors in innate immunity and inflammatory diseases. *Ann Med* 2007;39:581-593.
16. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Memet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* 2004;5:1166-1174.
17. Benko S, Philpott DJ, Girardin SE. The microbial and danger signals that activate Nod-like receptors. *Cytokine* 2008;43:368-373.
18. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature* 2006;442:39-44.
19. Mariathasan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol* 2007;7:31-40.
20. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911-1912.
21. Le Bourhis L, Benko S, Girardin SE. Nod1 and Nod2 in innate immunity and human inflammatory disorders. *Biochem Soc Trans* 2007;35:1479-1484.
22. Franchi L, Park JH, Shaw MH, Marina-Garcia N, Chen G, Kim YG, Nunez G. Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell Microbiol* 2008;10:1-8.
23. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783-801.
24. Baccala R, Hoebe K, Kono DH, Beutler B, Theofilopoulos AN. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med* 2007;13:543-551.
25. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guiet C, Briere F, Vlach J, Lebecque S, Trinchieri G, Bates EE. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* 2005;174:2942-2950.
26. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004;303:1522-1526.
27. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 2007;7:353-364.

28. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 2002;169:6668-6672.
29. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 2003;4:161-167.
30. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003;301:640-643.
31. Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci* 2002;27:474-482.
32. Beutler B. Innate immunity: an overview. *Mol Immunol* 2004;40:845-859.
33. Beutler B, Hoebe K, Georgel P, Tabeta K, Du X. Genetic analysis of innate immunity: identification and function of the TIR adapter proteins. *Adv Exp Med Biol* 2005;560:29-39.
34. Kawai T, Akira S. Pathogen recognition with Toll-like receptors. *Curr Opin Immunol* 2005;17:338-344.
35. Zingarelli B. Nuclear factor-kappaB. *Crit Care Med* 2005;33:S414-416.
36. Papadimitraki ED, Bertsias GK, Boumpas DT. Toll like receptors and autoimmunity: a critical appraisal. *J Autoimmun* 2007;29:310-318.
37. Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S. Toll-like receptor signal transduction. *Exp Mol Med* 2007;39:421-438.
38. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-680.
39. Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. *Nature* 2005;438:364-368.
40. Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J Exp Med* 2007;204:3095-3101.
41. Xu D, Komai-Koma M, Liew FY. Expression and function of Toll-like receptor on T cells. *Cell Immunol* 2005;233:85-89.
42. O'Neill LA. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* 2008;29:12-20.
43. Kufer TA, Sansonetti PJ. Sensing of bacteria: NOD a lonely job. *Curr Opin Microbiol* 2007;10:62-69.
44. Uehara A, Sugawara Y, Kurata S, Fujimoto Y, Fukase K, Kusumoto S, Satta Y, Sasano T, Sugawara S, Takada H. Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via toll-like receptors, NOD1 and NOD2 in human oral epithelial cells. *Cell Microbiol* 2005;7:675-686.
45. van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BM, Mengin-Lecreux D, Playford RJ. Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol* 2005;35:2471-2476.
46. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 2004;5:800-808.
47. Hawlisch H, Kohl J. Complement and Toll-like receptors: key regulators of adaptive immune responses. *Mol Immunol* 2006;43:13-21.
48. Rietschel ET, Brade L, Brandenburg K, Flad HD, de Jong-Leuveninck J, Kawahara K, Lindner B, Loppnow H, Luderitz T, Schade U, et al. Chemical structure and biologic activity of bacterial and synthetic lipid A. *Rev Infect Dis* 1987;9 Suppl 5:S527-536.
49. Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR. Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* 2004;53:735-740.
50. Caroff M, Karibian D, Cavaillon JM, Haeffner-Cavaillon N. Structural and functional analyses of bacterial lipopolysaccharides. *Microbes Infect* 2002;4:915-926.
51. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635-700.
52. Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. *Microbes Infect* 2002;4:837-851.
53. Darveau RP. Lipid A diversity and the innate host response to bacterial infection. *Curr Opin Microbiol* 1998;1:36-42.
54. Brandenburg K, Wiese A. Endotoxins: relationships between structure, function, and activity. *Curr Top Med Chem* 2004;4:1127-1146.

55. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ, Lee JO. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* 2007;130:906-917.
56. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999;162:3749-3752.
57. Qureshi ST, Gros P, Malo D. The Lps locus: genetic regulation of host responses to bacterial lipopolysaccharide. *Inflamm Res* 1999;48:613-620.
58. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;189:1777-1782.
59. Tobias PS, Soldau K, Ulevitch RJ. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J Biol Chem* 1989;264:10867-10871.
60. Visintin A, Latz E, Monks BG, Espevik T, Golenbock DT. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J Biol Chem* 2003;278:48313-48320.
61. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431-1433.
62. Gioannini TL, Teghanemt A, Zhang D, Levis EN, Weiss JP. Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. *J Endotoxin Res* 2005;11:117-123.
63. Ohto U, Fukase K, Miyake K, Satow Y. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. *Science* 2007;316:1632-1634.
64. Schromm AB, Lien E, Henneke P, Chow JC, Yoshimura A, Heine H, Latz E, Monks BG, Schwartz DA, Miyake K, Golenbock DT. Molecular genetic analysis of an endotoxin nonresponder mutant cell line: a point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. *J Exp Med* 2001;194:79-88.
65. Henneke P, Golenbock DT. Innate immune recognition of lipopolysaccharide by endothelial cells. *Crit Care Med* 2002;30:S207-213.
66. Re F, Strominger JL. Monomeric recombinant MD-2 binds toll-like receptor 4 tightly and confers lipopolysaccharide responsiveness. *J Biol Chem* 2002;277:23427-23432.
67. Viriyakosol S, Tobias PS, Kirkland TN. Mutational analysis of membrane and soluble forms of human MD-2. *J Biol Chem* 2006;281:11955-11964.
68. Visintin A, Halmen KA, Latz E, Monks BG, Golenbock DT. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. *J Immunol* 2005;175:6465-6472.
69. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB, Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L428-437.
70. Lenoir C, Sapin C, Broquet AH, Jouniaux AM, Bardin S, Gasnereau I, Thomas G, Seksik P, Trugnan G, Masliah J, Bachelet M. MD-2 controls bacterial lipopolysaccharide hyporesponsiveness in human intestinal epithelial cells. *Life Sci* 2008;82:519-528.
71. Ohnishi T, Muroi M, Tanamoto K. The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. *FEMS Immunol Med Microbiol* 2007;51:84-91.
72. Tissieres P, Dunn-Siegrist I, Schappi M, Elson G, Comte R, Nobre V, Pugin J. Soluble MD-2 is an acute-phase protein and an opsonin for Gram-negative bacteria. *Blood* 2008;111:2122-2131.
73. Jain V, Halle A, Halmen KA, Lien E, Charrel-Dennis M, Ram S, Golenbock DT, Visintin A. Phagocytosis and intracellular killing of MD-2 opsonized gram-negative bacteria depend on TLR4 signaling. *Blood* 2008;111:4637-4645.
74. Hooper LV, Gordon JL. Commensal host-bacterial relationships in the gut. *Science* 2001;292:1115-1118.
75. van der Waaij D, Berghuis-de Vries JM, Lekkerkerk L-v. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J Hyg (Lond)* 1971;69:405-411.
76. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 2004;4:478-485.
77. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009;9:313-323.

78. Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, Eastburn KK, Madara JL. Tight junctions are membrane microdomains. *J Cell Sci* 2000;113:1771-1781.
79. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of the apical junctional complex: mechanisms and possible roles in regulation of epithelial barriers. *Bioessays* 2005;27:356-365.
80. Viswanathan VK, Hecht G. Innate immunity and the gut. *Curr Opin Gastroenterol* 2000;16:546-551.
81. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. *Am J Physiol* 1991;260:C183-193.
82. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 2000;288:2222-2226.
83. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 2004;303:1662-1665.
84. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 2003;124:993-1000.
85. Wehkamp J, Schaubert J, Stange EF. Defensins and cathelicidins in gastrointestinal infections. *Curr Opin Gastroenterol* 2007; 23:32-38.
86. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389-395.
87. Ouellette AJ. Paneth cell alpha-defensin synthesis and function. *Curr Top Microbiol Immunol* 2006;306:1-25.
88. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 2000;1:113-118.
89. Ouellette AJ, Bevins CL. Paneth cell defensins and innate immunity of the small bowel. *Inflamm Bowel Dis* 2001;7:43-50.
90. Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted Paneth cell. *Cell Mol Life Sci* 2002;59:156-170.
91. Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 2003;422:522-526.
92. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 2008;105:20858-20863.
93. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol* 2001;167:1609-1616.
94. Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, Zhou Y, Hu B, Arditi M, Abreu MT. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* 2003;170:1406-1415.
95. Ortega-Cava CF, Ishihara S, Rumi MA, Kawashima K, Ishimura N, Kazumori H, Udagawa J, Kadowaki Y, Kinoshita Y. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. *J Immunol* 2003;170:3977-3985.
96. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 2001;167:1882-1885.
97. Burns K, Clatworthy J, Martin L, Martinon F, Plumpton C, Maschera B, Lewis A, Ray K, Tschopp J, Volpe F. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat Cell Biol* 2000;2:346-351.
98. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* 2004;126:1054-1070.
99. Rahman SH, Ammori BJ, Holmfield J, Larvin M, McMahon MJ. Intestinal hypoperfusion contributes to gut barrier failure in severe acute pancreatitis. *J Gastrointest Surg* 2003;7:26-35; discussion 35-26.
100. Axelsson I, Jakobsson I, Lindberg T, Polberger S, Benediktsson B, Raiha N. Macromolecular absorption in preterm and term infants. *Acta Paediatr Scand* 1989;78:532-537.
101. Rouwet EV, Heineman E, Buurman WA, ter Riet G, Ramsay G, Blanco CE. Intestinal permeability and carrier-mediated monosaccharide absorption in preterm neonates during the early postnatal period. *Pediatr Res* 2002;51:64-70.

102. Galan JE. Molecular genetic bases of Salmonella entry into host cells. *Mol Microbiol* 1996;20:263-271.
103. Zhou D, Galan J. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect* 2001;3:1293-1298.
104. Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest* 2004;84:282-291.
105. Chakravorty D, Kumar KS. Modulation of barrier function of small intestinal epithelial cells by lamina propria fibroblasts in response to lipopolysaccharide: possible role in TNF α in inducing barrier dysfunction. *Microbiol Immunol* 1999;43:527-533.
106. Ferrier L, Mazelin L, Cenac N, Desreumaux P, Janin A, Emilie D, Colombel JF, Garcia-Villar R, Fioramonti J, Bueno L. Stress-induced disruption of colonic epithelial barrier: role of interferon-gamma and myosin light chain kinase in mice. *Gastroenterology* 2003;125:795-804.
107. Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM. TNF- α -induced increase in intestinal epithelial tight junction permeability requires NF- κ B activation. *Am J Physiol Gastrointest Liver Physiol* 2004;286:G367-376.
108. Meddings JB, Sutherland LR, May GR. Intestinal permeability in patients with Crohn's disease. *Gut* 1994;35:1675-1676.
109. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, Riecken EO, Schulzke JD. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology* 1999;116:301-309.
110. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. *Shock* 2007;27:124-133.
111. Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002;347:417-429.
112. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002;20:495-549.
113. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet* 2006;368:1271-1283.
114. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004;126:1620-1633.
115. Meddings J. The significance of the gut barrier in disease. *Gut* 2008;57:438-440.
116. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 1993;75:253-261.
117. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 1998;66:5224-5231.
118. Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC, Owen MJ. T cell receptor- α β -deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol* 1997;150:91-97.
119. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263-274.
120. Rath HC, Schultz M, Freitag R, Dieleman LA, Li F, Linde HJ, Scholmerich J, Sartor RB. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infect Immun* 2001;69:2277-2285.
121. Card T, Logan RF, Rodrigues LC, Wheeler JG. Antibiotic use and the development of Crohn's disease. *Gut* 2004;53:246-250.
122. Isaacs KL, Sartor RB. Treatment of inflammatory bowel disease with antibiotics. *Gastroenterol Clin North Am* 2004;33:335-345.
123. Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL. Enteric colonization in sporadic neonatal necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 1985;4:591-595.
124. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *FASEB J* 2001;15:1398-1403.
125. Bury RG, Tudehope D. Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. *Cochrane Database Syst Rev* 2001;CD000405.
126. Krediet TG, van Lelyveld N, Vijlbrief DC, Brouwers HA, Kramer WL, Fleeer A, Gerards LJ. Microbiological factors associated with neonatal necrotizing enterocolitis: protective effect of early antibiotic treatment. *Acta Paediatr* 2003;92:1180-1182.

127. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 2000;68:7010-7017.
128. Hausmann M, Kiessling S, Mestermann S, Webb G, Spottl T, Andus T, Scholmerich J, Herfarth H, Ray K, Falk W, Rogler G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* 2002;122:1987-2000.
129. Medzhitov R, Janeway C, Jr. Innate immune recognition: mechanisms and pathways. *Immunol Rev* 2000;173:89-97.
130. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006;6:823-835.
131. Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 2007;19:3-10.
132. Vabulas RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, Hacker H, Wagner H. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem* 2001;276:31332-31339.
133. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem* 2002;277:15107-15112.
134. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, Malle E, Schaefer RM, Grone HJ. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J Clin Invest* 2005;115:2223-2233.
135. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 2006;26:174-179.
136. Maslinska D, Laure-Kamionowska M, Maslinski S. Toll-like receptors in rat brains injured by hypoxic-ischaemia or exposed to staphylococcal alpha-toxin. *Folia Neuropathol* 2004;42:125-132.
137. Shen XD, Ke B, Zhai Y, Gao F, Busuttill RW, Cheng G, Kupiec-Weglinski JW. Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. *Am J Transplant* 2005;5:1793-1800.
138. Hua F, Ha T, Ma J, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, Li C. Blocking the MyD88-dependent pathway protects the myocardium from ischemia/reperfusion injury in rat hearts. *Biochem Biophys Res Commun* 2005;338:1118-1125.
139. O'Neill LA. TLRs play good cop, bad cop in the lung. *Nat Med* 2005;11:1161-1162.
140. Frost RA, Nystrom GJ, Lang CH. Multiple Toll-like receptor ligands induce an IL-6 transcriptional response in skeletal myocytes. *Am J Physiol Regul Integr Comp Physiol* 2006;290:R773-784.
141. Oyama J, Blais C, Jr., Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T. Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. *Circulation* 2004;109:784-789.
142. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest* 2005;115:2894-2903.
143. Shigeoka AA, Holscher TD, King AJ, Hall FW, Kiosses WB, Tobias PS, Mackman N, McKay DB. TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J Immunol* 2007;178:6252-6258.
144. Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, Stolz DB, Geller DA, Rosengart MR, Billiar TR. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med* 2007;204:2913-2923.
145. Sakata Y, Dong JW, Vallejo JG, Huang CH, Baker JS, Tracey KJ, Tacheuchi O, Akira S, Mann DL. Toll-like receptor 2 modulates left ventricular function following ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2007;292:H503-509.
146. Shimamoto A, Pohlman TH, Shomura S, Tarukawa T, Takao M, Shimpo H. Toll-like receptor 4 mediates lung ischemia-reperfusion injury. *Ann Thorac Surg* 2006;82:2017-2023.
147. Shimamoto A, Chong AJ, Yada M, Shomura S, Takayama H, Fleisig AJ, Agnew ML, Hampton CR, Rothnie CL, Spring DJ, Pohlman TH, Shimpo H, Verrier ED. Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury. *Circulation* 2006;114:1270-274.
148. Hua F, Ma J, Ha T, Xia Y, Kelley J, Williams DL, Kao RL, Browder IW, Schweitzer JB, Kalbfleisch JH, Li C. Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. *J Neuroimmunol* 2007;190:101-111.

149. Ziegler G, Harhausen D, Schepers C, Hoffmann O, Rohr C, Prinz V, König J, Lehrach H, Niefeld W, Trendelenburg G. TLR2 has a detrimental role in mouse transient focal cerebral ischemia. *Biochem Biophys Res Commun* 2007;359:574-579.
150. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. *Biochem Biophys Res Commun* 2007;353:509-514.
151. Pulsikens WP, Teske GJ, Butter LM, Roelofs JJ, van der Poll T, Florquin S, Leemans JC. Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS ONE* 2008;3:e3596.
152. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007;8:741-752.
153. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci* 2007;32:37-43.
154. Zong WX, Thompson CB. Necrotic death as a cell fate. *Genes Dev* 2006;20:1-15.
155. Duprez L, Wirawan E, Berghe TV, Vandenabeele P. Major cell death pathways at a glance. *Microbes Infect* 2009.
156. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;281:1312-1316.
157. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269-290.
158. Saikumar P, Venkatachalam MA. Role of apoptosis in hypoxic/ischemic damage in the kidney. *Semin Nephrol* 2003;23:511-521.
159. Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000;6:513-519.
160. Adrain C, Martin SJ. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* 2001;26:390-397.
161. Bernardi P, Petronilli V, Di Lisa F, Forte M. A mitochondrial perspective on cell death. *Trends Biochem Sci* 2001;26:112-117.
162. Antonsson B. Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. *Cell Tissue Res* 2001;306:347-361.
163. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-257.
164. Wyllie AH. Apoptosis: an overview. *Br Med Bull* 1997;53:451-465.
165. Platt N, da Silva RP, Gordon S. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol* 1998;8:365-372.
166. Silverberg SJ, Shane E, Jacobs TP, Siris E, Bilezikian JP. A 10-year prospective study of primary hyperparathyroidism with or without parathyroid surgery. *N Engl J Med* 1999;341:1249-1255.
167. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 2000;12:1539-1546.
168. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 2000;191:423-434.
169. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002;418:191-195.
170. Helena Erlandsson Harris AR. Alarmin(g) news about danger: Workshop on Innate Danger Signals and HMGB1. *EMBO reports* 2006;8:774-778.
171. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tor M, Billiar T. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev* 2007; 220:60-81.
172. Keller M, Ruegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 2008;132:818-831.
173. Chen G, Li J, Ochani M, Rendon-Mitchell B, Qiang X, Susarla S, Ulloa L, Yang H, Fan S, Goyert SM, Wang P, Tracey KJ, Sama AE, Wang H. Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14- and TNF-dependent mechanisms. *J Leukoc Biol* 2004;76:994-1001.
174. Qin S, Wang H, Yuan R, Li H, Ochani M, Ochani K, Rosas-Ballina M, Czura CJ, Huston JM, Miller E, Lin X, Sherry B, Kumar A, Larosa G, Newman W, Tracey KJ, Yang H. Role of HMGB1 in apoptosis-mediated sepsis lethality. *J Exp Med* 2006;203:1637-1642.

175. Daemen MA, van 't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenabeele P, Buurman WA. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest* 1999;104:541-549.
176. Bonegio R, Lieberthal W. Role of apoptosis in the pathogenesis of acute renal failure. *Curr Opin Nephrol Hypertens* 2002;11:301-308.
177. Padanilam BJ. Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. *Am J Physiol Renal Physiol* 2003;284:F608-627.

Chapter 2

In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN- γ and TNF- α mediated up-regulation during inflammation

Tim GAM Wolfs, Wim A Buurman, Annemarie van Schadewijk, Bart de Vries,
Marc ARC Daemen, Pieter S Hiemstra, Cornelis van 't Veer

The Journal of Immunology 2002;168:1286-1293

Abstract

The reported requirement of functional TLR4 for resistance to Gram-negative pyelonephritis prompted us to localize the expression of TLR2 and TLR4 mRNA in the kidney at the cellular level by in situ hybridization. The majority of the constitutive TLR2 and TLR4 mRNA expression was found to be strategically located in the renal epithelial cells.

Assuming that the TLR mRNA expression is representative of apical protein expression, this suggests that these cells are able to detect and react with bacteria present in the lumen of the tubules.

To gain insight in the regulation of TLR expression during inflammation, we employed a model for renal inflammation. Renal inflammation evoked by ischemia markedly enhanced synthesis of TLR2 and TLR4 mRNA in the distal tubular epithelium, the thin limb of Henle's loop and collecting ducts. The increased renal TLR4 mRNA expression was associated with significant elevation of renal TLR4 protein expression as evaluated by Western blotting. Using RT-PCR, the enhanced TLR2 and TLR4 mRNA expression was shown to be completely dependent on the action of IFN- γ and TNF- α . These results indicate a potential mechanism of increased immunosurveillance during inflammation at the site in which ascending bacteria enter the kidney tissue, i.e. the collecting ducts and the distal part of the nephron.

Introduction

At least two members of the Toll-like receptor (TLR) family are involved in the innate defense mechanism against bacterial infections^{1,2}. TLR4 recognizes Gram-negative bacteria via the lipopolysaccharide moiety that is present in the surface of these microorganisms³⁻⁵. Another member of this receptor family, TLR2, induces responsiveness to bacterial lipoproteins and components of Gram-positive bacteria such as peptidoglycan, indicating the involvement of TLR2 in the resolution of infections⁶⁻⁹. After stimulation by bacterial products both receptors trigger the cell to produce inflammatory mediators. This process is mediated via a MyD88 dependent intracellular signaling pathway that causes translocation of NF- κ B. The latter induces the transcription of the genes encoding for cytokines, chemokines and adhesion molecules crucial to the inflammatory process aimed at clearance of invading bacteria^{1,10}.

Both TLR2 and TLR4 are predominantly expressed by monocytes/macrophages and neutrophils¹¹. Lower expression of TLR is observed *in vitro* by several other leukocytes, endothelial cells, epithelial cells and fibroblasts¹²⁻¹⁶. *In vitro* experiments indicate that TLR expression is modulated by bacterial products and cytokines¹¹. Accordingly, an IFN- γ responsive element is found in the promoter region of the gene encoding for TLR4¹⁷. Although the above indicates putative pathways that regulate TLR expression, the factors which mediate TLR expression *in vivo* remain to be elucidated.

Functional TLR4 expression is required for the resistance to experimental pyelonephritis induced by a bladder inoculum with Gram-negative bacteria. This is evidenced by the persistence of Gram-negative bacteria in the kidney after pyelonephritis induction in C3H/HeJ mice¹⁸ that carry a malfunctioning TLR4³, while mice bearing intact TLR4 rapidly clear bacteria from the urinary tract. The cellular origin of TLR expression in the kidney responsible for resistance to bacteria is unknown. Considering that the kidney is an important port of entry for bacteria, we set out to localize TLR expression in the kidney and to study the role of cytokines in the regulation of TLR expression *in vivo*. We used a renal ischemia/reperfusion (I/R) model that leads to transient tissue damage that is associated with an inflammatory process that develops rapidly during reperfusion¹⁹. The enhanced expression of MIP-2, KC, TNF- α , IFN- γ , and MHC class I and II molecules in this renal sterile inflammation model^{20,21,22} resembles the pattern of production of immunological mediators after bacterial infections to a large extent. This model enabled us to study the modulation of the expression of TLR2 and TLR4 mRNA by cytokines *in vivo* during inflammation.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-murine IFN- γ mAb F3 and anti-murine TNF- α mAb TN3 were kindly provided by HBT (Uden, The Netherlands) and Celltech (Slough, United Kingdom) respectively; polyclonal rabbit anti-murine TLR4 serum was kindly provided by Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA); peroxidase-conjugated goat anti-rat IgG and peroxidase conjugated goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA); anti-Tamm Horsfall protein was from (Cappel, Durham, NC, sheep-anti-dig/alkaline phosphatase was from Roche (Basel, Switzerland) and biotinylated rabbit anti-goat IgG was from Dako (Glostrup, Denmark). Other reagents were proteinase K (Life technologies, Paisley, United Kingdom), dextran sulfate (Pharmacia, Uppsala, Sweden), streptABComplex/AP (Dako), polyvinylalcohol (PVA), formamide and β -mercaptoethanol (Merck, Darmstadt, Germany), dithiothreitol (DTT) and salmon sperm DNA (Sigma, St. Louis, MO), T7 and SP6 RNA polymerase, tRNA, Nitro Blue Tetrazolium (NBT) and bicholyindolyl phosphate (BCIP) (Boehringer, Mannheim, Germany).

Animal model and protocol

All experiments were approved by the Institutional Animal Care Committee of the University of Maastricht (Maastricht, The Netherlands). Renal ischemia was induced as described^{20,21}. In brief, male Swiss mice (Charles River Breeding Laboratories, Heidelberg, Germany) were anesthetized and body temperature was maintained at 39°C. After laparotomy ischemia was induced by clamping the left renal pedicle for 45 minutes during which the wound was covered. Subsequent to removal of the clamp, the contralateral kidney was removed and stored. After closing the abdomen, mice were supplemented with prewarmed PBS to maintain fluid balance. The animals were sacrificed at one and six hours as well as 1, 3 and 5 days after ischemia. The experimental left kidney was harvested immediately and divided into specimens for assays described below. Mice subjected to ischemia were treated 10 minutes before reperfusion with 0.5 ml of PBS i.p. containing either 300 μ g of anti-IFN- γ mAb F3, or 1 mg of anti-TNF- α mAb TN3 or PBS only. Previously we have shown that mice receiving an isotype-matched control Ab did not differ in inflammatory parameters or renal injury when compared with the ischemia/PBS control group^{20,21}, indicating the specific effects of the respective antibodies.

In situ hybridization

Oligonucleotide primers were designed for the specific PCR amplification of a fragment of murine TLR2 and TLR4. The TLR2 primers were 5'-TCT GGG CAG TCT TGA ACA TTT-3', (sense primer) and 5'-AGA GTC AGG TGA TGG ATG TCG-3' (antisense primer), yielding a 321-bp fragment. The TLR4 primers were 5'-GCA ATG TCT CTG GCA

GGT GTA-3' (sense primer) and 5'-CAA GGG ATA AGA ACG CTG AGA-3' (antisense primer), yielding a product of 406-bp (all primers were synthesized by Eurogentec, Seraing, Belgium).

TLR2 and TLR4 cDNA fragments were amplified from murine kidney cDNA prepared from total RNA by reverse transcription (RT) as described below. The fragments were both TA-cloned into pGEM-Teasy (Promega, Madison, WI). All clones obtained, contained the correct TLR sequences that were evaluated using the Big Dye termination cycle sequencing kit (Perkin Elmer/Cetus, Emeryville, CA), according to the manufacturer's instructions. DH5- α competent cells were transformed by heat-shock procedures. Subsequently, clones containing plasmids with the TLR insert were selected, isolated and purified using the Qiafilter Plasmid midi kit (Qiagen, Hilden, Germany). Sense probes were prepared after linearization of the plasmid with NcoI and transcription with SP6 polymerase, antisense probes were prepared from the plasmid after linearisation with SpeI by transcription with T7 polymerase. cRNA probes were labeled by incorporation of digoxigen-labeled UTP following the manufacturers protocol (Boehringer). In situ hybridization was performed as described by de Boer *et al.*²³ using 3 μ m paraffin sections placed on coated slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany). Briefly, after prewarming the sections and subsequent rehydration with an decreasing xylene and ethanol gradient, the sections were hybridized with 30 ng probe in 300 μ l for 16 hours. The hybridization was performed at 50°C for TLR2 and at 55°C for TLR4 using a solution containing 50% formamide, 1 mg/ml tRNA, 10% dextran sulfate, 10 mM DTT, 0.25 mg/ml salmon sperm DNA and 4x standard saline citrate (SSC). Subsequently, sections were first washed in 2x SSC with 50% formamide at 37°C, secondly in 0.1x SSC with 20 mM β -mercaptoethanol at 42°C and finally, sections were treated with 100 U/ml RNase T1 in 2x SSC with 1 mM EDTA at 37°C. Digoxigen-labeled hybrids were detected with alkaline phosphatase conjugated sheep-anti-digoxigenin using NBT as chromogen and BCIP as coupling agent. Polyvinylalcohol was used to enhance the signal.

Evaluation of mRNA levels by RT-PCR

For RT-PCR total RNA was extracted from kidneys using the SV Total RNA isolation system (Promega) and treated with RQ1 RNase-Free DNase (Promega). Total RNA was reverse transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Life technologies) according to the supplier's recommendations. cDNA samples were standardized based on the content of β -actin cDNA as house-keeping gene. β -actin cDNA was evaluated by performance of a β -actin PCR on multiple dilutions of each cDNA sample. The amount of amplified product was estimated by densitometry of ethidiumbromide stained 1.2% agarose gels using a CCD camera and Imagemaster VDS software (Pharmacia). The TLR primers used for the generation of the cRNA probes were also used for PCR amplification. Primers for murine β -actin were: 5'-TAA AAC GCA GCT CAG TAA CAG TCG G-3' (sense primer) and

5'-TGC AAT CCT GTG GCA TCC ATG AAA C-3' (antisense primer); primers used for the amplification of murine TNF- α mRNA: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' and 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3' (antisense primer); primers designed for amplification of murine IFN- γ mRNA: 5'-AGC GGC TGA CTG AAC TCA GAT TGT AG-3' (sense primer) and 5'-GTC ACA GTT TTC AGC TGT ATA GGG-3' (antisense primer). All primers were used for PCR amplification of murine cDNA kidney samples from mice exposed to the interventions. PCR reactions with TLR2, TLR4, β -actin, IFN- γ or TNF- α specific primers were performed using appropriate dilutions of the cDNA. PCR reactions were performed in a total volume of 25 μ l in PCR buffer (Perkin Elmer), in the presence of 0.2 mM dNTP (Pharmacia), 1.0 μ M of each primer, 0.3 mM MgCl₂ and 0.5 U Taq polymerase (Perkin Elmer). PCR conditions for each primer couple were as follows: β -actin: 95°C for 30 sec, 60°C for 45 sec and 72°C for 30 sec during 21 cycles; TLR2: 95°C for 30 sec, 57°C for 30 sec and 72°C for 45 sec during 33 cycles; TLR4 : 95°C for 45 sec, 61°C for 45 sec and 72°C for 45 sec during 36 cycles; IFN- γ : 95°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec during 40 cycles; TNF- α : 95°C for 30 sec, 63°C for 45 sec and 72°C for 30 sec during 38 cycles. Levels of TLR2, TLR4, TNF- α and IFN- γ RNA expression were evaluated by densitometric image analysis as described above. Relative TLR mRNA levels were calculated by comparison of band intensities of the TLR RT-PCR products with standard curves prepared by PCR amplifications on dilution series of a highly concentrated murine kidney cDNA.

Immunohistochemistry

Staining with the anti-Tamm Horsfall protein on paraffin sections was conducted to discriminate between distal tubules, proximal tubular epithelium, the loop of Henle and collecting ducts. Immunohistochemistry was carried out on sections adjacent to those used for in situ hybridization. Paraffin sections treated as described above, were incubated with appropriate dilutions of primary antibody, washed and developed using biotinylated rabbit-anti-goat IgG, and alkaline phosphatase labeled strep-ABComplex and visualized as described above.

TLR4 Western blotting

Renal tissue samples for evaluation of TLR4 protein expression were obtained from mice of which the renal blood vessels were flushed, immediately after the mice were sacrificed, with ice-cold PBS containing nitroprusside and heparin by canulation of the left ventricle and opening of the vena cava. This was done to remove TLR4 expressing blood leukocytes. Western blotting on renal tissue was performed using 8% polyacrylamide SDS gels. Aliquots (50 μ l) of kidney homogenates (50 mg/ml) in 2% SDS sample buffer were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA). After transfer of the proteins membranes were blocked with 5% non-fat dry milk in 50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4 (TBS-T). Membranes were then incubated with

polyclonal rabbit anti-murine TLR4 serum²⁴, kindly provided by Dr. B. Beutler (Scripps Research Institute, La Jolla, CA), at a 1:2500 dilution in TBS-T with 0.5% non-fat dry milk. Positive bands were visualized by chemiluminescence technology (Supersignal, Pierce, Rockford, IL) using peroxidase conjugated goat anti-rabbit IgG at a 1:5000 dilution.

Results

Basal expression of TLR2 and TLR4 in the kidney

To gain insight into renal expression of TLR, we evaluated mRNA expression of both TLR2 and TLR4 by RT-PCR in cDNA samples of healthy kidneys. Constitutive expression of both renal TLR2 and TLR4 mRNA was observed (Figure 2.1). To determine the cellular origin of this constitutive expression TLR mRNA was localized by in situ hybridization. As depicted in Figure 2.2A, 2.2B and 2.3A, 2.3B basal expression of TLR2 and TLR4 mRNA was observed predominantly in the epithelial cells of the distal and proximal tubules and in Bowman's capsular epithelium. Expression in glomeruli and endothelial cells was minor. While macrophages are reported to express relatively abundant levels of TLR2 and TLR4 mRNA, it appeared that epithelial cells express the majority of the TLR mRNA in the kidney. Only few resident macrophage like cell types were observed in the renal tissue and these cells stained to a similar extent as tubular epithelium. Proximal epithelial cells in these healthy kidneys stained slightly more intensely for TLR4 mRNA compared with the distal epithelium. Control incubations using sense riboprobes were negative for healthy and experimental kidneys (Figure 2.3G).

Figure 2.1 Constitutive and post-ischemic renal TLR2 and TLR4 mRNA expression. Renal TLR 2 and TLR4 mRNA was evaluated by specific RT-PCR amplification of renal cDNA samples of healthy control mice and mice subjected to renal I/R. TLR2 and TLR4 mRNA was constitutively present in healthy renal tissue. Ischemia followed by one or six hours reperfusion resulted in abrogation of TLR expression. At 24 hours after ischemia, mRNA expression of both TLR returned to detectable levels. TLR2 and TLR4 expression was markedly enhanced at days 3 and 5 post ischemia. Results shown are representative for 3 independent RT-PCR reactions on renal cDNA samples of two mice in each treatment group or more. cDNAs were standardized for β -actin content.

Figure 2.2 Localization of constitutive and inflammation induced renal TLR2 mRNA by in situ hybridization. Renal TLR2 localization is shown in medulla (A) and cortex (B) of healthy kidneys, and in mice subjected to renal ischemia followed by one day (C,D) and five days (E,F) of reperfusion respectively. Constitutive expression for TLR2 was moderate and diffuse in cortex (B) and relatively low in the medulla (A). One day post-ischemia staining for TLR2 mRNA was reduced (C,D). Five days post-ischemia TLR2 expression is strongly enhanced (E,F) and predominantly located in collecting ducts, distal tubular epithelium and Henle's loop. The arrow indicates a macrophage like cell. See page 157 for colour figure.

Figure 2.3 Localization of constitutive and inflammation induced renal TLR4 mRNA by in situ hybridization. TLR4 localization is shown by incubation with the anti-sense riboprobe in medulla (A) and cortex (B) in healthy kidneys, and in mice subjected to renal ischemia followed by one day (C,D) and five days (E,F) of reperfusion respectively. Control incubations using TLR4 sense riboprobe were negative (G). Constitutive expression for TLR4 was moderate and diffuse in cortex, mainly by the tubular epithelial cells (B), and relatively low in the medulla (A). One day after ischemia TLR4 expression was primarily observed in collecting ducts, distal tubules and the thin limb of Henle's loop (C,D). Five days after ischemia TLR4 mRNA staining was strongly enhanced in collecting ducts, the distal tubules and the thin limb of the loops of Henle (E,F). A small number of macrophage like cells was observed, which stained all positive. Arrows indicate macrophage like cells. Distal tubules were identified by Tamm Horsfall protein staining (H). See page 158 for colour figure.

Effects of renal injury on TLR2 and TLR4 gene expression

Mice were exposed to unilateral renal I/R, a process that leads to tissue damage and a subsequent sterile inflammatory reaction. This inflammatory process is characterized by initial tubular apoptosis, followed by KC, MIP-2, and TNF- α expression and subsequent neutrophil influx with apoptosis, necrosis and organ dysfunction peaking at day 1^{19,20,22}. Thereafter a strongly enhanced IFN- γ mediated MHC-I and II expression occurs from day 3 onwards^{21,25}. The effect of this sterile inflammatory process on TLR2 and TLR4 mRNA levels was assessed by RT-PCR analysis. Levels of renal TLR2 mRNA decreased to undetectable levels in animals sacrificed at one and six hours after ischemia. TLR2 mRNA returned to basal level after one day (Figure 2.1). A similar mRNA expression pattern was observed for TLR4 (Figure 2.1): TLR4 mRNA expression was undetectable at one hour, from one to six hours the TLR4 synthesis returned to detectable levels. After 24 hours of reperfusion TLR4 expression was enhanced when compared to controls. TLR2 and TLR4 mRNA expression were similar in healthy and sham operated controls, indicating that the surgical procedure did not influence TLR expression (data not shown). The results of densitometric quantification of TLR2 and TLR4 mRNA levels at 1, 6 and 24 hours after ischemia are depicted in Figure 2.4A and 2.4B. At days 3 and 5 after renal ischemia, a significant increase of both TLR2 and TLR4 mRNA was detected in the kidney (Figure 2.1). A 4-5-fold increase of TLR2 mRNA was observed when compared to healthy controls (Figure 2.5A). Similarly a 4 and 5.5 fold enhancement of TLR4 mRNA was measured at three and five days post-ischemia respectively (Figure 2.5B).

To elucidate the cellular origin of the described enhanced TLR2 and TLR4 expression in this renal inflammation model, TLR mRNA was localized by in situ hybridization. Histology showed that renal injury was clearly present one day after ischemia, which is associated with the influx of neutrophils^{20,25}. In this inflamed tissue, reduced TLR2 mRNA expression was observed in the cortex compared to healthy controls (Figure 2.2D). In the medulla TLR2 transcripts appeared upregulated in the thin limb of Henle's loop (Figure 2.2C). Moreover, TLR2 transcription was detected in macrophage like cells in the interstitial tissue (Figure 2.2C, 2.2D). One day after ischemia TLR4 expression was predominantly observed in the epithelial cells of the distal tubules, the thin limb of Henle's loop and collecting ducts, while low expression was observed in the proximal tubules, glomeruli and capsular epithelium. A small number of macrophage like cells were present and stained positive. In concordance to the enhanced TLR mRNA expression as measured by RT-PCR at five days after ischemia, intense staining for both TLR2 and TLR4 mRNA was detected by in situ hybridization at the same timepoint. The intense staining was predominantly localized in the collecting ducts, the thin limb of Henle's loop and distal tubules (Figure 2.2E, 2.2F, 2.3E, 2.3F). Identification of distal tubules in these experiments was performed by Tamm Horsfall protein staining (Figure 2.3H). At five days post-ischemia, a small number of macrophage like cells were present which stained positive for TLR2 and TLR4. However, compared to the staining in collecting ducts, the loop of Henle and distal

tubules, this TLR expression by macrophage like cells constituted only a minority of the total expression (Figure 2.2E, 2.2F, 2.3E, 2.3F). At day 5 only moderate TLR expression was detected in the glomeruli and proximal tubules (Figure 2.2E, 2.2F, 2.3E, 2.3F). Taken together, renal I/R leads to marked enhancement of both TLR2 and TLR4 mRNA levels, which appeared to be predominantly present in epithelial cells of the distal tubules, the collecting ducts and Henle's thin loop.

Figure 2.4 I/R-induced changes in both TLR2 and TLR4 mRNA synthesis during the early reperfusion phase. Renal TLR 2 (A) and TLR4 (B) mRNA was evaluated by specific RT-PCR amplification of renal cDNA samples of healthy control mice and mice subjected to renal I/R. cDNA's were standardized for β -actin content. Quantitative data were obtained by densitometric evaluation of RT-PCR products which were compared to a standard curve obtained by amplification of a serial dilution of highly concentrated cDNA. TLR2 expression (A) after one hour ($n=2$) and six hours ($n=2$) following ischemia was not detectable. TLR2 expression returned to basal levels ($n=4$) at 24 hours following ischemia ($n=3$) (A). A similar expression pattern following I/R was observed for TLR4 (B). However, increased renal TLR4 mRNA expression was observed at 24 hours. A representative graph is shown for the outcome of 3 separate PCR experiments on the indicated samples.

Role of IFN- γ and TNF- α in regulation of renal TLR expression

Renal I/R results in TNF- α modulated renal inflammation that is followed by IFN- γ dependent up-regulation of MHC class I and II molecules, indicating an active cytokine mediated immune response in the organ^{20,21,25}. The role of these cytokines in the observed upregulation of TLR mRNA expression in our I/R induced renal inflammation model was evaluated by blocking TNF- α and IFN- γ with inhibitory antibodies. The observed enhancement of renal TLR2 mRNA expression five days after ischemia was completely prevented in animals treated with anti IFN- γ or anti TNF- α antibodies (Figure 2.6A, 2.6B). Moreover, blocking of either IFN- γ or TNF- α reduced the TLR2 mRNA expression at day 5 to below the constitutive level (Figure 2.6B). In contrast to this major inhibitory effect of anti TNF- α on TLR2 mRNA synthesis in our renal inflammation model, blocking of TNF- α resulted only in a partial reduction of TLR4 mRNA expression (Figure 2.6A, 2.6C). Similar to the effect of anti IFN- γ on TLR2 mRNA

expression, blocking of IFN- γ completely prevented the enhancement of TLR4 mRNA synthesis at five days after ischemia, resulting in TLR4 mRNA expression below the constitutive level. Our findings indicate that TLR2 and TLR4 expression are regulated differentially by TNF- α and IFN- γ . Because both IFN- γ and TNF- α are involved in the enhanced TLR mRNA expression we determined the effect of cytokine inhibition on cytokine mRNA expression. Anti IFN- γ treatment decreased IFN- γ mRNA translation in the kidneys five days after the ischemic insult whereas TNF- α mRNA was not decreased by inhibition of IFN- γ (Figure 2.7A). Anti TNF- α antibody administration did not decrease the renal TNF- α and renal IFN- γ mRNA levels when compared with PBS treated animals (Figure 2.7B). These findings show that IFN- γ does not enhance TLR mRNA expression in our model by induction of TNF- α and that TNF- α does not enhance TLR mRNA expression by induction of IFN- γ .

Figure 2.5 Renal I/R induced inflammation enhances TLR2 and TLR4 mRNA expression. Renal TLR 2 (A) and TLR4 (B) mRNA was evaluated by specific RT-PCR amplification of renal cDNA samples of healthy control mice and mice subjected to renal I/R. cDNA's were standardized for β -actin content. The means of densitometric evaluations are shown. Strongly enhanced TLR2 (A) mRNA levels were detected at three days ($n=3$) and five days ($n=4$) after ischemia, compared to levels detected one day after ischemia ($n=3$) and controls ($n=4$). Similarly, strongly enhanced TLR4 (B) expression was observed at three and five days following ischemia. A representative graph is shown for the outcome of three separate PCR experiments on the indicated samples.

TLR4 protein expression after renal I/R

Renal TLR4 expression was evaluated by Western blotting using specific anti-murine TLR4 antiserum. Whole renal tissue samples, and liver as positive control, were homogenized in SDS and proteins were separated by SDS-PAGE. Subsequent immunoblotting with the anti-murine TLR4 antiserum revealed positive bands with an apparent molecular weight of ~ 95 kD and ~ 120 kD in normal liver and in kidney tissue five days after renal I/R (Figure 2.8). The calculated molecular weight of mature TLR4 based on its amino acid sequence is 93.5 kD and is consistent with the anti-TLR4

positive bands at ~95 kD in the Western blot. The band at a molecular weight of ~120 kD in renal tissue after I/R and in normal liver probably represents a glycosylated form of the TLR4 protein. The observed molecular weight form of 120 kD of murine TLR4 is consistent with the reported apparent molecular weight of human recombinant TLR4²⁶. Basal TLR4 protein expression was not detectable with the used Western blotting technique in healthy murine renal tissue (Figure 2.8). These observations show that the upregulation of TLR4 mRNA expression during renal inflammation induced by I/R is accompanied by a significant increased TLR4 protein expression in the kidney.

Figure 2.6 Inflammation induced renal TLR2 and TLR4 expression is IFN- γ and TNF- α dependent. Renal TLR2 (A,B) and TLR4 (A,C) transcripts were detected in mice five days after ischemia by RT-PCR and quantitated as described for Figure 1 and 5. Mice were treated with inhibitory anti TNF- α or anti IFN- γ antibodies at the time of reperfusion. TLR2 (A,B) upregulation is prevented completely by both anti IFN- γ (n=2) and anti TNF- α (n=2) when compared to PBS treated animals (n=4). Enhancement of TLR4 (A,C) mRNA expression is only partially blocked by anti TNF- α but completely abrogated by anti IFN- γ . Representative graphs are shown for the outcome of 3 separate PCR experiments on the indicated samples.

Figure 2.7 Effect of cytokine inhibition on I/R induced renal cytokine expression. Renal IFN- γ (A) and TNF- α (B) mRNA levels were detected at day 5 after renal ischemia by RT-PCR in mice treated with anti TNF- α or anti IFN- γ . cDNA was standardized for β -actin content. Shown are the means of densitometric values. IFN- γ mRNA (A) expression at five day after ischemia is reduced by anti IFN- γ (n=2) but not by anti TNF- α (n=2) when compared with PBS treated animals (n=4). Treatment with anti TNF- α or anti IFN- γ did not affect TNF- α (B) mRNA levels. Representative graphs are shown for the outcome of 3 separate PCR experiments on the indicated samples.

Figure 2.8 I/R induced renal TLR4 protein expression. Murine TLR4 protein expression was evaluated by Western blotting using polyclonal rabbit anti-murine TLR4 serum. Tissue samples were homogenized in SDS-sample buffer and proteins were separated on 8% polyacrylamide SDSgels and immunoblotted using PVDF membranes. Lane 1, liver; lane 2, control kidney; lane 3, kidney five days after I/R.

Discussion

In this work, we describe the mRNA localization for TLR2, the apparent receptor for products of Gram-positive bacteria²⁷, and for TLR4, which recognizes LPS present on Gram-negative bacteria³, in renal tubular epithelial cells. Our description of TLR2 and TLR4 mRNA localization in the kidney is the first to show constitutive TLR2 and TLR4 *in*

vivo expression in the epithelial cells of the proximal and distal tubules. Moreover, we observed that a non-microbial inflammation as induced by I/R causes remarkably enhanced TLR2 and TLR4 mRNA expression by epithelial cells of the distal tubules, the thin limb of the loops of Henle, and collecting ducts. The enhanced TLR mRNA expression during renal inflammation was found to be mediated by both IFN- γ and TNF- α and is associated with a major increase of renal TLR4 protein expression. While *in vitro* TLR4 mRNA expression and responsiveness had been reported for several cell types such as leukocytes, endothelial cells, epithelial cells and fibroblasts, but most abundantly for macrophages¹¹⁻¹⁵, the differential expression of TLR4 *in vivo* remained to be elucidated. Our study indicates that murine renal epithelial cells express TLR2 and TLR4 *in vivo*. The observation that TLR2 and TLR4 mRNA expression in the kidney is predominantly located in renal tubular epithelium appears to have some implications for the understanding of the innate immune defense mechanism in the kidney against pyelonephritis as a result of ascending urinary tract infections (UTI). Gut derived Gram-negative and Gram-positive bacteria are the most prevalent microbial pathogens responsible for UTI, with *Escherichia coli* being the most common²⁸. Our data suggest that tubular epithelial cells can monitor the presence of both types of bacteria. In this context, Hagberg *et al.*¹⁸ showed already in 1984 that C3H/HeJ mice, who lack functional TLR4³, are highly susceptible to persistent Gram-negative pyelonephritis. TLR dependent cellular activation leads to the translocation of NF- κ B to the nucleus which leads to transcription of genes encoding for cytokines, chemokines, adhesion molecules and antimicrobial peptides^{4,3,10,29}. Consistently, TLR4 signaling, induction of CXC chemokine expression and CXC receptor signaling^{18,30}, and subsequent neutrophil recruitment are crucial for the clearance of Gram-negative bacteria from the kidney^{31,32}. Our results suggest that the functional role of TLR4 in prevention of pyelonephritis as described¹⁸ could be the result of functional TLR4 expression by the renal tubular epithelial cells. To monitor bacteria in the lumen of tubules and collecting ducts the TLR receptors should be expressed on the apical membrane of the renal epithelial cells. It should be noted that we located renal TLR expression at the mRNA level and obviously these results need to be confirmed by studies at the protein level by immunohistochemistry and with functional studies. In regard of the functionality of TLR4 in the kidney it is noteworthy that the mRNA for MD-2, the essential cofactor for TLR4, is abundantly present in the murine kidney³. We have confirmed the abundant renal MD-2 mRNA expression by RT-PCR analysis, and also observed elevation of MD-2 mRNA after I/R (data not shown). We applied a renal I/R model to elucidate the regulation of TLR expression during inflammation. This model allowed us to evaluate cytokine mediated TLR mRNA expression in an inflammatory process that is not influenced by bacterial products. Most interestingly, a shift occurs from the observed diffuse proximal and distal staining for TLR2 and TLR4 mRNA in healthy kidneys to a predominant and enhanced expression five days after ischemia in the distal epithelial tubular cells, the thin limb of the loops of Henle and collecting ducts. Following renal I/R injury monocyte and T-cell

influx has been reported from day 3 onwards³³. We observed a small number of macrophage like cells that stained all positive for both TLR2 and TLR4, however, compared to the enhanced expression in collecting ducts, the thin limb of the loops of Henle and distal tubules this constituted only a minority of the total TLR expression. The increased TLR2 and TLR4 mRNA expression in our renal inflammation model by epithelial cells in the distal part of the nephron and the medulla, suggests the mobilization of TLR dependent anti-microbial potential during inflammation to the site where ascending bacteria may enter the kidney.

The I/R induced TLR mRNA expression in the later phase of reperfusion (day 3-5) was dependent on the action of IFN- γ and TNF- α . Previously we demonstrated that anti TNF- α reduces kidney neutrophil influx and deterioration of renal function in our murine model of renal I/R²⁰, whereas anti IFN- γ did not affect these parameters²¹. The inhibition of TLR upregulation by anti TNF- α is not caused by a major effect on the IFN- γ mRNA synthesis (Figure 2.7). This observation is supported by data showing that anti TNF- α does not block the upregulation of MHC molecules in this model, while this process is largely IFN- γ dependent^{20,25}. Since blocking of either TNF- α or IFN- γ results in inhibition of the majority of TLR2 expression it appears that the observed enhancement of TLR2 expression is elicited by a synergistic action of these cytokines. TLR4 synthesis is only partially blocked by anti TNF- α and almost completely by anti IFN- γ , it appears therefore that the upregulation of TLR4 in our inflammation model is primarily mediated by IFN- γ . Thus, the regulation of TLR4 mRNA expression discerns with TLR2 in our *in vivo* model in respect to the dependence on TNF- α . Consistently, also *in vitro* studies indicate that TLR2 and TLR4 expression are regulated via distinct pathways¹¹. Our *in vivo* observation of IFN- γ mediated TLR4 expression is in line with the presence of a functional interferon response factor (IRF) motif in the promoter region of the human and mouse TLR4 gene¹⁷. It should be mentioned that we cannot exclude the possibility that TNF- α or IFN- γ act indirectly by stimulation of a putative modulator of TLR expression.

In general IFN- γ is involved in bacterial clearance during the late stages of infection with virulent Gram-negative or Gram-positive bacteria³⁴⁻³⁶. Furthermore, IFN- γ deficiency is found to be associated with infection by poorly pathogenic mycobacterium strains and Salmonella³⁷. Our observations suggest that IFN- γ augments enhanced reactivity to bacteria *in vivo* by upregulation of TLR2 and TLR4 mRNA. Indeed, increased expression of TLR4 by IFN- γ may explain the enhanced LPS induced lethality of IFN- γ treated rabbits and the enhanced LPS sensitivity of monocytes/macrophages induced by IFN- γ ³⁸⁻⁴⁰. It appears that increased TLR2 and TLR4 expression may play a role in the mechanisms involved in IFN- γ mediated resistance to virulent bacteria.

Although an obvious role for IFN- γ in urinary tract infections in humans is not reported it must be mentioned that the epithelial cells of the kidney stain strongly positive with anti IFN- γ receptor antibodies in immunohistochemistry⁴¹, indicating the responsiveness of these cells to IFN- γ . IFN- γ deficiency was observed to increase the

susceptibility of mice to experimental UTI urinary tract infection with uropathogenic *E. coli*⁴². The latter is in line with the IFN- γ mediated TLR4 expression (this study) and increased susceptibility of TLR4 deficient mice for Gram-negative UTI¹⁸. Next to behavior and anatomical impairments of the urinary tract, genetic factors also seem to be involved in human ascending urinary tract infections²⁷. However, the genetic influence in human UTI however needs further investigation (see ref³⁰).

In conclusion, TLR2 and TLR4 are constitutively expressed in both proximal and distal tubular renal epithelial cells *in vivo*. During renal inflammation TLR2 and TLR4 mRNA synthesis is enhanced by the action of TNF- α and IFN- γ , and this increased expression is mainly localized in distal tubules, the thin limb of the loops of Henle and collecting ducts. The epithelial localization of TLR mRNA expression suggests a role for epithelial derived TLR signaling in the inflammatory response observed during ascending UT infections.

References

1. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394-397.
2. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U-S-A* 1998;95:588-593.
3. Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.
4. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;189:1777-1782.
5. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 1999;274:10689-10692.
6. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 1999;163:1-5.
7. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem* 1999;274:33419-33425.
8. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;11:443-451.
9. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 2000;165:618-622.
10. May MJ, Ghosh S. Signal transduction through NF-kappa B. *Immunol Today* 1998;19:80-88.
11. Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, van 't Veer C, Penton Rol G, Ruco LP, Allavena P, Mantovani A. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol* 2000;164:5998-6004.
12. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;25:187-191.
13. Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, Polentarutti N, Muzio M, Arditi M. Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J Biol Chem* 2000;275:11058-11063.
14. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* 2000;164:966-972.
15. Mokuno Y, Matsuguchi T, Takano M, Nishimura H, Washizu J, Ogawa T, Takeuchi O, Akira S, Nimura Y, Yoshikai Y. Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by *Escherichia coli* infection in mice. *J Immunol* 2000;165:931-940.
16. Su GL, Klein RD, Aminlari A, Zhang HY, Steinstraesser L, Alarcon WH, Remick DG, Wang SC. Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 2000;31:932-936.
17. Rehli M, Poltorak A, Schwarzfischer L, Krause SW, Andreesen R, Beutler B. PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human Toll-like receptor 4 gene. *J Biol Chem* 2000;275:9773-9781.
18. Hagberg L, Hull R, McGhee JR, Michalek SM, Svanborg Eden C. Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. *Infect Immun* 1984;46:839-844.
19. Daemen MARC, van 't Veer C, Denecker G, Heemskerk VH, Wolfs TGAM, Clauss M, Vandenabeele P, Buurman WA. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest* 1999;104:541-549.

20. Daemen MARC, Van de Ven MWCM, Heineman E, Buurman WA. Involvement of endogenous interleukin-10 and tumor necrosis factor- α in renal ischemia-reperfusion injury. *Transplantation* 1999;67:792-800.
21. Daemen MARC, van 't Veer C, Wolfs TGAM, Buurman WA. Ischemia/reperfusion-induced IFN- γ up-regulation: Involvement of IL-12 and IL-18. *J Immunol* 1999;162:5506-5510.
22. Daemen MARC, de Vries B, van 't Veer C, Wolfs TGAM, Buurman WA. Apoptosis and chemokine induction after renal ischemia reperfusion. *Transplantation* 2001;71:1007-1011.
23. De Boer WJ, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JHJM. Transforming Growth Factor β_1 and Recruitment of Macrophages and Mast Cells in Airways in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 1998;158:1951-1957.
24. Nattermann J, Du X, Wei Y, Shevchenko D, Beutler B. Endotoxin-mimetic effect of antibodies against Toll-like receptor 4. *J Endotoxin Res* 2000;6:257-264.
25. Goes N, Urmson J, Vincent D, Ramassar V, Halloran PF. Induction of major histocompatibility complex markers and inflammatory cytokines after ischemic injury to the kidney: lessons from interferon-gamma gene knockout mice. *Transplantation* 1995;27:771-773.
26. da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. *J Biol Chem* 2001;276:21129-21135.
27. Takeuchi O, Hoshino K, Akira S. TLR2-Deficient and MyD88-Deficient Mice Are Highly Susceptible to *Staphylococcus aureus* Infection. *J Immunol* 2000;165:5392-5396.
28. Warren JW. Clinical presentations and epidemiology of urinary tract infections. In *Urinary tract infections: molecular pathogenesis and clinical management*. H.L.T. Mobley and J.W. Warren, editors. ASM Press, Washington D.C., 1996:3.
29. O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF. Expression and regulation of the human β -defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* 1999;163:6718-6724.
30. Frendus B, Godaly G, Hang L, Karpman D, Lundstedt AC, Svanborg C. Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. *J Exp Med* 2000;192:881-890.
31. Shahin RD, Engberg I, Hagberg L, Svanborg-Eden C. Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local gram-negative infection. *J Immunol* 1987;138:3475-3480.
32. Haraoka M, Hang L, Frendus B, Godaly G, Burdick M, Strieter R, Svanborg C. Neutrophil recruitment and resistance to urinary tract infection. *J. Infect. Dis.* 1999;80:1220-1229.
33. Ibrahim S, Jacobs F, Zukin Y, Enriquez D, Holt D, Baldwin W, Sanfilippo F, Ratner LE. Immuno-histochemical manifestations of unilateral kidney ischemia. *Clin Transplant* 1996;10:646-652.
34. Zhao YX, Nilsson IM, Tarkowski A. The dual role of interferon-gamma in experimental *Staphylococcus aureus* septicaemia versus arthritis. *Immunology* 1998;93:80-85.
35. Nauciel C, Espinasse-Maes F. Role of gamma interferon and tumor necrosis factor alpha in resistance to *Salmonella typhimurium* infection. *Infect Immun* 1992;60:450-454.
36. Muotiala A, Makela PH. Role of gamma interferon in late stages of murine salmonellosis. *Infect Immun* 1993;61:4248-4253.
37. Ottenhof THM, de Boer T, Verhagen CE, Verreck FAW, van Dissel JT. Human deficiencies in type 1 cytokine receptors reveal the essential role of type 1 cytokines in immunity to intracellular bacteria. *Microbes and Infection* 2000;2:1559-1566.
38. Jurkovich GJ, Mileski WJ, Maier RV, Winn RK, Rice CL. Interferon gamma increases sensitivity to endotoxin. *J Surg Res* 1991;51:197-203.
39. Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide responses: Expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. *Infect Immun* 1993;61:3222-3227.
40. Hayes MP, Freeman SL, Donnelly RP. IFN-gamma priming of monocytes enhances LPS-induced TNF production by augmentation both transcription and mRNA stability. *Cytokine* 1995;7:427-435.
41. Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G, Garotta G. Distribution of interferon-gamma receptor in human tissues. *Eur J Immunol* 1992;22:2403-2412.
42. Jones-Carson J, Balish E, Uehling DT. Susceptibility of immunodeficient gene-knockout mice to urinary tract infection. *J Urology* 1999;161:338-341.

Chapter 3

Apoptotic cell-death is initiated during normothermic ischemia in human kidneys

Tim GAM Wolfs, Bart de Vries, Sarah J Walter, Carine J Peutz-Kootstra,
LW Ernest van Heurn, Gosse ON Oosterhof, Wim A Buurman

American Journal of Transplantation 2005;5:68-75

Abstract

Ischemic damage plays an important role in post-transplant organ failure. Activation of the apoptotic cascade is crucially involved in post-ischemic inflammation resulting in tissue damage and organ dysfunction. Here we investigate the initiation of the apoptotic cascade during normothermic ischemia in human kidneys using a model for normothermic ischemia with kidneys nephrectomized because of renal cell carcinoma. Ex vivo, kidneys were stored at 37°C and consecutive biopsies were taken from disease free tissue. Pro- and anti-apoptotic proteins were assessed by western blotting and immunofluorescence.

During normothermic ischemia the pro-apoptotic proteins Bax and activated caspase-9 increased with ischemia time, whereas caspase-8 was not activated. The anti-apoptotic proteins Bcl-2 and cFLIP decreased in time. Data on Bcl-2 and Bax were supported by immunofluorescence for Bcl-2 and activated Bax. However, activation of the central effector caspase-3, essential for execution of the apoptotic process, was not detected.

In conclusion, during normothermic ischemia the apoptotic cascade in the human kidney is initiated, but not fulfilled. Our data show that the duration of ischemia significantly correlates with activation of the apoptotic cascade. These findings provide insight in the initiation of apoptotic cell-death during warm ischemia and may be useful in the assessment of ischemic injury.

Introduction

Post-transplant acute renal failure (ARF) is an important determinant of renal transplant survival. The occurrence of ARF is substantial in organs derived from cadaveric donors and in particular non-heart beating (NHB) category 2 donors¹, as compared to living donors². The inevitable ischemia of such NHB donor kidneys explains these differences in graft failure to a large extent². Understanding the mechanisms of injury initiated by ischemia-reperfusion (I/R) is therefore of critical importance for defining strategies to improve post-transplant organ function.

In different experimental models it has been established that renal ischemia followed by reperfusion leads to apoptotic cell death^{3,4}. Moreover, activation of the apoptotic cascade is crucially involved in the induction of post-ischemic inflammation and subsequent organ failure³. In line, enhanced apoptotic cell death established by tunel-staining, was detected in post-transplant biopsies from cadaveric allografts as compared to transplants derived from living donors, indicating that ischemic injury induces apoptosis in clinical transplantation⁵.

Apoptosis is executed by a family of cysteine proteases, called caspases⁶. Two major pathways of caspase activation have been described; i.e. the extrinsic and intrinsic pathway⁷. Activation of the extrinsic pathway, triggered by binding of extracellular ligands to cell surface death receptors, results in recruitment of the adaptor molecule and Fas-associated death domain (FADD) and activation of caspase-8 upon recruitment to the death-inducing signaling complex (DISC). The latter are controlled by cFLIP, an intracellular protein that inhibits death receptor signaling by regulating both recruitment and processing of pro-caspase-8 within the DISC⁸.

The intrinsic pathway is activated by various intracellular stimuli, such as anoxia, growth factor deprivation and DNA damage, all affecting the mitochondria, where they promote mitochondrial membrane permeabilization and the release of caspase activating factors, among others cytochrome c, into the cytosol^{9,10}. Cytosolic cytochrome c interacts with apaf-1, leading to its oligomerization and the recruitment of pro-caspase-9, which autoactivates within the so called apoptosome¹¹. Members of the Bcl-2 family, among others pro-apoptotic Bax and anti-apoptotic Bcl-2, control activation of the intrinsic pathway. For any given apoptotic stimulus, the balance between death and survival seems to be determined by the ratio of the apoptosis-stimulating and -suppressing Bcl-2 family members^{12,13}. The activation of either extrinsic or intrinsic pathway participates in a cascade that culminates in the activation of effector caspases. Activation of primarily effector caspases-3, -6, and -7, results in cleavage of a wide range of substrates leading to chromosomal DNA fragmentation and cellular morphologic changes characteristic of apoptosis⁶.

Although it appears that apoptotic cell death plays a role in the induction of organ damage due to renal I/R in humans^{5,14}, it is currently unknown whether the apoptotic cascade is already initiated during ischemia or becomes initiated upon reperfusion. In this context, we developed a human model for renal normothermic ischemia. This

model was used to study proteins involved in the initiation of the apoptotic cascade during normothermic ischemia. In particular, we investigated the consumption of endogenous caspase inhibitors as well as the activation of initiator and effector caspases. Our data indicate that during normothermic ischemia the apoptotic cascade is initiated in the human kidney. These data implicate that during normothermic ischemia, the circumstances are created that facilitate execution of apoptosis upon reperfusion. Therefore our data may be useful in the development of methods to assess ischemic injury. Moreover, renewed insight in early ischemic changes provides important opportunities to prevent clinical manifestations of reperfusion injury in the kidney, and potentially in other organs.

Materials and methods

Antibodies

The following antibodies (ab) were used: anti-murine Bax ab P19 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bcl-2, anti-human caspase-9 and anti-human caspase-3 from Stressgen (Victoria, Canada); monoclonal murine anti-caspase-8 was kindly provided by Dr. Klaus Schulze-Osthoff (Institute of Molecular Medicine, University of Duesseldorf, Germany); anti human cFLIP (Biocarta; San Diego, CA); Goat anti rabbit Texas Red and peroxidase conjugated goat anti-rabbit IgG from Jackson Immuno-Research Laboratories (West Grove, PA) and peroxidase conjugated goat anti-mouse IgG from Caltag (Burlingame, CA). Anti-Bcl-2 and anti-Bax from Upstate biotechnology (Lake Placid, NY) and Lotus Tetragonolobus Lectin from Vector (Burlingame, CA) were used for immunohistochemistry.

Human model for renal normothermic ischemia

In the present study, kidneys used were nephrectomized because of a renal cell carcinoma. These patients did not have a family history of renal cell carcinomas, neither did they have other signs of hereditary diseases like von Hippel-Lindau (VHL). Taking into consideration the pathology, the age of the patients and the absence of a family history/patient anamnesis, the chance that a hereditary effect influences our data, is certainly far below 1%.

Normothermic ischemia started at ligation of the renal artery about 5 min before nephrectomy. Thereafter, kidneys (n=5 per group) were immediately stored at 37°C and rapidly inspected by the responsible pathologist. Full-thickness biopsies were taken from non-diseased tissue. Subsequently, normothermic renal ischemia was prolonged up to 85 min. Renal temperature was measured during storage and remained between 36 and 37.5°C. Approximately every 15 min full-thickness biopsies were taken. Control kidneys (n=4) were cooled at 0°C immediately upon

nephrectomy. Biopsies from these control kidneys were taken within 10 min after nephrectomy. All biopsies were divided in several parts and either immediately stored in liquid nitrogen for western blotting, or embedded in Tissue Tek (EMS; Washington, PA) and snap-frozen in isopentane at -80°C for immunohistochemistry. All biopsies were stored at -80°C until further processing.

Western blotting

Renal tissue samples were homogenized in lysis buffer (200 mM NaCl, 10 mM Tris base, 5 mM EDTA, 10% Glycerin, 1 mM PMSF, 0.1 U/ml Aprotinin and 1 $\mu\text{g}/\text{ml}$ Leupeptin). Homogenates were centrifuged at 300 rpm for 10 min, supernatants were collected and centrifuged again at 10,000 rpm for 3 min. Final supernatants were harvested and total protein concentrations were determined using the Bradford method. To confirm equal protein loading immunoblotting was performed with an anti-actin antibody (Sigma, Chicago, IL). Aliquots with equal amounts of protein were heated at 100°C for 5 min in SDS sample buffer, separated on 15% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). After transfer of proteins, blocking, ab incubation steps and washing of the membrane were performed in Phosphate buffered saline (PBS) supplemented with 3% nonfat dry milk and 0.05% Tween. Except for incubations with ab to cFLIP, which were blocked in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (TBST) supplemented with 5% milk and washed in TBST with 0.5% milk.

After the incubation with the primary ab and washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary ab to rabbit or mouse IgG. Positive bands were visualized using chemiluminescence's (Supersignal; Pierce, Rockford, IL).

Immunoreactive bands for all proteins were evaluated densitometrically using a computer-controlled display camera (Imagemaster; Pharmacia, Piscataway, NJ) and image analysis Sigma Gel software (SPSS). For each protein, the optical density of each ischemic sample was compared with the average value of the four control samples.

Immunohistochemistry

Cryostat sections (5 μm) of frozen tissue were cut and stained for Bcl-2 or active Bax. Briefly, slides were dried, fixed in acetone for 10 min, and air-dried. Slides were rehydrated in PBS for 5 min and subsequently incubated in 10% normal goat serum in PBS to block aspecific ab binding. Slides were stained for two hours at RT with either anti-Bcl-2 or anti-Bax primary ab in PBS with 1% bovine serum albumin. After three washes in PBS for 5 min each, slides were incubated for 30 min with the Texas Red labeled secondary ab diluted in the same buffer with the addition of FITC-labeled Lotus Lectin for identification of proximal tubular epithelium¹⁵. After three washes in PBS, the slides were mounted using glycerol-PBS with 1,4-diazabicyclo(2,2,2)octane and 4,6-diamidino(2)phenylindole (DAPI) and viewed with an immunofluorescence

microscope. No significant staining was detected in slides incubated with control ab instead of the primary ab indicating the absence of significant background staining. Cryostat sections (5 μ m) of frozen tissue were cut and stained for Bcl-2 or active Bax. Briefly, slides were dried, fixed in acetone for 10 min, and air-dried. Slides were rehydrated in PBS for 5 min and subsequently incubated in 10% normal goat serum in PBS to block aspecific ab binding. Slides were stained for two hours at RT with either anti-Bcl-2 or anti-Bax primary ab in PBS with 1% bovine serum albumin. After three washes in PBS for 5 min each, slides were incubated for 30 min with the Texas Red labeled secondary ab diluted in the same buffer with the addition of FITC-labeled Lotus Lectin for identification of proximal tubular epithelium¹⁵. After three washes in PBS, the slides were mounted using glycerol-PBS with 1,4-diazabicyclo(2,2,2)octane and 4,6-diamidino(2)phenylindole (DAPI) and viewed with an immunofluorescence microscope. No significant staining was detected in slides incubated with control ab instead of the primary ab indicating the absence of significant background staining.

Statistical analysis

We used the Mann-Whitney test to compare Bax protein expression between the control and the “early” ischemia group. The Spearman analysis was used to determine correlation between the duration of normothermic ischemia and initiation of apoptosis. A *P* value of less than 0.05 was considered significant.

Results

Initiation of the intrinsic pathway during normothermic renal ischemia

To determine whether apoptogenic processes are initiated during ischemia, we evaluated the effect of normothermic ischemia on proteins involved in activation of both the intrinsic and extrinsic pathway. Since activation of the intrinsic pathway is regulated by members of the Bcl-2 protein family, we first determined the pro-apoptotic Bax protein content during ischemia by western blotting. As described by others, we observed that monomeric Bax (21 kDa) is constitutively expressed in human renal tissue¹⁶ (Figure 3.1). A short period of renal ischemia (15 until 35 min) led to a significant increase of Bax protein ($P < 0.02$, Mann-Whitney test) (Figure 3.1). Bax levels remained clearly elevated up to 60 min. Further prolongation of ischemia reduced Bax expression back to basal levels. Next the localisation of active Bax was studied using immunohistochemistry. For this purpose, an ab directed to the N-terminal region of active Bax that is inaccessible in the inactive conformation was applied. The data show that active Bax protein is nearly absent in control tissue (Figure 3.2). In concordance with the enhanced Bax protein content, as detected by western blotting, cytoplasmic active Bax staining increased in the early ischemic

phase. After 30 min of ischemia, activated Bax was predominantly localised in distal tubular epithelial cells. At this timepoint, also some focal staining was observed in proximal tubules. Proximal tubules were identified using Lotus Lectin staining. Similarly, after 80 min of ischemia, the majority of active Bax was detected in distal tubules (Figure 3.2).

Figure 3.1 Bax protein shows a rapid increase followed by decrease during normothermic ischemia. A) Representative immunoblot analysis of Bax. B) Densitometric analysis of Bax immunoblotting of ischemic (n=5) and control kidneys (n=4). Data of ischemic kidneys are expressed as the fraction of mean of control data. Individual ischemic kidneys are displayed by different symbols (■, ▲, ○, □, △) and control kidneys by a filled circle. Constitutive Bax expression (21 kDa) was detected in control tissue. After a short period of ischemia renal Bax expression levels were significantly increased ($P < 0.02$, Mann-Whitney test). Bax protein levels remained elevated up to 60 min. Dotted line represents trendline of all data.

Subsequently, we investigated the effect of ischemia on the regulation of the anti-apoptotic protein Bcl-2. Abundant monomeric Bcl-2 expression (26 kDa) was observed in control tissue (Figure 3.3). Bcl-2 western blot analysis revealed that during ischemia Bcl-2 levels were reduced. Moreover, this reduction of Bcl-2 significantly correlated with the duration of the ischemic insult ($r = 0.86$, $P < 0.001$, Spearman correlation analysis) (Figure 3.3). Immunohistology for Bcl-2 resulted in similar data; a decrease of Bcl-2 with time. Bcl-2 was primarily detected at the basolateral side of tubular epithelial cells. The expression was predominantly observed in proximal tubules while a lower expression was detected in distal tubules (Figure 3.4).

Collectively, enhanced Bax protein levels combined with decreased Bcl-2 levels suggests that normothermic ischemia enhances susceptibility for the activation of the intrinsic pathway. In this context we determined the activation of initiator caspase-9. We observed a significant correlation between activation of caspase-9 (34/37 kDa) and the normothermic ischemia time ($r = 0.79$, $P < 0.001$, Spearman correlation analysis) (Figure 3.5). Taken together, these data show that already during normothermic ischemia, activation of the intrinsic pathway of the apoptotic cascade occurs.

Figure 3.2 Activated Bax protein is predominantly expressed by distal tubular epithelial cells. While hardly detectable in control kidneys (A), Bax protein was increasingly expressed (red) in distal tubules in kidneys exposed to 30 min (B) or 80 min (C) of ischemia. In the ischemic sections also some focal staining of proximal tubules was observed (B,C). Proximal tubules were identified by FITC Lotus Lectin staining (green). Red staining, active Bax protein (Texas Red); blue staining, nuclei (4,6-diamidino(2)phenylindole). Magnification: x200. See page 159 for colour figure.

Figure 3.3 Depletion of renal Bcl-2 during normothermic ischemia. A) Representative immunoblot analysis of Bcl-2. B) Densitometric analysis of Bcl-2 immunoblotting with ischemic (n=5) and control kidneys (n=4). Data of ischemic kidneys are expressed as fraction of the mean of control data. Individual ischemic kidneys are displayed by different symbols (■, ▲, ○, □, △) and control kidneys by a filled circle. A significant correlation was detected between renal 26 kDa Bcl-2 levels and the length of normothermic ischemia ($r=0.86$, $P<0.001$, Spearman correlation analysis). Dotted line gives trendline of all data.

Figure 3.4 Immunofluorescence staining of Bcl-2 during renal normothermic ischemia. Renal Bcl-2 staining was most abundant (red) in proximal tubules of control tissue (A) and in kidneys rendered ischemic for 35 min (B) and 80 min respectively (C). Less staining was detected in distal tubules in both control and ischemic tissue. Proximal tubules were identified by FITC Lotus Lectin staining (green). Red staining, Bcl-2 protein (Texas Red); green staining, Lotus Lectin (fluorescein isothiocyanate); blue staining, nuclei (4,6-diamidino(2)phenylindole). Magnification: x200. See page 160 for colour figure.

Figure 3.5 Ischemia induces intrarenal caspase-9 activation. A) Representative immunoblot analysis of activated caspase-9. B) Densitometric analysis of caspase-9 immunoblotting with ischemic (n=5) and control kidneys (n=4). Data of ischemic kidneys are expressed as fraction of the mean of control data. Individual ischemic kidneys are displayed by different symbols (■, ▲, ○, □, △) and control kidneys by a filled circle. Activation of caspase-9 (34/37 kDa) is significantly correlated with the ischemia time ($r=0.79$, $P<0.001$, Spearman correlation analysis). Dotted line gives trendline of all data.

Initiation of the extrinsic pathway during normothermic renal ischemia

Next, we examined whether, besides initiation of the intrinsic pathway, also the extrinsic pathway was activated during ischemia. Activation of this route is regulated by the cytoplasmic protein cFLIP, a competitive inhibitor of caspase-8¹⁷. We therefore measured cFLIP levels during ischemia. Here we show that cFLIP (55 kDa) was normally expressed in the human kidney and got depleted during normothermic ischemia (Figure 3.6). Interestingly, the reduction of cFLIP levels significantly correlated with the duration of ischemia ($r=0.78$, $P<0.001$, Spearman correlation analysis). Subsequently, we determined whether cFLIP depletion results in activation of caspase-8, the initiator caspase of the intrinsic pathway. Similar levels of the pro-form of caspase-8 (43/44 kDa) were found in control and ischemic tissue (Figure 3.7A). In line, activated caspase-8 fragments were not detectable during normothermic ischemia (data not shown). Thus, although cFLIP was depleted during ischemia, no active caspase-8 was detected.

Figure 3.6 Depletion of cFLIP during normothermic renal ischemia. A) Representative immunoblot analysis of intrarenal cFLIP. B) Densitometric analysis of cFLIP immunoblotting with ischemic ($n=5$) and control kidneys ($n=4$). Data of ischemic kidneys are expressed as fraction of the mean of control data. Individual ischemic kidneys are displayed by different symbols ($\blacksquare, \blacktriangle, \circ, \square, \triangle$) and control kidneys by a filled circle. cFLIP (55 kDa) expression levels reduces in time with induction of the length of ischemia and this correlation is statistically significant ($r=0.78$, $P<0.001$, Spearman correlation analysis). Dotted line gives trendline of all data.

Figure 3.7 Processing of caspase-8 and caspase-3 during renal ischemia. A) The 54/55 kDa inactive isoforms of caspase-8 are detected in ischemic and control tissue. The 43/44 kDa fragment comprising the intermediate caspase-8 cleavage fragments are not detected (data not shown). A representative blot is shown. B) 34 kDa pro-form caspase-3 was present in healthy and ischemic tissue. Activation of caspase-3 could not be detected in any samples (data not shown). A representative blot is shown.

Activation of effector caspases during normothermic renal ischemia

To evaluate whether the observed activated intrinsic pathway leads to execution of the apoptotic process, processing of the central effector caspase, caspase-3 was determined. The caspase-3 proform (34 kDa) was detected in control tissue. The basal expression levels did not change during ischemia (Figure 3.7B). In line, fragments representing active caspase-3 were not observed. Taken together, our data implicate that during ischemia the circumstances are created for activation of the apoptotic cascade upon reperfusion.

Discussion

In the current study, we investigated the initiation of apoptotic cell-death during normothermic renal ischemia. In renal transplantation, normothermic warm ischemia occurs in NHB donation, especially in category 2 donors. This warm ischemia is considered to be the major cause of delayed graft function and or primary non-function. Also cold ischemia as occurs during preservation of donor organs can lead to organ damage. In cadaveric donors, cold ischemia time was associated with the numbers of apoptotic cells, present one hour after onset of reperfusion/transplantation^{5,14}. *In vitro* experiments showed that cold storage of proximal tubular cells for 48h resulted in activation of apoptosis, only after rewarming. The apoptotic process was not activated during cold storage¹⁸. Here we investigate initiation of the apoptotic process during normothermic ischemia in an ex vivo model. Initiation of the apoptotic cascade occurs either via activation of the extrinsic or the intrinsic pathway. Because regulation of the intrinsic pathway is accomplished in part through the stoichiometric balance of pro-apoptotic and anti-apoptotic Bcl-2 proteins^{12,19}, we studied alterations in levels of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) members of this protein family. Using a human model for renal normothermic ischemia we demonstrated that depletion of monomeric Bcl-2, that is able to prevent dimerization of the pro-apoptotic Bax, significantly correlated with the extent of ischemia. Our findings further extend recent data from Schwarz et al.²⁰. They showed that low mRNA levels for Bcl-2 in proximal tubular epithelial cells in pre-transplant cadaveric donor kidney biopsies are associated with apoptosis and subsequent ARF and delayed allograft function²⁰. These data support our findings that epithelial cells of the proximal tubules are the predominant site of renal Bcl-2 protein expression and that ischemia leads to reduced Bcl-2 levels. Next, we showed that protein levels of monomeric Bax are strongly enhanced by normothermic ischemia, predominantly in the early ischemic phase. These data are supported by findings from the group of Hébert, who showed in an experimental rat model elevation of renal Bax protein expression, 10 min after the induction of cardiac arrest²¹. Moreover, these western blotting data are completed by immunohistochemistry experiments with an ab that

detects active Bax. Immunohistochemistry data revealed that renal ischemia also resulted in activated Bax protein expression. Activated Bax was primarily detected in distal tubules. This increased distal active Bax expression and the decreased proximal Bcl-2 expression suggests a pro-apoptotic state in both distal and proximal tubular epithelium. At this stage there is no explanation for the observed diffuse expression of apoptotic proteins. Many mechanisms could be responsible such as differences in “stress” in the different parts of the tubule during ischemia. It is generally said that the proximal part is more sensitive to ischemia and reperfusion with more necrotic cell-death while the distal tubules suffer more from apoptotic cell-death⁹. However, it is not completely elucidated as yet which part of the nephron suffers the most severe after an ischemic insult^{22,23}.

Next, for the first time we showed that a significant correlation exists between activation of caspase-9 and the duration of normothermic renal ischemia. The observed activation of caspase-9 in the present study did however not result in activation of the downstream caspase-3 during normothermic ischemia. Activation of this central effector caspase-3 is essential for cleavage of different substrates resulting in execution of the apoptotic process. Previous published findings in experimental models have been contradictory as to whether caspase-3 is induced during ischemia or rather during reperfusion²⁴⁻²⁷. We do not have an explanation for the observed data that activated caspase-9 fails to activate caspase-3 at this stage. However, it is known that next to caspases, also XIAP, an inhibitor of caspase-3, is recruited to the apoptosome²⁸. How the inhibitory effect of XIAP is overcome and cellular caspase-3 activation and cell death can take place is at this stage unknown. Possibly reperfusion is necessary as major insult to overcome the protective effect of XIAP, resulting in cell death.

Besides activation of the intrinsic pathway, we also demonstrate that the extrinsic pathway is already initiated during ischemia. In particular, we show that depletion of the competitive caspase-8 inhibitor, cFLIP, significantly correlated with the length of normothermic renal ischemia. These findings further extend data from Rasper et al. who showed significant cFLIP depletion after ischemia in the reperfused heart¹⁷. Since cFLIP prevents death-receptor mediated apoptosis by precluding recruitment of caspase-8 to the DISC^{17,29}, reductions of the cFLIP content facilitate caspase-8 activation. However, in our model cFLIP depletion was not accompanied by caspase-8 activation during ischemia, indicating that low cFLIP levels alone are insufficient to activate the intrinsic pathway. Presumably, activation of the extrinsic pathway needs conditions that are only met during reperfusion. Indeed, in a rat cardiac I/R model it has been demonstrated that caspase-8 activation occurs in the reperfusion phase, but is not processed by ischemia alone³⁰.

Taken together, we show in a human model for renal normothermic ischemia that the intrinsic as well as the extrinsic pathway of apoptosis are initiated during ischemia. These data indicate that during ischemia the circumstances are created that facilitate execution of apoptosis upon reperfusion. This hypothesis is supported by data

obtained in our murine model of renal I/R. In this model, we confirmed the observation that initiation of the apoptotic process emerges during ischemia. Furthermore, the length of the ischemic period corresponds with the extent of apoptosis and the concomitant organ function during reperfusion (unpublished findings). Potentially, assessment of proteins involved in the early apoptotic process in pre-transplant kidney biopsies might be helpful to estimate ischemic damage and predict concomitant graft function. Evidence that apoptosis is crucially involved in clinical transplantation has been provided by different groups, showing that apoptotic cell death is enhanced in post-reperfusion biopsies from cadaveric allografts compared with living related renal transplants^{5,14}. However, these studies report apoptotic cell death in brain death donors. In these donors, besides ischemia, other factors such as periods of diminished organ perfusion, hemodynamic instability and activation of the immune-system affect post-transplant organ function³¹. In contrast, our model is restricted to the effect of ischemia itself, thereby reflecting the situation in NHB organ donation, in which warm ischemia forms the major determinant of graft outcome. Therefore, our novel findings that ischemia alone, already initiates the apoptotic process, might explain the increased risk for NHB graft dysfunction. In conclusion, in a human model for renal normothermic ischemia we demonstrate that the apoptotic cascade is initiated during ischemia. For the first time, the time dependent regulation of proteins responsible for the onset of the apoptotic cascade in the course of normothermic ischemia is shown. Determination of these processes might be beneficial in assessing the degree of ischemic injury in NHB donor kidneys and other organs. Furthermore, these new insights can be explored for the development of specific anti-apoptotic treatment to prevent organ damage upon transplantation due to ischemic injury.

References

1. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. *Transplant Proc* 1995;27:2893-2894.
2. Nicholson ML, Metcalfe MS, White SA, Waller JR, Doughman TM, Horsburgh T, Feehally J, Carr SJ, Veitch PS. A comparison of the results of renal transplantation from non-heart-beating, conventional cadaveric, and living donors. *Kidney Int* 2000;58:2585-2591.
3. Daemen MA, van 't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenabeele P, Buurman WA. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest* 1999;104:541-549.
4. Nogae S, Miyazaki M, Kobayashi N, Saito T, Abe K, Saito H, Nakane PK, Nakanishi Y, Koji T. Induction of apoptosis in ischemia-reperfusion model of mouse kidney: possible involvement of Fas. *J Am Soc Nephrol* 1998;9:620-631.
5. Burns AT, Davies DR, McLaren AJ, Cerundolo L, Morris PJ, Fuggle SV. Apoptosis in ischemia/reperfusion injury of human renal allografts. *Transplantation* 1998;66:872-876.
6. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;281:1312-1316.
7. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269-290.
8. Krueger A, Baumann S, Krammer PH, Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* 2001;21:8247-8254.
9. Saikumar P, Venkatachalam MA. Role of apoptosis in hypoxic/ischemic damage in the kidney. *Semin Nephrol* 2003;23:511-521.
10. Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000;6:513-519.
11. Adrain C, Martin SJ. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* 2001;26:390-397.
12. Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. Apoptosis: definition, mechanisms, and relevance to disease. *Am J Med* 1999;107:489-506.
13. Antonsson B. Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. *Cell Tissue Res* 2001;306:347-361.
14. Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, Feuerstein D, Kaskel FJ, Tellis V, Devarajan P. Activation of mitochondrial apoptotic pathways in human renal allografts after ischemia reperfusion injury. *Transplantation* 2003;76:50-54.
15. Markowitz GS, Radhakrishnan J, Kambham N, Valeri AM, Hines WH, D'Agati VD. Lithium nephrotoxicity: a progressive combined glomerular and tubulointerstitial nephropathy. *J Am Soc Nephrol* 2000;11:1439-1448.
16. Wever PC, Aten J, Rentenaar RJ, Hack CE, Koopman G, Weening JJ, et al. Apoptotic tubular cell death during acute renal allograft rejection. *Clin Nephrol* 1998;49:28-34.
17. Rasper DM, Vaillancourt JP, Hadano S, Houtzager VM, Seiden I, Keen SL, Tawa P, Xanthoudakis S, Nasir J, Martindale D, Koop BF, Peterson EP, Thornberry NA, Huang J, MacPherson DP, Black SC, Hornung F, Lenardo MJ, Hayden MR, Roy S, Nicholson DW. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ* 1998;5:271-288.
18. Salahudeen AK, Huang H, Joshi M, Moore NA, Jenkins JK. Involvement of the mitochondrial pathway in cold storage and rewarming-associated apoptosis of human renal proximal tubular cells. *Am J Transplant* 2003;3:273-80.
19. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322-1326.
20. Schwarz C, Hauser P, Steininger R, Regele H, Heinze G, Mayer G, Oberbauer R. Failure of BCL-2 up-regulation in proximal tubular epithelial cells of donor kidney biopsy specimens is associated with apoptosis and delayed graft function. *Lab Invest* 2002;82:941-948.
21. Bellemare S, Vigneault N, Madore F, Raymond MA, Caillier JF, Hebert MJ. Enhanced development of caspase-independent cortical cell death during cold storage in kidneys of non-heart-beating donors. *Transplantation* 2002;73:1742-1751.
22. Lieberthal W, Nigam SK. Acute renal failure. I. Relative importance of proximal vs. distal tubular injury. *Am J Physiol* 1998;275:F623-631.

23. Padanilam BJ. Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. *Am J Physiol Renal Physiol* 2003;284:F608-627.
24. Borutaite V, Jekabsone A, Morkuniene R, Brown GC. Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome c release and apoptosis induced by heart ischemia. *J Mol Cell Cardiol* 2003;35:357-366.
25. Soeda J, Miyagawa S, Sano K, Masumoto J, Taniguchi S, Kawasaki S. Cytochrome c release into cytosol with subsequent caspase activation during warm ischemia in rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001;281:G1115-1123.
26. Benchoua A, Guégan C, Couriaud C, Hosseini H, Sampaio N, Morin D, Onténiente B. Specific caspase pathways are activated in the two stages of cerebral infarction. *J Neurosci* 2001;21:7127-7134.
27. Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J. Reperfusion induces myocardial apoptotic cell death. *Cardiovasc Res* 2000;45:651-660.
28. Bratton SB, Walker G, Srinivasula SM, Sun XM, Butterworth M, Alnemri ES, Cohen GM. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *Embo J* 2001;20:998-1009.
29. Tschopp J, Irmeler M, Thome M. Inhibition of fas death signals by FLIPs. *Curr Opin Immunol* 1998;10:552-558.
30. Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, Latchman DS. Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. *Circ Res* 2002;90:745-748.
31. van der Hoeven JA, Molema G, Ter Horst GJ, Freund RL, Wiersema J, van Schilfgaarde R, Leuvenink HG, Ploeg RJ. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. *Kidney Int* 2003;64:1874-1882.

Chapter 4

Increased release of sMD-2 during human endotoxemia and sepsis; a role for endothelial cells

Tim GAM Wolfs, Irène Dunn-Siegrist, Cornelis van 't Veer, Caroline MIM Hodin, Wilfred TV Germeraad, Marieke AD van Zoelen, Robert-Jan van Suylen, Carine J Peutz-Kootstra, Greg Elson, Jérôme Pugin, Wim A Buurman

Molecular Immunology 2008;45:3268-77

Abstract

MD-2 is the crucial cofactor of TLR4 in the detection of LPS. Here, we show that soluble MD-2 (sMD-2) circulates in plasma of healthy individuals as a polymeric protein. The total amount of sMD-2 in septic plasma was strongly elevated and contained both sMD-2 polymers and monomers, the latter representing the putative biologically active form of MD-2. Moreover, during experimental human endotoxemia, the monomeric and total sMD-2 content in plasma increased with the kinetics of an acute phase protein. The increase in sMD-2 monomers was paralleled by enhanced TLR4 costimulatory activity. The presence of functional sMD-2 during endotoxemia and sepsis was confirmed by immunodepletion. Immuno-histochemistry revealed that MD-2 expression in septic patients was strongly enhanced on endothelium and multiple inflammatory cells in lung and liver. *In vitro* studies showed that sMD-2 release appears to be restricted to endothelial cells and dendritic cells. Release of sMD-2 by endothelial cells was strongly enhanced by LPS and TNF- α stimulation. Taken together, this study demonstrates the increase of both circulating polymeric and functional monomeric sMD-2 during endotoxemia and sepsis, and evidence is provided that the endothelium is involved in this process.

Introduction

Recognition of invading microbial pathogens by the innate immune system leads to an inflammatory response aimed at elimination of the infecting microbes until the adaptive immunity becomes functional. This innate immune reaction towards pathogens is predominantly initiated and controlled by Toll-like receptors, a family of proteins that specifically recognizes and senses microbial products. LPS, produced by Gram-negative bacteria, is a very potent and abundant bacterial product that is believed to be one of the central mediators in Gram-negative sepsis. LPS activates the host innate immune system via TLR4, thereby initiating a cascade of serine/threonine kinases that ultimately results in the transcription of genes involved in inflammation¹. It has been clearly demonstrated that MD-2 is the crucial cofactor of TLR4 mediated LPS signaling, a process that is greatly enhanced by CD14 and the serum component LPS-binding protein²⁻⁴. Although MD-2 has been reported to be required for TLR4 membrane expression, a recent study indicates that TLR4 is also transported to the cell surface in the absence of MD-2⁵. TLR4/MD-2 transfection experiments have shown that MD-2 produced in excess is secreted as a soluble protein (sMD-2)^{6,7} and exists as a heterogeneous collection of disulfide-linked oligomers⁸.

Recently, direct evidence for the presence of circulating sMD-2 has been provided⁹ and an earlier study suggested that plasma derived sMD-2 could function as costimulatory molecule in the response of TLR4 transfected cells to LPS¹⁰. Furthermore, *in vitro* studies have shown that sMD-2 can directly bind LPS with high affinity and that such LPS/MD-2 complexes form an active ligand capable of activating TLR4 positive cells¹¹⁻¹³. Interestingly, evidence has been provided that only monomeric sMD-2 can associate with TLR4¹⁴⁻¹⁶. This finding has been confirmed and further extended by a report showing that only monomeric sMD-2 is able to bind LPS¹⁷.

The current study was designed to evaluate the biochemical and functional properties of sMD-2 in human plasma during systemic inflammation, such as that encountered during sepsis and experimental human endotoxemia. To this end, high affinity monoclonal antibodies directed to MD-2 were developed that recognize both soluble MD-2 and TLR4-bound MD-2.

Materials and methods

Healthy controls and patients with severe sepsis and septic shock

Patients meeting the criteria for severe sepsis and septic shock were prospectively enrolled in the study¹⁸. Sepsis is as a systemic host response to infection, manifested by conditions such as temperature >38°C or <36°C; heart rate >90 beats per minute and respiratory rate >20 breaths per minute or PCO₂. Severe sepsis is defined as sepsis associated with organ failure and septic shock is severe sepsis associated with

cardiovascular failure necessitating the administration of vasopressor agents¹⁹. The research protocol was approved by the Ethic Committee of the University Hospital of Geneva. An informed consent was provided according to the Declaration of Helsinki. In total, 34 subjects were included in the study. The study population comprised 12 healthy volunteers (six men, six women; mean age $\pm$ 1SD, 52 ± 12), 11 patients with severe sepsis (five men, six women; 59 ± 12) and 11 patients with septic shock (six men, five women; 61 ± 16).

Blood samples of patients and healthy controls were taken using evacuated blood collection tubes containing EDTA. Blood samples were immediately put on melting ice, and plasma was prepared by centrifugation at 4000 rpm for 5 min at 4°C. Supernatants were stored in aliquots at -20°C. Plasma samples of septic patients were obtained within 48 hours after admission to the medical ICU. The ICU mortality in patients with severe sepsis and septic shock was 36%. The main infectious source was severe community acquired pneumonia (n=7). Other sources were abdominal sepsis (n=5), nosocomial pneumonia (n=3), bacterial endocarditis (n=2), primary bacteremia (n=2), osteomyelitis (n=1), myositis (n=1) and urosepsis (n=1). Bacterial pathogens could be isolated in 7 out of 11 patients with sepsis (2 *S.aureus*, 2 *L.pneumophila*, 1 *E.coli*, 1 *B.fragilis*, and 1 *S.epidermidis*) and in 10 out of 11 patients with septic shock (3 *S.aureus*, 2 *H.influenzae*, 2 *E.coli*, 1 *S.epidermidis*, 1 *S.pneumoniae* and 1 *P.aeruginosa*).

Endotoxemia in human volunteers

The study was approved by the institutional scientific and Ethics Committee of the Academic Medical Center, University of Amsterdam. Written informed consent was obtained from each subject before the start of the study. Eight healthy male volunteers (25-35 years) were challenged with an i.v. bolus of 4 ng/kg LPS (*E.coli* LPS, lot G; US Pharmacopeia, Rockville, MD) as described before²⁰. Blood was obtained directly before LPS administration (0 hours), and 2, 4, 6, 8, 10, 23 hours thereafter.

Autopsy tissue from septic patients and controls for immunohistochemistry

Formalin fixed, paraffin embedded lung, liver, kidney, lymph node and spleen tissue from four patients that died because of a bronchopneumonia related sepsis was obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands". Tissues used in this study were collected within 24 hours after death of the patient. Normal tissue was sampled from four patients of each group who underwent resection for a carcinoma. Samples were taken from healthy parts of the organ, distant from the tumor.

Expression of human MD-2 IgGFc and MD-2-Flag fusion proteins

To avoid multimeric MD-2 formation after transfection, full length human MD-2 was amplified from a clone containing MD-2 cDNA with mutated cysteines kindly provided by Dr. M. Kennedy and Dr. D. Segal National Institutes of Health, Bethesda, MD). DNA encoding the hinge, CH2, and CH3 exons derived from human IgG1 was amplified from marathon ready spleen cDNA (Clontech, Palo Alto, CA). Primers used for amplification incorporated restriction sites enabling in frame ligation into the expression vector pcDNA3.1- (Invitrogen, Carlsbad, CA). In order to increase the mRNA translation efficiency, the Kozak consensus translation initiation site was introduced using the QuikChange XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Clones containing the correct DNA sequences were identified by sequencing using the Big dye termination cycle sequencing kit (Perkin Elmer/Cetus, Emeryville CA), according to the manufacturer's instructions. Next, COS-1 cells were transfected with the expression vector using lipofectamine (Roche, Basel, Switzerland) according to the manufacturer's instructions and stable lines were established by selection with 700 µg/ml G418 (Life Technologies, Palo Alto, CA). Supernatant from stable transfectants was collected and passed over a rat anti-human IgGfc1 column. Bound protein was eluted with 0.1 M glycine pH 2.6 and collected in 1/10 volume 1 M Tris pH 9.0 to neutralize the acidic pH. Purity was assessed by SDS PAGE and coomassie staining.

Recombinant MD-2-Flag fusion protein was produced by COS-1 cells, stably transfected with Dr. K. Miyake's pEFBOS-hMD-2-FLAG (kindly provided via Dr. C. Kirschning, Munich, Germany). After centrifugation, supernatant was removed and the pellet was washed three times in PBS and lysed in buffer containing 200 mM NaCl, 10 mM Tris base, 5 mM EDTA, 10% Glycerin, 1mM PMSF, 0.1 U/ml Aprotinin and 1 µg/ml Leupeptin. The protein concentrations of human MD-2-IgG and MD-2-Flag were determined using an anti-human IgG or MD-2 ELISA respectively. Equal quantities of MD-2-IgGfc and MD-2-Flag based on standardization of these ELISAs with purified MD-2-IgGfc displayed bands of identical intensity on Western blotting.

Generation of monoclonal antibodies

Balb/C mice, obtained from Charles River Breeding Laboratories (Heidelberg, Germany) were immunized repeatedly with 10 µg human MD-2-IgGfc according to a protocol approved by the Institutional Animal Care Committee of the University of Maastricht. MAbs for human MD-2 were produced by standard methods using SP2/0 cells as fusion myeloma. All mAbs (HM30-33) detected specifically MD-2 on TLR4/MD-2 transfected HEK cells and sMD-2 in ELISA and western blot and were determined as IgG1 isotypes using the IsoStrip mouse mAb isotyping kit (Hycult biotechnology, Uden, the Netherlands). HM30 and HM31 were used in ELISA, HM31 in western blotting, HM32 in immunohistochemistry and HM33 in flow cytometry.

Inhibition studies revealed that two different epitopes are recognized by our mAbs; HM30/32 react with the first and HM31/33 with a second epitope.

sMD-2 ELISA

A 96-well plate was coated overnight at 4°C with 5 µg of HM30 anti-human MD2 mAb. A standard titration curve was obtained by serial dilution of a known quantity of the human MD-2-IgGFc recombinant protein. Test samples were diluted at least six fold and incubated for 1h at room temperature. Next, the plates were incubated with biotin-conjugated anti-human MD2 mAb HM31 (2 µg/ml), followed by washing and incubation with streptavidin peroxidase for 1h. After adding 3,3',5,5'-tetramethylbenzidine (TMB) substrate, the reaction was stopped with acid and the OD was determined at 450 nm. The sandwich ELISA detected the MD-2 protein in cellular lysate and cellular supernatant of both MD-2 and TLR4/MD-2 transfected cells. The analysis of plasma samples with the ELISA was hampered by lack of consistency when compared with western blot. We considered that the formation of large complexes of sMD-2 disturbs the correct quantification of sMD-2 in plasma.

Western blotting

Plasma aliquots were heated in Laemmli buffer with or without 0.1 M DTT, separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). Membranes were incubated with 1 µg/ml biotinylated anti-human MD-2 (HM31), followed by streptavidin peroxidase. Positive bands were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Chemical Co., Rockford, IL) and transferred onto an X-ray film.

Quantitative sMD-2 analysis

For this assay, band intensities of the sMD-2 isoforms (17, 20 and 25 kDa), derived after reduced western blotting were determined using a FluorS Multi-Imager system and Quantity One software (Bio-Rad, Hercules, CA). A protein standard was made by lysis of MD-2 Flag transfected HEK cells which was quantified for MD-2 by the above described ELISA. For quantitative western blot, the total amount of sMD-2 in each plasma sample was calculated in the linear range of a serial dilution of the MD-2 standard with a range of 0.5 to 10 ng/ml.

Depletion of native sMD-2 from plasma

To deplete native sMD-2 from septic plasma in a single step by immunodepletion, the murine anti-human MD-2 mAb (HM32) was coupled to CNBr-Sepharose beads (750 µg/ml gel; Amersham, Uppsala, Sweden). Human septic plasma was diluted

2.5 fold in PBS and incubated at 4°C for 5 hours with the mAb coated beads. Depleted plasma was collected by centrifugation.

MD-2 functional analysis

TLR4-HEK293 cells were used for stimulation assays as described before¹⁰. Briefly, on the day of the assay, TLR4-HEK293 cells were collected by trypsin treatment, resuspended in DMEM (Gibco) containing 10% FCS and distributed into 96-well plates at the concentration of 60,000 cells per well. Plasma from controls, patients and LPS volunteers was added at a final concentration of 5%. Ultrapure *E.coli* K12LCD25 LPS (Invivogen) was added to the wells at a final concentration of 30 ng/ml and incubated at 37°C for 24 hours. Conditioned supernatants were then collected and assayed for human IL-8 protein by ELISA using paired monoclonal antibodies (Endogen, Woburn, MA).

Endothelial cells

HUVEC or immortalized HUVEC (ECRF-24) were cultured and stimulated in culture medium as described previously²¹. When both HUVEC and ECRF-24 cells were used in parallel experiments, similar results were obtained. In line with this, the phenotype of both endothelial cell types was previously shown to be similar²¹.

Isolation of peripheral blood leukocytes

Monocytes, lymphocytes and granulocytes were isolated on a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). The interface containing mononuclear cells was collected, as well as the pellet containing the granulocytes. The latter cells were isolated by lysis of erythrocytes with ammonium chloride and washed twice with PBS. Isolated monocytes were differentiated into immature (iDC) and mature (DC) dendritic cells respectively as previously described²².

Flow cytometric analysis

Flow cytometric analysis was performed on peripheral blood leukocytes and endothelial cells.

Phycoerythrin (PE), and allophycocyanin (APC) conjugated anti-human CD3 (PE), CD19 (APC) and CD14 (PE) monoclonal antibodies (all from BD Bioscience, Erembodegem, Belgium) were used to identify T-cells, B-cells and monocytes, respectively. Immature and mature DC populations were double-gated during analysis on forward scatter (FSC) vs. side scatter (SSC) and HLA-DR+++ vs. CD11c+++ expression and granulocytes were identified and gated on FSC vs. SSC. In each measurement 15.000 events were acquired, the live gate containing between 8.000 and 10.000 cells. The lineage vs FSC gate contained between 9000 cells (T-cells) and 1000 cells (B-cells). Cultured

endothelial cells were harvested by scraping and gated on FSC vs. SSC. For analysis of these cells, 15000 events were acquired and the live gate contained between 9000 and 10.000 cells. Anti-human MD2 18H10 mAb, generated as previously described¹⁰ and FITC labeled HM33 were used to detect the MD-2 protein in the above described cells. To demonstrate protein surface expression, cells were washed in FACS buffer with the predetermined appropriate antibody dilution or with the corresponding isotype controls. Analysis was performed on a FACSort cytometer (BD).

Immunohistochemistry

Paraffin sections of lung and liver tissues were incubated with the anti-human MD-2 HM31 mAb, followed by incubation with a rabbit anti-mouse biotinylated secondary antibody (DAKO, Glostrup, Denmark). Staining was obtained with a streptavidin-biotin HRP system (DAKO) and 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, Mo). Nuclei were counterstained with haematoxylin. Micro-photographs were made using a Nikon Eclipse E800 microscope and a Nikon digital camera DXM1200F.

Statistical analysis

Quantitative data are presented as mean $\pm$ SEM. The Mann-Whitney *U*-test was used to compare sMD-2 concentrations in plasma from healthy volunteers and septic patients. The unpaired Student's *t*-test was used to compare IL-8 concentrations in supernatant from *in vitro* experiments. The paired Student's *t*-test was used to compare plasma obtained at various timepoints after LPS infusion in healthy volunteers. Differences were considered statistically significant at $P < 0.05$.

Results

Assessment of sMD-2 in plasma of septic patients

Recombinant sMD-2 has been reported to multimerize, resulting in the formation of large, inactive oligomers^{6,14}, whilst monomeric sMD-2 is supposed to be the active form involved in LPS recognition and cell signaling. Figure 4.1A shows the molecular appearance of the native sMD-2 protein in plasma. Western blotting under non reduced conditions showed that sMD-2 in the circulation of healthy and septic individuals consists primarily of multimers. Interestingly, monomeric sMD-2 (17-25 kDa) was only present in plasma of septic patients (Figure 4.1A). Western blot analysis with lower quantities of healthy and septic plasma showed that all multimeric forms of sMD-2 in septic plasma are elevated when compared to control plasma, indicating that the increased amount of monomeric MD-2 as seen during sepsis is not primarily caused by a change from polymeric to monomeric MD-2 (data not shown).

To demonstrate the specificity of the antibodies, recombinant MD-2 Flag was included as a positive control (Figure 4.1A+B).

Reduction of the disulfide bonds of sMD-2 give rise to monomeric subunits of approximately 17, 20 and 25 kDa, corresponding with the calculated mass of monomeric MD-2. The 3 isoforms most likely correspond to the unglycosylated (17 kDa) and glycosylated (20 and 25 kDa) forms of MD-2. Reduction of sMD-2 allowed us to quantify the total amount in both control and septic plasma by western blotting (Figure 4.1B). Circulating sMD-2 levels were strongly elevated during sepsis (mean $\pm$ 1SD) 13.1 ± 5.67 ng/ml when compared with healthy controls 2.33 ± 0.52 ng/ml ($P=0.0001$) (Figure 4.1C). Next we analyzed, whether the sMD-2 protein detected in septic plasma conferred LPS responsiveness to TLR4 transfected HEK cells. Plasma of both septic patients and LPS volunteers lacked detectable amounts of LPS as previously reported²³. In concordance with earlier findings, these experiments clearly demonstrate the LPS stimulating capacity of septic plasma¹⁰ ($P<0.0001$, unpaired Student's t-test) (Figure 4.1D). To confirm the sMD-2 dependent TLR4 costimulatory effect of septic plasma, the protein was depleted using anti-MD-2 coated sepharose beads (Figure 4.1E). As depicted in Figure 4.1D, the LPS stimulating capacity of sMD-2 depleted septic plasma was significantly reduced ($P<0.0001$, unpaired Student's t-test). Taken together, the data show that plasma of healthy individuals contains only sMD-2 polymers while septic plasma also contains detectable amounts of sMD-2 monomers, which are most likely responsible for the TLR4 costimulatory capacity detected with septic plasma.

Kinetics of the sMD-2 release during experimental human endotoxemia

In order to examine the kinetics of the release of monomeric and total sMD-2, we made use of a human endotoxemia model. This well characterized model is used for proof of concept studies involving systemic inflammation triggered by the innate immune system in man. Healthy volunteers were administered with an i.v. endotoxin bolus of 4 ng/kg, and blood was collected at various time points before and after endotoxin administration. Reducing western blot analysis demonstrated in all individuals that circulating sMD-2 protein levels started to rise significantly ($P<0.001$, Paired Student's t-test), 6 hours after endotoxin infusion (Figure 4.2A+B). Also plasma from endotoxemic volunteers (as septic patients) showed enhanced levels of sMD-2 monomers (Figure 4.2C; non reducing conditions). Next, we investigated whether the rise in active monomeric sMD-2 also leads to increased MD-2 activity, using the above described LPS stimulated TLR4 assay. Levels of activation were significantly higher in plasma samples obtained from 6 till 23 hours post endotoxin infusion compared with plasma's sampled before LPS administration ($P<0.001$, Paired Student's t-test) (Figure 4.2D).

Figure 4.1 Detection of soluble MD-2 in plasma from healthy individuals and septic patients. Western blot analysis was performed to analyse the molecular appearance of sMD-2 in plasma. **A)** Under non-reduced conditions, sMD-2 in control (lane 1+2) and septic plasma (lane 3+4) is mainly present as multimers (≥ 50 kDa). Two representative samples are shown. Recombinant MD-2 Flag (P) was used as positive control. Hardly any monomeric (active) sMD-2 was ascertained in plasma of healthy individuals under non-reduced conditions (lane 1+2). In contrast, during sepsis monomeric sMD-2 was clearly present (lane 3+4). For both control ($n=7$) and septic plasma's ($n=12$), representative samples are shown. **B)** The total sMD-2 content in plasma was analysed by immuno-blotting under reduced circumstances. Small amounts of sMD-2 were detected in healthy controls (lane 1-3), whereas in plasma from septic patients (lane 4-6), sMD-2 protein levels were clearly elevated. For both control ($n=12$) and septic plasma's ($n=22$), representative samples are shown. Recombinant MD-2 Flag (P) was used as positive control. **C)** The total sMD-2 concentration in randomly selected control ($n=7$) and septic ($n=10$) plasma was estimated by quantitative western blot analysis and results are depicted in a scatter plot. **D)** Activation of TLR4 positive HEK cells by LPS is significantly (*) promoted by plasma of septic patients with IL-8 as read out. IL-8 secretion upon LPS activation of TLR4 transfected HEK cells is significantly (**) inhibited in the presence of sMD-2 depleted septic plasma when compared with untreated plasma of septic patients. **E)** Reduced western blot analysis confirmed a strong decrease of the total sMD-2 content, after sMD-2 depletion of septic plasma. Three samples before (1,3,5) and after immuno-depletion of sMD-2 (2,4,6) are shown.

Figure 4.2 Release of functional sMD-2 in experimental human endotoxemia. Reduced western blot analysis demonstrates that i.v. administration of LPS results in the enhanced release of the total sMD-2 content in plasma. A) A representative sample (n=8) is shown and time in hours in relation to LPS injection is given. B) The total sMD-2 content during endotoxemia was analysed by quantitative immuno-blotting under reduced circumstances. Statistical significance (*) was reached from 6 hours post LPS infusion. C) Western blotting under non reduced circumstances showed enhanced release of MD-2 monomers in plasma, starting 6 hours post LPS infusion. A representative sample is shown (n=8) and time in hours in relation to LPS injection is given. D) Plasma samples of LPS volunteers support LPS activation of TLR4 transfected HEK cells. Functional analysis shows that the detected increase of sMD-2 in plasma during endotoxemia is mirrored by the LPS supporting activity of TLR4 transfected HEK cells. Delta IL-8 is the difference between the IL-8 concentrations in supernatants of LPS stimulated versus unstimulated TLR4 positive HEK cells in the presence of volunteer plasma for each individual timepoint. Statistical significance (*) was reached from 6 hours post LPS infusion. The average for the outcome of six different experiments is shown and time in hours in relation to LPS injection is given.

Cellular source of sMD-2

To identify the source responsible for the enhanced (active) sMD-2 content during human endotoxemia and sepsis, we first examined the cell specific expression of MD-2 in the healthy and septic lung and liver, organs critically involved in the response to LPS^{24,25}. In lung sections from septic patients, MD-2 expression was abundantly expressed by endothelial cells covering small and medium sized arteries and arterioles (Figure 4.3A). MD-2 expression was also localized in different subsets of inflammatory cells and pericytes. In healthy control tissue, moderate MD-2 expression

by endothelial and inflammatory cells was occasionally observed (Figure 4.3B). Incubations using isotype matched control ab's were negative for healthy and septic lung tissues (Figure 4.3C). In the healthy liver, MD-2 positive cells were sporadically detected (Figure 4.3D). During sepsis, MD-2 protein was predominantly located in endothelial cells lining hepatic sinusoids, Kupffer cells and inflammatory cells (Figure 4.3E). In addition, the distribution of MD-2 in the kidney, spleen and lymph nodes was assessed (data not shown). In line with the constitutive pulmonary and hepatic MD-2 expression, multiple inflammatory cells appeared to be weakly positive for MD-2 in these organs. In kidneys, moderate epithelial staining was detected. During sepsis, MD-2 staining was not increased in the lymph nodes. In the septic spleen, some MD-2 positive endothelial cells were detected. Although during sepsis, a moderate increase of the renal and splenic MD-2 expression (mainly renal epithelial cells and some inflammatory cells) was detected, by far the strongest increase was observed in the liver and lung.

Since the immunohistochemical analysis revealed that MD-2 *in vivo* is predominantly expressed by different subsets of inflammatory cells and the endothelium, we further analyzed the protein distribution by flow cytometry. Expression was found on monocytes, B-lymphocytes, granulocytes, immature and mature dendritic cells and endothelial cells whereas no MD-2 expression by T-lymphocytes was detected (Figure 4.4). The increase in mean fluorescence intensity (MFI) for MD2 vs Isotype are:

iDCs: 6.9x (mean = 267.4 vs. 38.7), mDCs: 4.3x (mean = 168.3 vs. 39.1), monocytes: 2.7x (mean = 17.8 vs. 6.6), granulocytes: 2.3x (mean = 7.9 vs. 3.4), B cells: 1.7x (8 vs. 4.7), endothelial cells: 6.1 (mean = 72.5 vs. 11.8) and T cells: no increase (4 vs. 4).

Next, reduced western blotting was used to demonstrate the presence of sMD-2 in culture supernatants from the cells described above. The data showed that basal sMD-2 secretion was restricted to endothelial cells and dendritic cells (Figure 4.5A+B). The latter finding confirms and extends a report from Visintin who suggested the release of sMD-2 by human monocyte-derived immature DCs (iDCs)⁶. Next, we investigated the release of sMD-2 by cells present in circulating blood and endothelial cells in response to LPS in order to further define the cellular source responsible for the enhanced sMD-2 release as seen during endotoxemia and sepsis. Cultures of whole blood, isolated peripheral blood mononuclear cells (PBMC's) and endothelial cells incubated for 0, 2, 6 and 24h with or without 100 ng/ml LPS were analyzed for sMD-2 release. Small amounts of sMD-2 were measured in supernatants from unstimulated endothelial cells while exposure to LPS resulted in increased sMD-2 release, in a time-dependent-fashion up to 24h (Figure 4.5C). We subsequently investigated the sMD-2 production by endothelial cells after treatment with the LPS response related cytokines TNF- α and IL-1 β ²⁶. Stimulation of endothelial cells with TNF- α raised sMD-2 levels over time, in a pattern similar to that observed upon LPS activation (Figure 4.5D). In a similar fashion, IL-1 β was observed to induce the release of sMD-2 by endothelial cells (Figure 4.5E). Without stimulation, sMD-2 did not

accumulate over time (data not shown). IL-8 release was monitored as positive control for endothelial cell activation (Figure 4.5F).

Figure 4.3 *In vivo* MD-2 expression in lung and liver. Paraffin sections of autopsy lung and liver tissue from patients that died of bronchopneumonia related sepsis (n=4) were immunostained with anti MD-2. A) In septic lungs MD-2 was clearly detected in endothelial cells (indicated by arrows) and pericytes (arrow heads). Asterix denote MD-2 positive inflammatory cells. B) In healthy lung (n=4), some endothelial (arrows) and inflammatory cells (arrow heads) stained weakly positive for MD-2. C) No AEC reaction product in septic lung tissue was detected using a mouse isotype control. D) In the healthy liver (n=4), MD-2 positive cells were hardly detected. E) MD-2 positive staining in the septic liver is represented by endothelial cells (arrow), Kupffer cells (arrow head) and inflammatory cells (inset) (original magnification 200x). See page 161 for colour figure.

In contrast to endothelial cells, isolated PBMC's cultured for up to 24 hours in the presence and absence of LPS, did not release detectable amounts of sMD-2 (Figure 4.5G). In line, sMD-2 levels did not increase in whole blood cultures with LPS (Figure 4.5H).

The apparent capacity of endothelial cells to release enhanced levels of sMD-2 upon LPS stimulation prompted us to determine the MD-2 surface expression of LPS treated endothelial cells by Flow cytometric analysis. The extracellular MD-2 expression of unstimulated cells (mean = 72.5 vs. 11.0) (Figure 4.6A) remained unaltered after 2h of LPS stimulation (mean = 73.1 vs. 11.3) (Figure 4.6B). In contrast, after 4 hours of exposure to LPS (Figure 4.6C), 30% decrease of the MD-2 surface expression was observed (mean = 49.5 vs 11.3). Although 22h post LPS treatment the MD-2 expression remained reduced when compared with untreated cells, the expression levels tended to recover (mean = 57.2 vs. 12.1) (Figure 4.6D). Thus, at timepoints when sMD-2 levels increase in the endothelial cell supernatant (between 2 and 6 hours post LPS treatment), MD-2 surface expression starts to decrease.

Figure 4.4 MD-2 expression by hematopoietic cells. Flow cytometric analysis of MD-2 expression on peripheral blood cells, cultured monocyte derived dendritic cells and endothelial cells (n=4). The histograms show MD-2 expression stained with anti-MD-2 antibody (HM33) (black lines). Gray lines denote isotype control staining.

Figure 4.5

Activation of endothelial cells by LPS and TNF- α leads to sMD-2 release. sMD-2 in supernatants ($n=4$) of cultured endothelial cells (A) and iDCs (B) was detected by reduced western blot analysis. Enhanced release in a time dependent fashion of sMD-2 in supernatants of LPS (C) or TNF- α (D) activated HUVEC's was shown by reduced western blotting ($n=4$). E) A smaller sMD-2 increase in cell supernatant of IL-1 β activated endothelial cells was observed, at 24 hours after stimulation. F) Activation of endothelial cells was ascertained by measuring the interleukin-8 release. Statistical significance was reached from 6 hours for all stimuli. G) PBMC's ($n=4$) cultured for up to 24 hours in the absence and presence of LPS, did not release detectable amounts of sMD-2. H) Stimulation of whole blood cultures ($n=4$) with LPS for up to 24 hours, did not lead to enhanced release of sMD-2, compared with non-stimulated cultures.

Figure 4.6 Reduction of MD-2 surface expression on endothelial cells after LPS stimulation. Endothelial surface MD-2 expression after exposure to LPS for 0 hours (A), 2 hours (B), 4 hours (C) and 22 hours (D) is shown. Dotted line: isotype control; solid line anti MD-2 antibody 18H10. Numbers shown on top right represent the mean fluorescence intensity of MD-2 and isotype respectively. Representative data of four independent experiments are shown.

Discussion

Since the discovery of MD-2, evidence has been accumulating that MD-2 is an indispensable protein for LPS recognition and signaling by TLR4. To answer unresolved questions concerning the function of MD-2, unique monoclonal antibodies were developed. We discovered that under reducing circumstances, circulating sMD-2 is mainly present as a doublet of ~20 and 25 kDa, representing differentially glycosylated forms. The smallest observed and weakest expressed unit was approximately 17 kDa, consistent with the calculated molecular mass of the non glycosylated peptide. Interestingly, conflicting reports have been published about the functionality of such unglycosylated MD-2. Earlier studies report that lack of glycosylation impaired MD-2 function in a LPS reporter system^{27,28}. In contrast, a recent study shows that glycosylation is not important for membrane MD-2 function, while it rather plays a role in the function of soluble MD-2 in LPS stimulation of TLR4 positive HEK cells¹⁶. Non-reduced analysis shows primarily large sMD-2 multimers in control and septic plasma. These high molecular weight sMD-2 isoforms correspond probably to inactive

disulfide-linked MD-2 multimers. Although we cannot exclude the possibility that sMD-2 multimers contain MD-2 ligands, the complexes appear not to activate the TLR4 positive cells. Interestingly, monomeric sMD-2, representing the active form, was only detected in septic plasma. In line with this observation, septic but not normal plasma was found to support LPS activation of TLR4 positive epithelial cells. Direct evidence that sMD-2 in septic plasma is functionally involved in LPS responses was demonstrated by immunodepletion of sMD-2. These combined findings confirm and extend earlier observations which suggested that functional sMD-2 is present in the circulation during sepsis¹⁰.

The absence of monomeric MD-2 in control plasma could be due to either the rapid formation of polymers, or the effective clearance from the circulation in combination with a lower production of monomeric sMD-2. This remains to be elucidated.

Assessment of sMD-2 concentrations in plasma of control and septic individuals resulted in average sMD-2 concentrations in the low ng/ml range which is in agreement with a recent report⁹. In addition, in septic plasma we estimated concentrations of the active monomeric form to be in the pg/ml range. In concordance, very low concentrations of sMD-2 were reported to exhibit a remarkable capacity for restoring LPS responsiveness to TLR4 expressing reporter cells⁶. Moreover, recent studies describe the generation of defined LPS:MD-2 complexes at very low concentrations (pM) of endotoxin and soluble MD-2 and demonstrate that these complexes, at pM concentrations, activate cells in a TLR4-dependent fashion^{13,29}.

The fact that circulating sMD-2 is predominantly polymeric raises questions regarding the function of sMD-2 multimers. Aggregation into large polymers might protect sMD-2 from renal clearance, resulting in the formation of a reservoir that when necessary, could be activated by cleavage. Of interest, a recent study suggests that the molecular structure and the concomitant function of sMD-2 are influenced by an intestinal serine protease³⁰. Whether a comparable system is present in the circulatory system or in local tissues during inflammation remains to be determined.

sMD-2 multimers have also been suggested to function as LPS decoy, facilitating LPS clearance from the circulation, as demonstrated for the other LPS-binding proteins CD14 and LBP¹⁴. This suggestion is in agreement with an earlier report, showing that high amounts of sMD-2 inhibit cellular responses of cells expressing the TLR4 MD-2 complex to LPS¹¹. This decoy function could be explained by a report showing that besides very efficient binding of LPS by sMD-2 monomers, also low affinity interactions between polymeric sMD-2 and LPS can occur¹⁶. Increased concentrations of sMD-2 polymers can thus compete for LPS. However, others have reported that sMD-2 multimers do not bind LPS¹⁷. The role of circulating MD-2 multimers remains therefore to be elucidated.

In order to determine the kinetics of (monomeric) sMD-2 release *in vivo* as observed during sepsis, we made use of a standardised human endotoxemia model. LPS administration to healthy volunteers induces a well defined temporal release of

overlapping pro- and anti-inflammatory signals²⁶. In these subjects, an increase of circulating monomeric and total sMD-2 was observed, starting 6 hours after LPS administration. Plasma sMD-2 levels remained elevated up to 23 hours post LPS infusion. Consistently, this enhancement of sMD-2 monomers was largely paralleled by an increase of TLR4 costimulatory activity. Remarkably, elevated release and LPS supporting activity of sMD-2 occurs when manifestations of systemic inflammation generally abate. The time-related change in plasma concentrations of sMD-2 upon LPS infusion, mirrors the expression pattern of typical acute phase reactants such as CRP and SAA in this endotoxemia model³¹. Therefore, and also based on the increased levels of sMD-2 in septic patients as discussed above, one may argue that sMD-2 is an acute-phase protein.

The potential cellular sources of circulating sMD-2 were studied. The immunohistochemical demonstration of MD-2 expression by endothelial and inflammatory cells in liver and lung of septic patients led us to analyze the MD-2 releasing capacity of leukocytes and endothelial cells. These experiments revealed that MD-2 was mainly expressed by endothelial cells and dendritic cells and to a lesser extend by monocytes, granulocytes and B-lymphocytes. In line, TLR4 expression and LPS reactivity by myeloid cells, B cells and endothelial cells have been extensively reported³²⁻³⁴. In addition, for the first time we demonstrate that sMD-2 release of the MD-2 expressing cells studied in this work was restricted to dendritic and endothelial cells. LPS exposure of *in vitro* cultured endothelial cells led to a strong induced release of sMD-2, whereas LPS treatment of hematopoietic cells did not result in sMD-2 release. Besides LPS, also TNF- α appeared to be a potent inducer of the sMD-2 release by endothelial cells, suggesting that MD-2 protein expression by endothelial cells can be regulated at sites of acute inflammation. This is supported by previous studies demonstrating upregulation of MD-2 mRNA by endothelial and epithelial cells in multiple organs by cytokines, including TNF- α ³⁵⁻³⁸. Although this study does not exclude the possibility that other sources such as epithelial cells are capable of producing sMD-2 *in vivo*, our findings combined with the fact that numerous endothelial cells cover the immense network of blood vessels suggest that endothelial cells are a source of the enhanced circulating MD-2 levels during acute systemic inflammatory diseases such as endotoxemia and sepsis.

Recent studies may help to understand the functional importance of sMD-2 *in vivo*. In particular, previous reports strongly suggest that sMD-2 can complement cells that express TLR4 but lack mMD-2 such as pulmonary epithelial cells³⁸. This suggestion is extended by Ohnishi *et al.* showing that LPS can induce the formation of CD14-TLR4 complexes on the cell surface in the absence of mMD-2 while subsequent binding of sMD-2 is essential for activation of these cells³⁹.

Here, we show for the first time a strongly enhanced expression of MD-2 by pulmonary endothelial cells during sepsis. This increased MD-2 expression could be responsible for enhanced sensitivity to LPS, resulting in the production of chemokines and adhesion molecules and could explain the extreme susceptibility of lung tissue

during endotoxemia and sepsis¹⁰. This is supported by a murine endotoxemia study, showing that the pulmonary endothelium is the dominant regulator of neutrophil accumulation and concomitant pulmonary failure⁴⁰. Interestingly, we also observed in the septic lung enhanced MD-2 expression by pericytes. These cells have been reported to be capable to respond to LPS by enhanced TLR4 expression and cytokine release in the rat⁴¹.

In the liver of septic patients a significant MD-2 expression was predominantly localized in endothelial cells covering hepatic sinusoids and Kupffer cells while MD-2 was undetectable in healthy control liver. These human data closely resemble the hepatic distribution and regulation of TLR2 in control and endotoxemic mice⁴², suggesting that during inflammation, hepatic TLR's and MD-2 are strategically located to respond to gut-derived bacteria and bacterial toxins.

In conclusion, in this report we show that during sepsis total sMD-2 plasma levels are elevated. In these patients but not in controls, the presence of monomeric sMD-2, representing the active form, was detected. In endotoxemic volunteers, a time related increase of the total sMD-2 content and sMD-2 monomers occurred.

Release of sMD-2 was found to be restricted to dendritic cells and endothelial cells, while both LPS and TNF- α were potent inducers for MD-2 release by endothelial cells. Our study suggests a novel role of the endothelium in mediating the host response during inflammation.

References

1. Beutler B. Innate immunity: an overview. *Mol Immunol* 2004;40:845-859.
2. Perera PY, Vogel SN, Detore GR, Haziot A, Goyert SM. 1997. CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. *J Immunol* 1997;158:4422-4429.
3. Wurfel MM, Monks BG, Ingalls RR, Dedrick RL, Delude R, Zhou D, Lamping N, Schumann RR, Thieringer R, Fenton MJ, Wright SD, Golenbock D. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med* 1997;186:2051-2056.
4. Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002;3:667-672.
5. Visintin A, Halmen KA, Khan N, Monks BG, Golenbock DT, Lien E. MD-2 expression is not required for cell surface targeting of Toll-like receptor 4 (TLR4). *J Leukoc Biol* 2006;80:1584-1592.
6. Visintin A, Mazzoni A, Spitzer JA, Segal DM. Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. *Proc Natl Acad Sci U S A* 2001;98:12156-12161.
7. Kennedy MN, Mullen GE, Leifer CA, Lee C, Mazzoni A, Dileepan KN, Segal DM. A complex of soluble MD-2 and lipopolysaccharide serves as an activating ligand for Toll-like receptor 4. *J Biol Chem* 2004;279:34698-34704.
8. Mullen GE, Kennedy MN, Visintin A, Mazzoni A, Leifer CA, Davies DR, Segal DM. 2003. The role of disulfide bonds in the assembly and function of MD-2. *Proc Natl Acad Sci U S A* 2003;100:3919-3924.
9. Viriyakosol S, McCray PB, Ashbaugh ME, Chu J, Jia HP, Weiss J, Kirkland TN. Characterization of monoclonal antibodies to human soluble MD-2 protein. *Hybridoma (Larchmt)* 2006;25:349-357.
10. Pugin J, Stern-Voeffray S, Daubeuf B, Matthay MA, Elson G, Dunn-Siegrist I. Soluble MD-2 activity in plasma from patients with severe sepsis and septic shock. *Blood* 2004;104:4071-4079.
11. Viriyakosol S, Tobias PS, Kitchens RL, Kirkland TN. MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem* 2001;276:38044-38051.
12. Mancek M, Pristovsek P, Jerala R. Identification of LPS-binding peptide fragment of MD-2, a toll-receptor accessory protein. *Biochem Biophys Res Commun* 2002;292:880-885.
13. Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, Weiss JP. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A* 2004;101:4186-4191.
14. Re F, Strominger JL. Monomeric recombinant MD-2 binds toll-like receptor 4 tightly and confers lipopolysaccharide responsiveness. *J Biol Chem* 2002;277:23427-23432.
15. Visintin A, Latz E, Monks BG, Espevik T, Golenbock DT. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J Biol Chem* 2003;278:48313-48320.
16. Viriyakosol S, Tobias PS, Kirkland TN. Mutational analysis of membrane and soluble forms of human MD-2. *J Biol Chem* 2006;281:11955-11964.
17. Visintin A, Halmen KA, Latz E, Monks BG, Golenbock DT. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. *J Immunol* 2005;175:6465-6472.
18. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992;101:1644-1655.
19. Vincent JL, Jacobs F. Infection in critically ill patients: clinical impact and management. *Curr Opin Infect Dis* 2003;16:309-313.
20. Branger J, van den Blink B, Weijer S, Madwed J, Bos CL, Gupta A, Yong CL, Polmar SH, Olszyna DP, Hack CE, van Deventer SJ, Peppelenbosch MP, van der Poll T. Anti-inflammatory effects of a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *J Immunol* 2002;168:4070-4077.

21. Fontijn R, Hop C, Brinkman HJ, Slater R, Westerveld A, van Mourik JA, Pannekoek H. Maintenance of vascular endothelial cell-specific properties after immortalization with an amphotrophic replication-deficient retrovirus containing human papilloma virus 16 E6/E7 DNA. *Exp Cell Res* 1995;216:199-207.
22. Cloosen S, Thio M, Vanclee A, van Leeuwen EB, Senden-Gijsbers BL, Oving EB, Germeaad WT, Bos GM. Mucin-1 is expressed on dendritic cells, both in vitro and in vivo. *Int Immunol* 2004;16:1561-1571.
23. von der Mohlen MA, Kimmings AN, Wedel NI, Mevissen ML, Jansen J, Friedmann N, Lorenz TJ, Nelson BJ, White ML, Bauer R, et al. Inhibition of endotoxin-induced cytokine release and neutrophil activation in humans by use of recombinant bactericidal/permeability-increasing protein. *J Infect Dis* 1995;172:144-151.
24. Welbourn CR, Young Y. Endotoxin, septic shock and acute lung injury: neutrophils, macrophages and inflammatory mediators. *Br J Surg* 1992;79:998-1003.
25. Munford RS. Detoxifying endotoxin: time, place and person. *J Endotoxin Res* 2005;11:69-84.
26. Lowry SF. Human endotoxemia: a model for mechanistic insight and therapeutic targeting. *Shock* 2005;24 Suppl 1:94-100.
27. da Silva Correia J, Ulevitch RJ. MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *J Biol Chem* 2002;277:1845-1854.
28. Ohnishi T, Muroi M, Tanamoto K. N-linked glycosylations at Asn(26) and Asn(114) of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. *J Immunol* 2001;167:3354-3359.
29. Prohinar P, F. Re F, Widstrom R, Zhang D, Teghanemt A, Weiss JP, Gioannini TL. Specific high affinity interactions of monomeric endotoxin.protein complexes with Toll-like receptor 4 ectodomain. *J Biol Chem* 2007;282:1010-1017.
30. Cario E, Golenbock DT, Visintin A, Runzi M, Gerken G, Podolsky DK. 2006. Trypsin-sensitive modulation of intestinal epithelial MD-2 as mechanism of lipopolysaccharide tolerance. *J Immunol* 2006;176:4258-4266.
31. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP. New insights into the biology of the acute phase response. *J Clin Immunol* 1999;19:203-214.
32. Muzio M, Natoli G, Sacconi S, Levrero M, Mantovani A. The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J Exp Med* 1998;187:2097-2101.
33. Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, Mantovani A, Rothe M, Muzio M, Arditi M. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J Biol Chem* 1999;274:7611-7614.
34. Elson G, Dunn-Siegrist I, Daubeuf B, Pugin J. Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. *Blood* 2007;109:1574-1583.
35. Faure E, Thomas L, Xu H, Medvedev A, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. *J Immunol* 2001;166:2018-2024.
36. Abreu MT, Arnold ET, Thomas LS, Gonsky R, Zhou Y, Hu B, Arditi M. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem* 2002;277:20431-20437.
37. Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, van 't Veer C. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J Immunol* 2002;168:1286-1293.
38. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L428-437.
39. Ohnishi T, Muroi M, Tanamoto K. The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. *FEMS Immunol Med Microbiol* 2007;51:84-91.
40. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytniuk L, Raharjo E, Kubes P. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 2003;111:1011-1020.

41. Edelman DA, Jiang Y, Tyburski JG, Wilson RF, Steffes CP. Cytokine production in lipopolysaccharide-exposed rat lung pericytes. *J Trauma* 2007;62:89-93.
42. Ojaniemi M, Liljeroos M, Harju K, Sormunen R, Vuolteenaho R, Hallman M. TLR-2 is upregulated and mobilized to the hepatocyte plasma membrane in the space of Disse and to the Kupffer cells TLR-4 dependently during acute endotoxemia in mice. *Immunol Lett* 2006;102:158-168.

Chapter 5

**The localization of the LPS-
recognition complex in the human
healthy and inflamed premature and
adult gut**

Tim GAM Wolfs, Joep PM Derikx, Caroline MIM Hodin, Joris Vanderlocht,
Ann Driessen, Adriaan P de Bruïne, Charles L Bevins, Felix Lasitschka, Nikolaus Gassler,
Wim G van Gemert, Wim A Buurman

Inflammatory Bowel Diseases 2010;16:68-75

Abstract

Microbiota in the intestinal lumen provide an abundant source of potentially detrimental antigens, including lipopolysaccharide (LPS), a potent immunostimulatory product of Gram-negative bacteria recognized by the host via TLR-4 and MD-2. An aberrant immune response to LPS or other bacterial antigens has been linked to inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC).

We investigated which cells express MD-2 in the normal and inflamed ileum from neonates and adults by immunohistochemistry. Moreover, MD-2 and TLR4 mRNA expression in normal adult ileum was studied by RT-PCR on cells isolated by laser capture microdissection.

Premature infants did not show MD-2 expression neither in epithelial cells nor in the lamina propria. Similarly, MD-2 was absent in epithelial cells and lamina propria inflammatory cells in preterm infants with NEC. MD-2 protein in the healthy term neonatal and adult ileum was predominantly expressed by Paneth cells and some resident inflammatory cells in the lamina propria. MD-2 and TLR-4 mRNA expression was restricted to crypt cells. Also in IBD, Paneth cells were still the sole MD-2 expressing epithelial cells whereas inflammatory cells (mainly plasma cells) were responsible for the vast majority of the MD-2 expression.

In conclusion, the absence of MD-2 in the immature neonatal gut suggests impaired LPS sensing, which could predispose neonates to NEC upon microbial colonization of the immature intestine. The apparent expression of MD-2 by Paneth cells supports the critical concept that these cells respond to luminal bacterial products in order to maintain homeostasis with the intestinal microbiota *in vivo*.

Introduction

Microbiota are abundantly present in the intestinal lumen and provide a continuous source of antigens and toxins capable of activating the host immune system. A dysregulated immune response to bacterial derived molecules can result in excessive mucosal inflammation. Such an inappropriate intestinal inflammatory response is considered to underlie intestinal disorders such as ulcerative colitis, Crohn's disease and necrotizing enterocolitis (NEC)^{1,2}, the latter predominantly affecting premature infants. NEC is assumed to be caused by an immature immune response to commensal organisms that initially colonize the gut after birth^{1,3,4}. In contrast, the inflammatory bowel diseases ulcerative colitis and Crohn's disease are considered to be the consequence, in part, of an excessive response of the intestinal immune system to the host microbiota^{2,5}.

The healthy intestine is populated with various cells capable of responding to microbiota and their immunostimulatory products. First, antigen presenting cells as dendritic cells in the lamina propria monitor the luminal contents and coordinate an appropriate immune response^{6,7}. Second, enterocytes are considered to actively participate as innate immune sensors of microbial pathogens and commensal organisms⁸. Finally, Paneth cells release their antimicrobial peptides in the crypt lumen in response to the entry of bacteria⁹⁻¹².

Host recognition of microbial components is achieved by so-called pattern recognition receptors (PRRs), such as the NOD-like (NLRs) and Toll-like receptors (TLRs). Muramyl dipeptide (MDP), derived from peptidoglycan, is present in the cell wall of virtually all bacteria and recognized by NOD2, a PRR expressed in intestinal epithelial cells⁸ including Paneth cells¹³. The activity of another PRR, TLR9 was implicated by the degranulation observed in response to its ligand bacterial CpG DNA¹⁴. In addition, functional studies suggest that human lamina propria dendritic cells in the intestine respond to TLR ligands¹⁵.

Several experimental studies in rodent and porcine models suggest a role for Gram-negative organisms in the development of intestinal inflammatory pathology¹⁶⁻¹⁸. The capacity to induce proinflammatory responses by sensing conserved bacterial structures via TLRs, might therefore be important for aberrant immune reactions in the intestinal mucosa. This prompted us to investigate the expression of TLR4 and MD-2, two molecules responsible for the pattern recognition and signaling of Gram-negative derived LPS, in the healthy and inflamed gut of preterms, term infants and adults. The distribution of MD-2 in the healthy and inflamed intestine of premature neonates and adults was determined by immunohistochemistry (IHC). In addition, MD-2 and TLR4 mRNA expression was assessed in the healthy adult intestine in villus and crypt epithelial cells that were isolated by laser capture microdissection.

Materials and methods

Patients

Some of the intestinal samples were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage and use of tissue and patient data were performed in agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands”. In addition, fresh tissue samples were collected during routine gastrointestinal surgery (such as closure of stoma) after appropriate informed consent of the patients and/or their parents or caretakers. The study was approved by the medical ethics committee of the Maastricht University Medical Centre.

Immunohistochemistry

Staining for MD-2 was performed using the anti-MD-2 mab HM31 which was earlier characterized as MD-2 specific¹⁹. To ascertain the suitability of HM31 for immunohistochemistry on paraffin sections, staining was performed on MD-2 and TLR4 transfected CHO cells that were fixed in 4% formaldehyde, embedded in 2% agarose and subsequently in paraffin according to the AgarCyto cell block procedure²⁰. Sections of paraffin-embedded tissue or transiently transfected CHO cells were dewaxed and rehydrated through xylene and a decreasing ethanol gradient, followed by blocking the endogenous peroxidase with 0.3% H₂O₂ in methanol. After blocking with 5% bovine serum albumin, sections were incubated for 1 hour with the anti-MD-2 antibody HM31 followed by incubation with a goat anti mouse biotinylated secondary antibody (Dako, Glostrup, Denmark). Binding of the primary antibody was demonstrated by the streptavidin-biotin system (Dako) and visualized by applying 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, Mo). Nuclei were counterstained with haematoxylin. The slides were photographed by a Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F and were analysed by the computer program, Lucia G/F color image analysis.

To identify cell types that expressed MD-2, an immunohistochemical procedure for sequential double-antigen localization was applied. Paraffin sections were pretreated as described above. Antibodies directed against human defensin-5 (antibody 8C8)²¹, human lysozyme (Dako) or human CD138 (Dako) were used to perform IHC in combination with anti MD-2. In this procedure, sections were incubated for 1 hour with the anti-MD-2 antibody HM31 followed by incubation with a goat antimouse biotinylated secondary antibody. MD-2-positive cells were demonstrated by the streptABComplex/HRP detection system (Dako) and visualized by 3,3'-diaminobenzidine (DAB) (Dako).

Before incubation with the second primary antibody for detection of the cellular marker of interest, slides were blocked again with 5% bovine serum albumin for 30 minutes. Cells that stained positive for human defensin-5, lysozyme or CD138 were

counterstained by HistoGreen (Linaris, Wertheim, Germany). Control experiments were performed with isotype control IgG.

Laser microdissection and pressure catapulting

Fresh ileum mucosa samples were cut into 18 µm thick sections using a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) and processed as follows: the sections were mounted on membrane slides (PEN-membrane, 1 mm glass, Carl Zeiss MicroImaging, Bernried, Germany) and incubated for 10 min at -20°C in RNAlater-ICE (Ambion, Austin, TX). For further preservation, samples were fixed in ethanol and stained in cresyl violet acetate (1% (w/v) in ACS-grade ethanol (Sigma) for 15 sec. Subsequently, the slides were washed in ethanol and incubated for 5 min in xylene. After air-drying the slides were mounted on the stage of an inverse microscope that is a component of a Microbeam LMPC System (Carl Zeiss MicroImaging). We employed the RoboLPC method to microdissect and capture the appropriate tissue fragments (approx. 8 mm² crypt epithelium and epithelium lining the tips of the villi each, ~ 100,000 – 250,000 cells). For sample collection we applied 0.5 ml AdhesiveCaps opaque (Carl Zeiss MicroImaging).

RNA Isolation of microdissected samples

Total RNA was isolated from each sample using the Chomczynski/Sacchi method²² according to the manufacturer's instructions (peqGOLD TriFast kit, PeqLab Biotechnology, Erlangen Germany). For precipitation, 2 µl of Pellet Paint Co-Precipitant (Novagen, San Diego, CA) per sample was used. Subsequently, isolated RNA was purified using an Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA), using an elution volume of 12 µl. Quality of the RNA was assessed on RNA 6000 Nano microfluidics chips (Agilent Technologies, Waldbronn, Germany) on an Agilent 2100 BioAnalyzer according to the manufacturer's instructions.

Evaluation of mRNA levels by RT-PCR

RNA samples (10 µl of purified mRNA) were reverse-transcribed into cDNA using VersoTM Reverse Transcriptase and a blend of random hexamers and anchored oligo-dT 3/1 (v/v) as primer (VersoTM cDNA Kit; Thermo Fisher Scientific, ABgene House, UK) according to the manufacturer's protocol (Abgene). PCR reactions were performed in a total volume of 25 µl with specific primers for cytokeratin-18, TLR4, MD-2, alpha-defensin-5, lysozyme or CD45 using 1 µl of 5x diluted cDNA. Next, a nested PCR was conducted using 2.5 µl of the first reaction as a template. Amplified products were visualized on ethidiumbromide stained 1.2% agarose gels and photographed with a CCD camera (Pharmacia, Uppsala, Sweden). Primers for cytokeratin-18 were: 5'- GAG ACT GGA GCC ATT ACT TCA AG-3' (sense primer), 5'-GCC TTT TAC TTC CTC TTC CTG-3' (antisense primer), 5'-GAT CTT CGC AAA TAC TGT GGA C-3' (nested sense primer) and 5'-CTT GGC GAG GTC CTG AGA TT-3' (nested antisense primer). Primers for TLR4 were:

5'-TGC ATG GAG CTG AAT TTC TAC-3' (sense primer), 5'-TGA GCC ACA TTA AGT TCT TTC-3' (antisense primer), 5'-ACC TGG ACC TGA GCT TTA ATC-3' (nested sense primer) and 5'-CCA ATG GGG AAG TTC TCT AG-3' (nested antisense primer). Primers used for the amplification of MD-2 were: 5'-CCT GTT TTC TTC CAT ATT TAC TG-3' (sense primer), 5'-CTT GAA GGA GAA TGA TAT TGT TG-3' (antisense primer), 5'-TGG GTC TGC AAC TCA TCC G-3' (nested sense primer) and 5'-AAT AAC TTC TTT GCG CTT TGG-3' (nested antisense primer). Primers designed for amplification of alpha-defensin-5 mRNA were: 5'-TCG CCA TCC TTG CTG CCA-3' (sense primer), 5'-GTA GAG GCG GCC ACT GAT TTC-3' (antisense primer), 5'-ATT CTC CTG GTG GCC CTG-3' (nested sense primer) and 5'-TCA CAC ACC CCG GAG AGG-3' (nested antisense primer). Primers designed for amplification of lysozyme were: 5'-CCC TCC TTT CTG TTA CGG TCC-3' (sense primer), 5'-CTA ACG CCT TGT GGA TCA CGG 3' (antisense primer), 5'-TGT TAC GGT CCA GGG CAA G-3' (nested sense primer) and 5'-AAG CAC TGC AGG ATA AAT GAC A-3' (nested antisense primer). Primers for CD45 were 5'-CCT CAG CCT TGC ACA CCA CAG-3' (sense primer), 5'-CTT TTC AAC CCC TGG TGG CAC-3' (antisense primer), 5'-GCC TTA CCT GCA CGC ACC-3' (nested sense primer) and 5'-ATG AGA TAT GGA AAC AGA CGC A-3' (nested antisense primer). All primers were synthesized by Eurogentec, Seraing, Belgium.

Results

Expression of MD-2 in the healthy intestine of preterm and term infants

To investigate the suitability of the anti-MD-2 mab for immunohistochemistry, MD-2-TLR4 transfected CHO cells were fixed, embedded in paraffin and stained for MD-2. These experiments showed positive staining in MD-2 expressing (Figure 5.1A), but not in mock transfected CHO cells (Figure 5.1B). Using this earlier characterized anti-MD-2 antibody¹⁹, intestinal tissue of preterm and term infants was investigated by immunohistochemical analysis. In the healthy ileum of term infants (n=10), the majority of the MD-2 expression was located at the base of the crypts of Lieberkühn (Figure 5.2B1, Table 5.1). The crypt position and cellular morphology of the MD-2 positive cells corresponded with Paneth cells. Consistent with this presumption, sequential double-antigen localization clearly showed colocalisation of MD-2 with the typical Paneth cell markers alpha-defensin-5 (Figure 5.3B, Table 5.1) and lysozyme (data not shown). No MD-2 staining was detectable in other epithelial cells in the healthy ileum of term infants (n=10) (Figure 5.2B1). In addition, some resident inflammatory cells in the lamina propria of the ileum stained positive for MD-2 (Figure 5.2B1, Table 5.1). No staining was observed after replacement of the primary Ab with the corresponding isotype control (Figure 5.2E1).

In line with the healthy ileum of term infants, Paneth cells of preterms (<39 weeks) (n=10) stained also positive for alpha-defensin-5 (Figure 5.3A) and lysozyme (data not shown). However, MD-2 was not detected immunohistochemically in the Paneth cells

or inflammatory cells of the healthy ileum of these premature infants (Figure 5.2A1+5.3A, Table 5.1).

Figure 5.1 Characterization of monoclonal antibodies to human MD-2. Paraffin sections of MD-2 transfected CHO cells, stained for the presence of human MD-2, using AEC as a substrate (red) showed that MD-2 positive staining was observed in transfected cells, being 50% of the cells (A). No positive cells were detectable in mock-transfected cells (B). See page 162 for colour figure.

Expression of MD-2 in the healthy mature intestine

Similar to the staining pattern observed in term infants, virtually all MD-2 positive cells in the adult ileum ($n=10$) were located at the base of the crypts of Lieberkühn (Figure 5.2C1) and these MD-2 expressing epithelial cells colocalized with the Paneth cells markers alpha-defensin-5 (Figure 5.3C, Table 5.1) and lysozyme (data not shown). MD-2 staining was not detectable in other epithelial cells in the healthy ileum or colon ($n=10$) (Figure 5.2C1+5.2D1). In addition, some resident inflammatory cells in the lamina propria of the ileum and colon stained positive for MD-2 (Figure 5.2C1+5.2D1, Table 5.1). The negative control with the replacement of the primary antibody with an isotype-matched control antibody showed no staining (data not shown).

Next, TLR4 and MD-2 mRNA expression was analyzed by RT-PCR in the healthy adult ileum, in either crypt or villus epithelial cell populations that were isolated by laser capture microdissection (LCM) ($n=7$). In concordance with our findings on protein level, MD-2 mRNA expression was detected exclusively in epithelial cells lining the bottom of the crypts (Figure 5.4). Interestingly, TLR4 mRNA was also restricted to epithelial cells located in the crypts (Figure 5.4). As a control, RT-PCR for cytokeratin-18 expression confirmed the epithelial identity of isolated cells in both villus and crypt samples. Isolation of epithelial cells from the crypt was confirmed by the presence of mRNA for lysozyme and alpha- defensin-5 (Figure 5.4). In addition, CD45 mRNA was undetectable in microdissected cells confirming that the detected MD-2 and TLR4 RT-PCR signals in the crypt epithelium could not be attributed to contaminant leukocytes (data not shown).

Figure 5.2 MD-2 expression in the healthy and inflamed intestine of preterm and term infants and adults.

MD-2 positive cells, visualized with AEC (red), were not detected in paraffin sections of the immature intestine (n=10) (A1). In the healthy ileum of term infants and adults (n=10), MD-2 expression is mainly located at the base of the crypts of Lieberkühn (B1+C1). Sporadically, MD-2 positive inflammatory cells were detected in the lamina propria (indicated by arrows) (B1+C1). No MD-2 staining was detected in epithelial cells in paraffin sections of the mature healthy colon (n=10) (D1). Occasionally, MD-2 expressing inflammatory cells were observed in the lamina propria (indicated by arrows) (D1). No staining could be observed when an isotype-matched primary control antibody was used (E1). MD-2 expressing cells were not detected in paraffin sections of the immature intestine of NEC patients (n=10) (A2). During intestinal inflammation of the mature ileum, expression of MD-2 is observed in Paneth cells, but most is expressed by a subset of characteristic inflammatory cells in the lamina propria (C2). In the inflamed mature colon (n=20), large numbers of MD-2 positive cells were detectable in the lamina propria whereas epithelial staining for MD-2 was absent (D2). A representative section is shown for the outcome of three separate immunohistochemical experiments on the indicated samples. See page 163 for colour figure.

Table 5.1 Patient characteristics and main findings.

Subgroup	Collected tissue	Surgical indication	Gender	MD-2 distribution
Healthy ileum of premature infants	MPTC (*) n=5 Fresh tissue n=5	Volvulus, malrotation, closure of stoma	5 men 5 women	Absent
Healthy ileum of term infants	MPTC n=5 Fresh tissue n=5	Volvulus, malrotation, closure of stoma	4 men 6 women	Primarily Paneth cells, some inflammatory cells
Healthy mature ileum	MPTC n=0 Fresh tissue n=10	closure of stoma	4 men 6 women	Primarily Paneth cells some inflammatory cells
Inflamed ileum of premature infants	MPTC n=9 Fresh tissue n=1	NEC (#) n=10	6 men 4 women	Absent
Inflamed adult colon	MPTC n=10 Fresh tissue n=10	Crohn's disease n=10, ulcerative colitis n=10	8 men 12 women	Multiple inflammatory cells
Inflamed adult ileum	MPTC n=4 Fresh tissue n=6	Crohn's disease n=10	5 men 5 women	Multiple inflammatory cells, Paneth cells

* MPTC = Maastricht Pathology Tissue Collection; # NEC = Necrotizing enterocolitis.

Figure 5.3 Sequential double-antigen localization of MD-2 and alpha-defensin-5. For antigen colocalisation, MD-2 positive cells were visualized with DAB (brown) and a classical Paneth cell marker alpha-defensin-5 with HistoGreen (green). Immunoreactivity for alpha-defensin-5 but not for MD-2 was seen in the ileum of preterm infants (A). In the ileum of term infants and adults (B+C), Alpha-defensin-5 colocalized with all MD-2 positive epithelial cells (n=10). Representative examples of the immunohistochemical staining patterns are shown. See page 165 for colour figure.

Lack of MD-2 expression in the inflamed gut of preterms

Specimens obtained from the ileum of NEC patients (n=10), showed an increased influx of inflammatory cells at the site of mucosal injury. However, these inflammatory cells lacked the MD-2 protein (Figure 5.2A2, Table 5.1) In line with the expression pattern in healthy preterms, MD-2 staining of intestinal epithelial cells was not observed during inflammation in these infants (Figure 5.2A2, Table 5.1).

Expression of MD-2 in the inflamed adult intestine

In inflamed mature ileal tissue (n=10), MD-2 was expressed by the Paneth cell (Figure 5.2C2, Table 5.1). However, inflammatory cells in the lamina propria were responsible for the majority of the MD-2 expression in these sections. In the inflamed colon of patients with Crohn's disease (n=10) or ulcerative colitis (n=10), MD-2 was not detected in epithelial cell populations, whereas multiple inflammatory cells in the

lamina propria stained positive for MD-2 (Figure 5.2D2, Table 5.1). Many of the MD-2 positive inflammatory cells in both inflamed ileal and colon were identified as CD138 positive plasma cells (Figure 5.5).

Figure 5.4 Basal TLR4 and MD-2 mRNA expression in microdissected epithelial cells lining the villi or the crypts of the adult ileum. Cytokeratin-18 mRNA expression was detected using cDNA from both villus and crypt samples obtained by LCM. Constitutive ileal TLR4 and MD-2 mRNA expression was confined to crypt epithelial cells. Crypt epithelial cells expressed mRNAs for antimicrobial peptides lysozyme and alpha-defensin-5 characteristic for Paneth cells.

Figure 5.5 Identification of cells expressing MD-2 in the inflamed mucosa of the adult gut. Many inflammatory cells, positive for MD-2 (brown), co-express the marker for plasma cells, CD138 (green). See page 165 for colour figure.

Discussion

This study shows that MD-2 is absent in the premature gut, indicating that the premature intestine lacks crucial molecules required for detection and sensing of LPS. Such a deficient innate immune defense might predispose preterm born infants to bacterial overgrowth once the gut becomes colonized. Several reports concerning maturation of TLR4 during fetal development in other organs support our findings that preterm infants have a reduced capacity to sense LPS. In particular, for mice and baboons, evidence was provided that TLR4 mRNA levels in the immature fetal lung are decreased in comparison to mature newborns^{23,24}. In addition, human premature infants express considerably lower levels of TLR4 on their monocytes than term born infants, and concomitantly, the cytokine production by monocytes upon LPS stimulation is increased in a gestational age-dependent manner²⁵.

In term infants as in healthy adults, ileal MD-2 was predominantly expressed by Paneth cells and some resident inflammatory cells. In addition, TLR4 and MD-2 mRNA was detected in small intestinal crypts isolated by laser capture microdissection. The TLR4 and MD-2 positive epithelial cell populations were also positive for lysozyme and alpha-defensin-5 mRNA, two Paneth cell markers. Together, these data strongly suggest that Paneth cells are the predominant, if not sole epithelial cells that express the molecules of the LPS recognition complex in the healthy ileum. We were not able to assess the distribution of the TLR4 protein since appropriate high affinity antibodies for immunohistochemistry are till now not available²⁶.

The present finding that Paneth cells harbor molecules required for the detection and sensing of LPS, extends previous studies showing that Paneth cells express PRRs, including TLR9 and NOD-2²⁷. In addition, our data might explain work from Ayabe *et al.* who showed that Paneth cells secrete their antimicrobial peptides following exposure to microbial components, such as LPS⁹. Moreover, our data are in agreement with a recent publication which showed that penetration of luminal commensal and pathogenic bacteria is controlled by Paneth cell intrinsic MyD88-signaling²⁸. Collectively, these data suggest that Paneth cells actively sense luminal bacterial products, potentially via microbial detector molecules such as TLR4/MD-2, resulting in the release of their antimicrobial peptides. On their turn, Paneth cells derived antimicrobial peptides are considered to play an essential role in maintaining host-microbial homeostasis at the mucosal interface²⁸.

The presence of MD-2 in the cytoplasm of Paneth cells might imply that Paneth cells secrete soluble MD-2 (sMD-2), which has recently been shown to exist *in vivo*^{19,29} in addition to TLR4-bound MD-2. Of interest, two recent reports demonstrated that sMD-2 binds tightly to the surface of live Gram-negative bacteria, enhancing cellular activation, bacterial internalization and intracellular killing, in a TLR4 dependent manner^{30,31}. Such a possible role of Paneth cell derived sMD-2 would be in line with the antibacterial defense function of these cells.

In contrast to the MD-2 expression pattern in the healthy adult intestine, our data in IBD patients reveal that many MD-2 positive inflammatory cells were present in the lamina propria. In line with these data, abundant expression of MD-2 by inflammatory cells located in the lamina propria was reported in the inflamed colon of IBD patients³². However, despite the presence of multiple inflammatory cells, minimal MD-2 expression was detected in the terminal ileum of patients with active IBD³². Consistent with our findings, previous studies show enhanced numbers of TLR4 positive inflammatory cells in the lamina propria during active IBD in man^{33,34}. In addition, we found that the lamina propria infiltrating plasma cells strongly stained for MD-2, indicating enhanced responsiveness to LPS in such tissue.

Of interest, data presented here show that during active IBD, Paneth cells were still the only MD-2 expressing epithelial cells in the gut. Although the debate regarding TLR4 and MD-2 expression by epithelial cells during active IBD is still open³²⁻³⁴, our data are consistent with those of Hausmann *et al.* that suggest that absorptive epithelial cells are not directly involved in the response to LPS.

In line with the absence of MD-2 expression in healthy preterms, MD-2 was not present in epithelial cells of preterm infants suffering NEC. Moreover, in NEC patients, MD-2 staining was also absent in the abundantly recruited inflammatory cells. A lack of MD-2 expression suggests reduced inflammatory signaling which could predispose these preterm infants to bacterial overgrowth after intestinal colonization and might therefore be a possible mechanism for the pathophysiology of the disease¹.

Paradoxically, C3H/HeJ mice that carry a malfunctioning TLR4, were reported to be protected to the development of NEC in a model of formula feeding and hypoxia³⁵. The authors suggest that TLR4 is involved in the pathogenesis of NEC, by disturbance of the balance between mucosal injury and repair in the small intestine. In addition, using neonatal rats and TLR4 mutant C3H/HeJ mice, Jilling and colleagues provided evidence that bacterial-derived products activate TLR4 on intestinal epithelial cells that are abnormally up-regulated following neonatal stress, resulting in downstream inflammatory gene expression and NEC¹⁶. The differences between experimental and clinical NEC needs further investigation.

Taken together, this study shows that the *in vivo* distribution of MD-2 is gestation and inflammation-dependent. The absence of MD-2 in the healthy and inflamed premature gut supports the hypothesis that an immature immune system underlies the pathophysiology of NEC^{1,3,36}. In contrast, the abundant increase of MD-2 positive inflammatory cells during IBD suggest an increased capacity to react to Gram negative derived LPS and this might be involved in the excessive response of the adult intestinal immune system.

References

1. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet*. 2006;368:1271-1283.
2. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007;448:427-434.
3. Neu J, Chen M, Beierle E. Intestinal innate immunity: how does it relate to the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg*. 2005;14:137-144.
4. Hunter CJ, Upperman JS, Ford HR, Camerini V. Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). *Pediatr Res*. 2008;63:117-123.
5. Torres MI, Rios A. Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy. *World J Gastroenterol*. 2008;14:1972-1980.
6. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*. 2005;307:254-258.
7. Becker C, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O, Frick J, Galle PR, Autenrieth I, Neurath MF. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest*. 2003;112:693-706.
8. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology*. 2003;124:993-1000.
9. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol*. 2000;1:113-118.
10. Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature*. 2003;422:522-526.
11. Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted Paneth cell. *Cell Mol Life Sci*. 2002;59:156-170.
12. Ouellette AJ, Bevins CL. Paneth cell defensins and innate immunity of the small bowel. *Inflamm Bowel Dis*. 2001;7:43-50.
13. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nuñez G, Keshav S. Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology*. 2003;125:47-57.
14. Rumio C, Besusso D, Palazzo M, Selleri S, Sfondrini L, Dubini F, Ménard S, Balsari A. Degranulation of paneth cells via toll-like receptor 9. *Am J Pathol*. 2004;165:373-381.
15. Oldenhove G, de Heusch M, Urbain-Vansanten G, Urbain J, Maliszewski C, Leo O, Moser M. CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. *J Exp Med*. 2003;198:259-266.
16. Jilling T, Simon D, Lu J, Meng FJ, Li D, Schy R, Thomson RB, Soliman A, Arditi M, Caplan MS. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J Immunol*. 2006;177:3273-282.
17. Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjornvad CR, Thymann T, Grondahl ML, Hansen AK, Jensen SK, Boye M, Moelbak L, Buddington RK, Weström BR, Holst JJ, Burrin DG. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology*. 2006;130:1776-1792.
18. Eckmann L. Animal models of inflammatory bowel disease: lessons from enteric infections. *Ann N Y Acad Sci*. 2006;1072:28-38.
19. Wolfs TG, Dunn-Siegrist I, van't Veer C, Hodin CM, Germeraad WT, van Zoelen MA, van Suylen RJ, Peutz-Kootstra CJ, Elson G, Pugin J, Buurman WA. Increased release of SMD-2 during human endotoxemia and sepsis: a role for endothelial cells. *Mol Immunol*. 2008;45:3268-3277.
20. Kerstens HM, Robben JC, Poddighe PJ, Melchers WJ, Boonstra H, de Wilde PC, Macville MV, Hanselaar AG. AgarCyto: a novel cell-processing method for multiple molecular diagnostic analyses of the uterine cervix. *J Histochem Cytochem*. 2000;48:709-718.
21. Shen B, Porter EM, Reynoso E, Shen C, Ghosh D, Connor JT, Drazba J, Rho HK, Gramlich TL, Li R, Ormsby AH, Sy MS, Ganz T, Bevins CL. Human defensin 5 expression in intestinal metaplasia of the upper gastrointestinal tract. *J Clin Pathol*. 2005;58:687-694.

22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162:156-159.
23. Harju K, Glumoff V, Hallman M. Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice. *Pediatr Res.* 2001;49:81-83.
24. Awasthi S, Cropper J, Brown KM. Developmental expression of Toll-like receptors-2 and -4 in preterm baboon lung. *Dev Comp Immunol.* 2008;32:1088-1098.
25. Förster-Waldl E, Sadeghi K, Tamandl D, Gerhold B, Hallwirth U, Rohrmeister K, Hayde M, Prusa AR, Herkner K, Boltz-Nitulescu G, Pollak A, Spittler A. Monocyte toll-like receptor 4 expression and LPS-induced cytokine production increase during gestational aging. *Pediatr Res.* 2005;58:121-124.
26. Fukata M, Abreu MT. TLR4 signalling in the intestine in health and disease. *Biochem Soc Trans.* 2007;35:1473-1478.
27. Wehkamp J, Stange EF. Paneth cells and the innate immune response. *Curr Opin Gastroenterol.* 2006;22:644-650.
28. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A.* 2008;105:20858-20863.
29. Viriyakosol S, McCray PB, Ashbaugh ME, Chu J, Jia HP, Weiss J, Kirkland TN. Characterization of monoclonal antibodies to human soluble MD-2 protein. *Hybridoma (Larchmt).* 2006;25:349-357.
30. Tissières P, Dunn-Siegrist I, Schäppi M, Elson G, Comte R, Nobre V, Pugin J. Soluble MD-2 is an acute-phase protein and an opsonin for Gram-negative bacteria. *Blood.* 2008;111:2122-211.
31. Jain V, Halle A, Halmen KA, Lien E, Charrel-Dennis M, Ram S, Golenbock DT, Visintin A. Phagocytosis and intracellular killing of MD-2 opsonized gram-negative bacteria depend on TLR4 signaling. *Blood.* 2008;111:4637-4645.
32. Cario E, Golenbock DT, Visintin A, Rünzi M, Gerken G, Podolsky DK. Trypsin-sensitive modulation of intestinal epithelial MD-2 as mechanism of lipopolysaccharide tolerance. *J Immunol.* 2006;176:4258-4266.
33. Hausmann M, Kiessling S, Mestermann S, Webb G, Spöttl T, Andus T, Schölmerich J, Herfarth H, Ray K, Falk W, Rogler G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology.* 2002;122:1987-2000.
34. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun.* 2000;68:7010-7017.
35. Leaphart CL, Cavallo J, Gribar SC, Cetin S, Li J, Branca MF, Dubowski TD, Sodhi CP, Hackam DJ. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J Immunol.* 2007;179:4808-4820.
36. Martin CR, Walker WA. Intestinal immune defences and the inflammatory response in necrotising enterocolitis. *Semin Fetal Neonatal Med.* 2006;11:369-377.

Chapter 6

Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep

Tim GAM Wolfs, Wim A Buurman, Bea Zoer, Rob MJ Moonen, Joep PM Derikx,
Geertje Thuijls, Eduardo Villamor, Markus Gantert, Yves Garnier, Luc JI Zimmermann,
Boris W Kramer

PLoS One 2009;4:e5837

Abstract

Chorioamnionitis is the most significant source of prenatal inflammation and preterm delivery. Prematurity and prenatal inflammation are associated with compromised postnatal developmental outcomes, of the intestinal immune defence, gut barrier function and the vascular system. We developed a sheep model to study how the antenatal development of the gut was affected by gestation and/or by endotoxin induced chorioamnionitis.

Chorioamnionitis was induced at different gestational ages (GA). Animals were sacrificed at low GA after 2d or 14d exposure to chorioamnionitis. Long term effects of 30d exposure to chorioamnionitis were studied in near term animals after induction of chorioamnionitis. The cellular distribution of tight junction protein ZO-1 was shown to be underdeveloped at low GA whereas endotoxin induced chorioamnionitis prevented the maturation of tight junctions during later gestation. Endotoxin induced chorioamnionitis did not induce an early (2d) inflammatory response in the gut in preterm animals. However, 14d after endotoxin administration preterm animals had increased numbers of T-lymphocytes, myeloperoxidase-positive cells and gammadelta T-cells which lasted till 30d after induction of chorioamnionitis in then near term animals. At early GA, low intestinal TLR-4 and MD-2 mRNA levels were detected which were further down regulated during endotoxin-induced chorioamnionitis. Predisposition to organ injury by ischemia was assessed by the vascular function of third-generation mesenteric arteries. Endotoxin-exposed animals of low GA had increased contractile response to the thromboxane A2 mimetic U46619 and reduced endothelium-dependent relaxation in responses to acetylcholine. The administration of a nitric oxide (NO) donor completely restored endothelial dysfunction suggesting reduced NO bioavailability which was not due to low expression of endothelial nitric oxide synthase.

Our results indicate that the distribution of the tight junctional protein ZO-1, the immune defence and vascular function are immature at low GA and are further compromised by endotoxin-induced chorioamnionitis. This study suggests that both prematurity and inflammation in utero disturb fetal gut development, potentially predisposing to postnatal intestinal pathology.

Introduction

Chorioamnionitis is a bacterial infection of the amniotic fluid, placenta and membranes that is very frequently diagnosed after preterm birth¹. The disorder is usually clinically silent which makes estimates about the onset of infection during gestation difficult². Chorioamnionitis results from the fetal exposure to bacteria and bacterial toxins in the contaminated amniotic fluid. Animal studies showed that fetal aspiration of contaminated amniotic fluid induces lung inflammation, resulting in profound changes of the pulmonary homeostasis as indicated by increases in surfactant lipids and transient injury to the microvasculature and alveolar septa³⁻⁶. These alterations in lung development are associated with increased risk of bronchopulmonary dysplasia⁷.

Swallowing of the bacterial contaminated amniotic fluid is likely to result in antenatal exposure of the premature intestine to bacteria and their toxins in utero. Whether this exposure results in inflammatory and or developmental changes of the gut of preterm babies is subject of the current study. We hypothesized that prematurity and inflammation, either alone or combined, disturb maturation of the gut barrier, the innate immune defense and vascular function thereby potentially increasing the risk to postnatal intestinal pathologic conditions. To test our hypothesis, chorioamnionitis was induced by intraamniotic endotoxin injection at low gestational age (GA) and animals were delivered either preterm or near term. In this model, the distribution of the tight junctional protein zonula occludens protein 1 (ZO-1) was determined since no data exists on its expression during gestation. Besides the TLR4 and MD-2 mRNA expression, also the distribution of either myeloperoxidase (MPO) expressing cells (polymorphonuclear leukocytes (PMN) as marker for early inflammation) or T-lymphocytes and gammadelta T-cells (cells known to be crucial in monitoring and maintaining integrity of epithelial tissues; late inflammation) were assessed. Finally, the contraction or relaxation of third-generation mesenteric arteries was studied. Relaxation was studied in the absence and presence of a NO donor and concomitantly the expression of endothelial nitric oxide synthase (eNOS) was assessed.

In this study, we show that a low gestational age and endotoxin induced chorioamnionitis both prevent maturation of the intestinal barrier function, the innate immune defense and vascular function.

Materials and methods

Animals

This study was performed according to the guidelines of the Animal Care Committee of the University of Maastricht, which approved the protocol. The study was conducted after approval and in compliance with the guidelines of the ethical

committee. Time-mated Texel ewes with singleton fetuses were randomly assigned to groups of four or five animals, to receive a single dose of 10 mg endotoxin (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) resuspended in saline or the equivalent volume of saline for control animals by ultrasound guided intraamniotic injections (Figure 6.1). Chorioamnionitis was induced at 110–111d GA and at 123d GA. Animals were sacrificed at low GA of 125d GA after 2d or 14d exposure to chorioamnionitis. The low GA of 125d GA is comparable with a human GA of approximately 27 weeks. Long term effects of 30d exposure to endotoxin were studied in near term animals at 140d GA (term gestation being 147d GA) after induction of chorioamnionitis at 110–111d (Figure 6.1). All animals were delivered by caesarean section. Lambs received a lethal i.v. injection of phenobarbiturate. The stomach content was collected for analysis. Samples of the small intestine were collected for snap freezing and fixation. Third generation mesenteric arteries of the small intestine were prepared for analysis of vascular reactivity.

Figure 6.1 Experimental design. Gestational development and effects of chorioamnionitis were studied at two different gestational ages. At 125d GA, fetuses were comparable to 27 weeks of human gestation. Lambs were almost near term at 140d GA since term gestation is 147d. Chorioamnionitis was induced by a single injection of endotoxin under ultrasound guidance at 111d GA and at 123d GA. Animals were delivered at 125d GA and animals of the control group underwent the same procedure with an injection of saline. Animals of 140d GA had a separate control group to assess gestational changes.

Antibodies and reagents

Intestinal tissue was analysed with the following antibodies: monoclonal antibody against endothelial nitric oxide synthase (eNOS) (BD Transduction laboratories, San Jose, CA); rabbit antibodies against human MPO and CD3 Dakocytomation (Glostrup, Denmark) or Zonula Occludens protein 1 (ZO-1) (Invitrogen, San Francisco, CA); monoclonal antibody IL-A29 for the detection of gammadelta T-cells (VMRD, Pullman,

WA). Secondary antibodies, biotin conjugated rabbit anti-mouse and Texas red conjugated goat anti-rabbit were purchased from Dakocytomation and peroxidase conjugated goat anti-rabbit from Jackson (West Grove, PA).

Immunohistochemistry

Small intestinal biopsies, fixed overnight in 4% formaldehyde, were embedded in paraffin and sectioned at 3 μm . For staining of CD-3 and eNOS expressing cells, slides were heated in 10 mM Na-citrate (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol and subsequently, slides were blocked with either normal goat serum (eNOS and MPO) or bovine serum albumin (CD-3) for 30 min at room temperature. Slides were incubated with the primary antibody of interest for one hour at RT (CD-3 and MPO) or overnight at 4°C (eNOS). After washing, sections were incubated with the appropriate secondary conjugated antibody. Binding of the eNOS antibody was demonstrated by the streptavidin-biotin system (Dakocytomation) and binding of antibodies against CD-3 and MPO was detected using a peroxidase conjugated secondary antibody. Positive staining was visualized by applying 3-amino-9-ethylcarbazole (AEC, Sigma); nuclei were counterstained with haematoxylin. The number of cells exhibiting immunostaining for MPO and CD-3 were counted per high power field (200x). Figures were expressed as number of MPO or CD-3 expressing cells per high power field.

Frozen tissue sections were first fixed in filtered, ice-cold acetone and next in 4% paraformaldehyde. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol. After blocking with 10% normal goat serum, sections were incubated for two hours with a mab against gamma delta T-cells. After washing, an appropriate biotin conjugated secondary antibody was used. Binding of the primary antibody was demonstrated by the streptavidin-biotin system (Dakocytomation) and visualized by AEC. Nuclei were counterstained with haematoxylin. All slides were photographed by a Nikon eclipse E800 microscope with a Nikon digital camera DXM1200F and were analysed by the computer program, Lucia G color image analysis.

Immunofluorescence

Tight junction distribution was examined by immunofluorescent staining of frozen sections (3 μm) with an antibody to Zonula Occludens protein 1 (ZO-1). Ileum sections were fixed with 4% paraformaldehyde. After blockage of non-specific binding sites with 10% goat serum, slides were incubated with anti-ZO-1 for one hour. Thereafter, the sections were incubated 45 min with Texas red conjugated goat anti-rabbit antibody, followed by 2 min incubation with 4',6-diamino-2-phenyl indole (DAPI), dehydrated in ascending ethanol series and mounted in Fluorescence Mounting Solution (Dakocytomation). The distribution of ZO-1 was recorded at a magnification of 200x using the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE

fluorescence microscope (Leica, Wetzlar, Germany). All images were taken at equal time-exposures after being normalized to negative control sections without primary antibody, to exclude non-specific binding of the secondary antibody or autofluorescence. At least 25 microscopic fields for each tissue section were studied.

Evaluation of mRNA levels by RT-PCR

For RT-PCR total RNA was extracted from ileal tissues using the SV Total RNA isolation system (Promega, Madison, WI). Next, isolated total RNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Life technologies, Paisley, United Kingdom) according to the supplier's recommendations. cDNA samples were standardized based on the content of GAPDH cDNA as housekeeping gene. GAPDH cDNA was evaluated by performance of a GAPDH PCR on multiple dilutions of each cDNA sample. The amount of amplified product was estimated by densitometry of ethidiumbromide stained 1.2% agarose gels using a CCD camera and Imagemaster VDS software (Amersham, Uppsala, Sweden). Primer sequences for GAPDH were: 5'-CGT CTT CAC CAC CAT GGA GA-3' (sense primer) and 5'-CGG CCA TCA CGC CAC AGT TT-3' (antisense primer); primers used for the amplification of TLR4 mRNA: 5'-CCT CTC CAC CTT GAT ACT GAC G -3' (sense primer) and 5'-GGT ACC TGG TTC AAT AAA GCC T-3' (antisense primer) and primers designed for amplification of MD-2 mRNA: 5'-CCT GTT TTC TTC CAT ATT TAC TG (sense primer) and 5'-AAT AAC TTC TTT GCG CTT TGG-3'. PCR reactions with GAPDH, TLR4 or MD-2 specific primers were performed using appropriate dilutions of the cDNA. PCR reactions were performed in a total volume of 25 µl in PCR buffer (Perkin Elmer/Cetus, Emeryville, CA) in the presence of 0.2 mM dNTP (Amersham), 1.0 µM of each primer, 0.3 mM MgCl₂ and 0.5 U Taq polymerase (Perkin Elmer). PCR conditions for each primer couple were as follows: GAPDH: 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec during 21 cycles; TLR4: 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec during 40 cycles; MD-2: 95°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec during 33 cycles. Levels of TLR4 and MD-2 RNA expression were evaluated by densitometric image analysis as described above. Relative TLR4 and MD-2 mRNA levels were calculated by comparison of band intensities of the RT-PCR products to standard curves prepared by PCR amplifications on dilution series of a highly concentrated ileal ovine cDNA.

Arterial reactivity

Since ischemia as a consequence of vascular compromise is one of the frequently proposed risk factors for impaired intestinal homeostasis, responsiveness to contractile and endothelium-dependent and -independent relaxant agonists was assessed in isolated third-generation mesenteric arteries. Artery rings were mounted in a wire myograph, bathed in Krebs solution (gassed with 95% air/5% CO₂, pH 7.4), and normalized to a resting pretension corresponding to an intraluminal pressure of

40 mm Hg (125d GA) or 50 mm Hg (140d GA). The contractions induced by KCl (62.5 mM), the thromboxane A2 mimetic U46619 (10^{-10} - 10^{-7} M) and the adrenergic receptor agonist norepinephrine (NE; 10^{-10} - 10^{-4} M) was assessed. In addition, the endothelium-dependent relaxation (evoked by acetylcholine, Ach; 10^{-9} - 10^{-4} M)⁸ and endothelium-independent relaxation evoked by the NO donor sodium nitroprusside (SNP; 10^{-9} - 10^{-4} M) were analyzed. For the study of the relaxant responses, vessels were pre-contracted with U46619 (10^{-7} M) as previously described⁹.

Statistical analysis

The number of cells exhibiting immunostaining for MPO and CD-3 were counted per high power field. A Mann-Whitney U-test was used for between-group comparisons. Vascular contractile and relaxation responses are represented as mean $\pm$ SEM and the 2-way unpaired t-test was used to test for significant differences between saline and endotoxin treated groups. TLR4 and MD-2 mRNA levels were represented as mean $\pm$ SEM and differences between preterm and term lambs or saline and endotoxin injected animals were compared using an unpaired t-test. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Chicago, IL) and differences were considered statistically significant at $P < 0.05$.

Results

Tight junctions

Tight junctions have an important role in maintaining the barrier integrity by connecting enterocytes^{10,11}. In order to analyse the maturation and the influence of endotoxin in the fetal gut on this essential component of the gut barrier, the distribution of the zonula occludens protein 1 (ZO-1) was determined. ZO-1 staining was fragmented in premature saline treated control animals of 125d GA (Figure 6.2A). Interestingly, endotoxin exposure for 2d did not change the pattern (Figure 6.2B). Fetuses exposed to endotoxin for 14d showed further fragmented tight junctional distribution at 125d GA (Figure 6.2C). In contrast, in near term saline injected animals of 140d GA, ZO-1 proteins formed a continuous ring, which is indicative for a normal tight junctional distribution (Figure 6.2D). Interestingly, in these near term lambs of 140d GA, exposure to endotoxin for 30d resulted in partial loss of the ZO-1 protein in comparison to the age-matched control animals with saline injection (Figure 6.2E).

Figure 6.2 Immunolocalisation of ZO-1 (red) in the fetal intestine. At 125d GA, a fragmented staining pattern for ZO-1 was seen in control animals (A) or lambs exposed to endotoxin for 2 (B) or 14d (C). At 140d GA, a normal ZO-1 distribution was detected in control animals (D) that became disrupted upon endotoxin exposure for 30d (E). Magnification 200x. For inset, 1000x magnification was used. See page 166 for colour figure.

Inflammatory parameters

In preterm control animals of 125d GA, hardly any MPO expressing cells were detected (data not shown). At this GA, endotoxin exposure for 2d did not result in a significant increase of infiltrating intestinal MPO positive cells (data not shown).

Interestingly, at 14 days post endotoxin treatment, large numbers of MPO positive cells were present in the lamina propria of the immature intestine (Figure 6.3A). Remarkably, 30d post endotoxin treatment, the number of MPO expressing cells reactivity was still significantly enhanced in the near term fetus (Figure 6.3B) when compared to saline treated control animals of this GA (Figure 6.3C). For each individual animal, the number of infiltrating MPO expressing cells was counted per high-powerfield and displayed in a scatter plot (Figure 6.3D).

Figure 6.3 At 14 and 30d after intraamniotic endotoxin injection, massive influx of PMNs was detected (A+B). At 140d GA, some MPO positive cells were detected in control sections (C). For each experimental group mean cell counts of MPO positive cells are depicted per high-power field (D). See page 167 for colour figure.

The recruitment of CD-3 positive T-cells into the fetal gut paralleled that of PMN: in preterm lambs exposed to endotoxin for 2d, no significant increase of CD-3 expressing T-cells in the lamina propria was observed when compared with time matched control tissues of 125d GA (data not shown). In contrast, increased numbers of CD-3 expressing cells were seen at 14d and 30d post endotoxin exposure in both preterm

and near term animals of 125 and 140d GA respectively, with the highest density in the 14d group (Figure 6.4A+B). For each individual section, the number of CD-3 positive cells was quantified per high-power-field and data are depicted in a scatter plot (Figure 6.4C).

Figure 6.4 Intraamniotic endotoxin results in increased ileal T-cell density. Significant increase of CD-3 immunoreactivity was seen at 14 and 30d after endotoxin exposure (A+B). For each experimental group mean cell counts of CD-3 positive cells are depicted per high-power field (C). See page 168 for colour figure.

Next, the distribution of gammadelta T-cells was evaluated, cells known to be crucial in monitoring and maintaining integrity of epithelial tissues¹². In saline treated preterm animals of 125d GA, hardly any gammadelta T-cells were present in the lamina propria or lower mucosa (data not shown). Accordingly, in these preterm lambs, migration of gammadelta T-cells from the lower to the upper mucosa occurred sporadically after 2 or 14d post endotoxin treatment (Figure 6.5A+B). In saline injected animals of 140d GA, gammadelta T-cells were also undetectable in the lamina propria (Figure 6.5C). However, in these near term lambs, the lymphoid follicles in the lower mucosa became populated with gammadelta T-cells (Figure 6.5D). Interestingly,

30d of intraamniotic endotoxin exposure in near term animals resulted in migration of gammadelta T-cells from the lower to the upper mucosa in close proximity to areas of damaged intestinal epithelial cells (Figure 6.5E).

TLR4 and MD-2 expression

The molecular complex responsible for the recognition and signaling of endotoxin is formed by TLR4 and its indispensable cofactor MD-2^{13,14}. In preterm lambs of 125d, weak constitutive TLR4 and MD-2 mRNA expression was detected and this expression even decreased within 2d following intraamniotic endotoxin treatment (Figure 6.6). Also intraamniotic exposure to endotoxin for 14d in preterms resulted in a downregulation of the TLR4 and MD-2 mRNA levels (Figure 6.6). In saline treated near term sheep of 140d GA, TLR4 and MD-2 expression was significantly increased when compared with preterm controls of 125d GA. However, intraamniotic endotoxin exposure for 30d also resulted in a strong reduction of the TLR4 and MD-2 expression in near term lambs (Figure 6.6).

Vascular contraction and relaxation responses

Contractions of third-generation mesenteric arteries, evoked by the thromboxane receptor agonist U46619 increased with gestational age (Figure 6.7A). After endotoxin-induced chorioamnionitis, the potency and the contractile efficacy of U46619 was significantly increased at early (125d) but not at late GA (140d) (Figure 6.7A). In contrast, the contractions induced by KCl (data not shown) and NE (Figure 6.7B) were not statistically different, both in control animals at different gestational ages and in lambs exposed to chorioamnionitis.

Either ACh (Figure 6.7C) or SNP (Figure 6.7D) relaxed U46619-contracted fetal mesenteric arteries in a concentration-dependent manner and these relaxations did not change with gestational age. Interestingly, ACh-evoked relaxation was almost abolished in the 125d fetuses exposed to endotoxin (Figure 6.7C). However this effect of endotoxin on ACh-induced relaxation was not further observed at 140d GA (Figure 6.7C).

Interestingly, the presence of SNP completely reversed the impaired relaxation of premature animals exposed to endotoxin (Figure 6.7D). These findings suggest a vascular impairment during chorioamnionitis at low GA on account of reduced release of NO by endothelial cells.

Figure 6.5 Endotoxin induced chorioamnionitis results in migration of gammadelta T-cells to sites of mucosal damage at late GA. After 2d or 14d of endotoxin exposure, hardly any gammadelta T-cells were detected in the upper mucosa of the preterm intestine (A+B). At 140d GA, gammadelta T-cells were not detected in the lamina propria whereas lymphoid follicles in the basal mucosa became populated with gamma delta T-cells (C+D). 30d after endotoxin injection, increased numbers of gammadelta T-cells were found within areas of mucosal damage (E). See page 169 for colour figure.

Figure 6.6

Ileal TLR4 and MD-2 mRNA was evaluated by ovine specific RT-PCR amplification of cDNA samples from healthy control lambs and lambs subjected to endotoxin induced chorioamnionitis. Representative DNA fragments are shown for each group (n=4). cDNAs were standardized for GAPDH content. Quantitative data were obtained by densitometric evaluation of RT-PCR products which were compared to a standard curve obtained by amplification of a serial dilution of highly concentrated cDNA. At 125d GA, TLR4 and MD-2 mRNA levels were significantly (*) lower compared to animals at 140d GA. TLR4 and MD-2 mRNA expression was significantly reduced following intraamniotic endotoxin injection for 2, 14 and 30d.

Figure 6.7 Thromboxane A₂ mimic U46619 induced contractions of jejunal arteries increased with gestational age and were significantly (*) ($P < 0.05$) augmented after intra-amniotic endotoxin injection in the 125d ($P < 0.05$) but not in the 140d GA animals (A). Concentration-dependent contractile effects of norepinephrine tend to increase ($P > 0.05$) during gestation but remained unaltered during chorioamnionitis (B). Concentration-dependent relaxation of mesenteric arteries induced by acetylcholine was unaltered during gestation and was significantly (*) inhibited at 14d but not after 30d of endotoxin exposure (C). The relaxation response curve for sodium nitroprusside remained identical during gestation or chorioamnionitis (D).

eNOS expression

Since the findings described above point at endothelial dysfunction due to impaired synthesis of NO, the distribution of endothelial nitric oxide synthase (eNOS) was studied in mesenteric arteries. Constitutive eNOS expression was absent in preterm and near term control tissues of 125 and 140d GA respectively (data not shown). However, two days after intraamniotic endotoxin injection in preterm animals, eNOS was weakly expressed by submucosal vascular endothelia (Figure 6.8A). At this GA, endothelial staining further enhanced at 14 days after endotoxin exposure (Figure 6.8B). eNOS immunoreactivity was also present in the vascular endothelium of the submucosa in near term animals that were exposed to endotoxin for 30d (Figure 6.8C).

Figure 6.8 Intraamniotic endotoxin induces eNOS expression. Weak eNOS staining was seen at 2d post endotoxin treatment (A) while expression increased in animals exposed to endotoxin for 14 or days 30d (B-C). See page 170 for colour figure.

Discussion

This study shows for the first time the effects of gestation either alone or combined with antenatal exposure to bacterial toxins on the development of the fetal gut. A sheep model was used that closely resembles the human situation of preterm babies that are in utero exposed to chorioamnionitis by bacterial toxins resulting in adverse outcomes¹⁵. Administration of endotoxin to the amniotic fluid resulted in increased concentrations of endotoxin activity in the stomach fluid of animals exposed for 2d to endotoxin (data not shown), indicating passage of endotoxin to the fetal gut. This experimental set up allowed us to investigate whether prematurity and inflammation, either alone or combined, influence the development of the intestine in utero.

To our knowledge, this is the first report showing that the cellular distribution of tight junction protein ZO-1 was underdeveloped at 125d GA in sheep. Although ZO-1 is not the only tight junction protein responsible for paracellular barrier sealing, it plays a central role in maintenance of paracellular intestinal barrier integrity by connection of the intercellular tight junction proteins (claudins and occludin) with the cell cytoskeleton.

The presence of fragmented tight junctions in the gut of preterms (125d GA) is inherent to immaturity and correlates with diminished gut barrier function in preterm infants, as assessed by sugar absorption tests¹⁶. In near term control tissue of 140d GA, staining of ZO-1 formed a continuous band lining the mucosal epithelium, indicating maturation of the gut barrier during gestation.

This study shows that bacterial toxins impair maturation of tight junctions during gestation, an effect which lasted as long as 30d post endotoxin administration. These data are in line with earlier in vitro and in vivo findings showing that the permeability of the intestinal epithelium is impaired after exposure to LPS¹⁷⁻¹⁹. An imperfect tight junctional distribution, either due to immaturity or inflammation suggests an easy access of microbial toxins to the mucosa and the inner layers of the gut, both in utero and after birth. This led us to investigate the mRNA levels of the molecules representing the endotoxin recognition complex during gestation and after intraamniotic endotoxin injection. A gestational dependent increase of intestinal TLR4 and MD-2 mRNA was detected. In line, recent findings from our group showed that the human MD-2 protein is expressed in term but absent in preterm infants²⁰. Combined, these findings suggest that the capacity to recognize and sense Gram-negative derived endotoxin by the fetal intestine is immature at low GA. Interestingly, within 48 hours after intraamniotic endotoxin, TLR4 mRNA became undetectable and concomitantly, also MD-2 expression levels were reduced. The nature of this rapid downregulation needs further investigation^{21,22}, but it might explain the here observed hyporesponsiveness of the fetal gut to endotoxin at low GA. In particular, an impaired recruitment of MPO-positive cells or T-lymphocytes was detected in the intestine at 2d after intraamniotic injection of endotoxin at 125d GA, likely due to absence of endotoxin recognition. This is consistent with earlier studies in this

chorioamnionitis model showing that proinflammatory cytokine mRNAs were unaltered in the fetal intestine, from five hours to 25d after intraamniotic injection of endotoxin²³. In contrast to the intestinal TLR4 and MD-2 mRNA expression data presented here, previous studies with this animal model showed that intraamniotic endotoxin induced pulmonary TLR4 mRNA expression within 2d²⁴. In line, endotoxin induced chorioamnionitis provoked a rapid inflammatory reaction in the respiratory system within 24 hours^{5,6,25,26}.

Interestingly, at 14 and even 30d after intraamniotic endotoxin injection, a strong intestinal inflammatory response was present with a massive influx of MPO positive cells and T-lymphocytes. Paradoxically, at 14 and 30d post endotoxin exposure, TLR4 mRNA was still undetectable while MD-2 expression levels remained reduced. Whether this sustained inflammatory process is the result of a broken or absent tolerance to endotoxin remains to be elucidated^{22,27}. In addition, activation via TLR4-MD-2 independent intestinal innate immune receptors cannot be excluded with these experiments. Alternatively, the delayed inflammatory response in the fetal gut might be the consequence of a systemic rather than a local response of the fetal intestine. Esophageal occlusion experiments need to be performed to define the site of stimulation of the massive influx of PMNs and T-cells in the fetal gut.

At 30d post endotoxin exposure, inflammatory parameters were decreased compared to the 14d group, and this was paralleled by migration of gammadelta T-cells from the lower mucosa towards compromised epithelial surfaces. These findings are supported by a report from Chen *et al.* who showed that gammadelta T-cells preserve the integrity of stressed or injured intestinal mucosa¹². Moreover, our study showed that the lymphoid follicles of the healthy fetal intestine became populated with gammadelta T-lymphocytes between 125 days and 140 days of gestational age. In addition to the fragmented tight junctional distribution as seen in preterms or following endotoxin treatment, the absence of gamma delta T-cells in the mucosa at 125d GA suggests a reduced capacity to preserve the already compromised epithelium.

This study shows that intrauterine exposure to endotoxin induced a transient increase in the mesenteric vascular responsiveness to the thromboxane receptor agonist U46619. In addition a profound suppression of ACh-induced endothelium-dependent relaxation in mesenteric arteries was found during chorioamnionitis at low GA a process that was completely reversed by administration of the NO donor SNP. Our results strongly suggest the presence of an endotoxin-evoked transient endothelial damage and it could be speculated that a similar mechanism of endothelial dysfunction and diminished NO production might be partially responsible for the vascular vulnerability of the intestine in preterm infants²⁸.

The decreased NO availability could have several explanations. First, clinical reports showed that the intestinal synthesis of the important NO precursor arginine is strongly reduced in premature infants²⁹⁻³². Second, a mechanism for the reduced NO biosynthesis would be a decreased expression of eNOS. Paradoxically, our data

showed an enhanced expression of eNOS in the chorioamnionitis exposed animals. Such an increase in eNOS expression indicates the presence of a compensatory mechanism, which would attempt to maintain normal NO level by upregulating eNOS^{33,34}.

In summary, in this study evidence is provided that both prematurity and chorioamnionitis are associated with impaired development of the intestinal innate immune defence, the tight junctional distribution and vascular function in utero. Although at present, we can only speculate about the postnatal consequences of these developmental disorders, immaturity of the parameters investigated in this study are all associated with neonatal intestinal pathology and NEC in particular³⁵⁻³⁸. The immature gut of preterm babies is a risk factor on its own which is further aggravated by the presence of antenatal inflammation. In addition, reduced expression of innate immune receptors as seen in the intestine of preterm lambs suggests that the capacity to recognize and sense Gram-negative derived endotoxin by the fetal intestine is immature at low GA. Such a lack of endotoxin sensing in the preterm intestine, against the background of a disrupted tight junctional distribution, supports the theory that an inadequate immune response contributes to the pathogenesis of NEC, potentially by allowing bacterial invasion and subsequent overgrowth in premature infants. Further research is needed to assess the importance of preterm delivery and the consequences of inflammation in utero on the development of neonatal intestinal pathologies such as NEC.

References

1. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* 2000;342: 1500-1507.
2. Saji F, Samejima Y, Kamiura S, Sawai K, Shimoya K, Kimura T. Cytokine production in chorioamnionitis. *J Reprod Immunol* 2000;47: 185-196.
3. Kramer BW, Kramer S, Ikegami M, Jobe AH. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. *Am J Physiol Lung Cell Mol Physiol* 2002;283: L452-459.
4. Kallapur SG, Jobe AH. Contribution of inflammation to lung injury and development. *Arch Dis Child Fetal Neonatal Ed* 2006;91: F132-135.
5. Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, Ikegami M, Jobe AH. Dose and time response after intraamniotic endotoxin in preterm lambs. *Am J Respir Crit Care Med* 2001;164: 982-988.
6. Kramer BW. Antenatal inflammation and lung injury: prenatal origin of neonatal disease. *J Perinatol* 2008;28 Suppl 1: S21-27.
7. Jobe AH. Antenatal factors and the development of bronchopulmonary dysplasia. *Semin Neonatol* 2003; 8: 9-17.
8. Shaul PW, Farrar MA, Zellers TM. Oxygen modulates endothelium-derived relaxing factor production in fetal pulmonary arteries. *Am J Physiol* 1992;262: H355-364.
9. Villamor E, Kessels CG, Fischer MA, Bast A, de Mey JG, Blanco CE. Role of superoxide anion on basal and stimulated nitric oxide activity in neonatal piglet pulmonary vessels. *Pediatr Res* 2003;54: 372-381.
10. Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, Eastburn KK, Madara JL. Tight junctions are membrane microdomains. *J Cell Sci* 2000;113 (Pt 10): 1771-1781.
11. Viswanathan VK, Hecht G. Innate immunity and the gut. *Curr Opin Gastroenterol* 2000;16: 546-551.
12. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 2002;99: 14338-14343.
13. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 2004;430: 257-263.
14. Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, Kitamura T, Kosugi A, Kimoto M, Miyake K. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* 2002;3: 667-672.
15. Been JV, Kramer BW, Zimmermann LJ. In utero and early-life conditions and adult health and disease. *N Engl J Med* 2008;359: 1523-1524; author reply 1524.
16. Rouwet EV, Heineman E, Buurman WA, ter Riet G, Ramsay G, Blanco CE. Intestinal permeability and carrier-mediated monosaccharide absorption in preterm neonates during the early postnatal period. *Pediatr Res* 2002;51: 64-70.
17. Spitz J, Yuhan R, Koutsouris A, Blatt C, Alverdy J, Hecht G. Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. *Am J Physiol* 1995;268: G374-379.
18. Philpott DJ, McKay DM, Sherman PM, Perdue MH. Infection of T84 cells with enteropathogenic *Escherichia coli* alters barrier and transport functions. *Am J Physiol* 1996;270: G634-645.
19. Hackam DJ, Upperman JS, Grishin A, Ford HR. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg* 2005;14: 49-57.
20. Wolfs TG, Derikx JP, Hodin CM, Vanderlocht J, Driessen A, de Bruïne AP, Bevins CL, Lasitschka F, Gassler N, van Gemert WG, Buurman WA. The localization of the LPS-recognition complex in the human healthy and inflamed premature and adult gut. *Inflamm Bowel Dis* 2009, in press.
21. Kramer BW, Ikegami M, Moss TJ, Nitsos I, Newnham JP, Jobe AH. Endotoxin-induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. *Am J Respir Crit Care Med* 2005;171: 73-77.
22. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hypo-responsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* 2004;126: 1054-1070.
23. Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol* 2001;280: L527-536.

24. Hillman NH, Moss TJ, Nitsos I, Kramer BW, Bachurski CJ, Ikegami M, Jobe AH, Kallapur SG. Toll-like receptors and agonist responses in the developing fetal sheep lung. *Pediatr Res* 2008;63: 388-393.
25. Moss TJ, Nitsos I, Kramer BW, Ikegami M, Newnham JP, Jobe AH. Intra-amniotic endotoxin induces lung maturation by direct effects on the developing respiratory tract in preterm sheep. *Am J Obstet Gynecol* 2002;187: 1059-1065.
26. Kallapur SG, Jobe AH, Ball MK, Nitsos I, Moss TJ, Hillman NH, Newnham JP, Kramer BW. Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. *J Immunol* 2007;179: 8491-8499.
27. Kramer BW, Kallapur SG, Moss TJ, Nitsos I, Newnham JP, Jobe AH. Intra-amniotic LPS modulation of TLR signaling in lung and blood monocytes of fetal sheep. *Innate Immun* 2009;15: 101-107.
28. Moonen RM, Paulussen AD, Souren NY, Kessels AG, Rubio-Gozalbo ME, Villamor E. Carbamoyl phosphate synthetase polymorphisms as a risk factor for necrotizing enterocolitis. *Pediatr Res* 2007;62: 188-190.
29. Wu G, Jaeger LA, Bazer FW, Rhoads JM. Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. *J Nutr Biochem* 2004;15: 442-451.
30. Zamora SA, Amin HJ, McMillan DD, Kubes P, Fick GH, Bützner JD, Parsons HG, Scott RB. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* 1997;131: 226-232.
31. Becker RM, Wu G, Galanko JA, Chen W, Maynor AR, Bose CL, Rhoads JM. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. *J Pediatr* 2000;137: 785-793.
32. Richir MC, Siroen MP, van Elburg RM, Fetter WP, Quik F, Nijveldt RJ, Heij HA, Smit BJ, Teerlink T, van Leeuwen PA. Low plasma concentrations of arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. *Br J Nutr* 2007;97: 906-911.
33. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;87: 840-844.
34. López-Ongil S, Hernández-Perera O, Navarro-Antolín J, Pérez de Lema G, Rodríguez-Puyol M, Lamas S, Rodríguez-Puyol D. Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br J Pharmacol* 1998;124: 447-454.
35. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet* 2006;368: 1271-1283.
36. Srinivasan PS, Brandler MD, D'Souza A. Necrotizing enterocolitis. *Clin Perinatol* 2008;35: 251-272, x.
37. Kawase Y, Ishii T, Arai H, Uga N. Gastrointestinal perforation in very low-birthweight infants. *Pediatr Int* 2006;48: 599-603.
38. Boston VE. Necrotising enterocolitis and localised intestinal perforation: different diseases or ends of a spectrum of pathology. *Pediatr Surg Int* 2006;22: 477-484.

Summary and discussion

Summary and discussion

The innate immune system is the first line of defense against infection and injury. Infectious microorganisms are predominantly detected by TLR, a family of PRR which recognize and sense conserved molecular structures shared by a broad range of microbes. In addition, evidence is accumulating that TLR are involved in the response against endogenous damage-associated molecular patterns (DAMPs) which are released following cellular injury. The inflammatory response mediated by TLR activation is elicited to combat infecting microbes, to remove cellular remnants and to initiate tissue regeneration. However, the intensity and duration of TLR responses must be tightly controlled since prolonged and excessive activation of these TLR can contribute to abundant inflammation, tissue damage, multiple organ dysfunction and eventually death. Such inappropriate immune activation is seen after trauma, following major surgery including vascular, cardiac and transplant surgery and in diseases related to surgery including sepsis, IBD and NEC.

The purpose of this thesis is to increase the understanding of TLR biology in surgical patients. Characterization of the molecular pathways involved in TLR mediated immune activation is crucial to develop therapeutic strategies to blunt, but not abrogate, cellular responses to TLR ligands.

Sterile injury following renal ischemia reperfusion

Ischemia reperfusion (I/R) induces an inflammatory reaction, illustrated by cytokine production and neutrophil influx in reperfused tissue. I/R injury is a major cause of acute renal failure after hemorrhagic shock or major cardiovascular surgery. In the context of renal transplant surgery, I/R-induced inflammatory injury is associated with delayed graft function that may affect long-term transplant outcome. The signals activating the innate immune system which underlie the inflammatory response following renal I/R include altered or injured cellular structures, molecules released by injured cells, or substances actively synthesized and secreted by cells upon I/R.

Hypothesizing that TLR mediate innate immune activation in the course of I/R, the distribution of TLR expressed in the kidney was analyzed (Chapter 2). In addition, the role of cytokines in the regulation of TLR expression following I/R was studied. To this end, a renal I/R model was used that causes transient tissue damage associated with inflammation developing rapidly after I/R. This model enabled us to study modulation of TLR2 and TLR4 mRNA expression by cytokines *in vivo* during inflammation.

TLR2 and TLR4 mRNA in the murine kidney was constitutively expressed by renal tubular epithelial cells (TECs) and epithelial cells in the Bowman capsule. During renal inflammation, TLR2 and TLR4 mRNA synthesis was enhanced by action of TNF- α and IFN- γ . This increased expression was mainly localized in TEC, the thin limb of the loops of Henle, and collecting ducts. Interestingly, endogenous TLR2 and TLR4 ligands

including high mobility group box 1, hyaluronan, biglycan and heat shock protein 70 that are claimed to activate TLR2 and TLR4, are known to be abundantly present upon I/R injury in TLR expressing renal epithelial cells^{1,2}. These combined data provide circumstantial evidence that activation of TLR by ligands released during I/R injury could be an important link between an inflammatory immune response and damage in the organ. Functional evidence that TLR play a central role in early tubular injury and the onset of inflammation following renal I/R, has been found in mice engineered to lack either TLR2 or TLR4³⁻⁵. Concordingly, human data showed that the pathogenesis of I/R injury following kidney transplantation involves signaling through TLR4 expressed in donor kidney cells⁶. Loss of function TLR4 mutations attenuated intragraft proinflammatory gene expression and these mutations were associated with improved early graft function⁶.

In conclusion, evidence was provided that TLR contribute to the inflammatory response occurring after renal I/R. Additional studies are required to reveal the mechanisms underlying TLR mediated immune activation following I/R. Defining such pathways might lead to the development of novel pharmacotherapies for I/R injury in the kidney and other organs.

Earlier work from our laboratory showed that the sterile inflammatory process following renal I/R could be prevented with specific inhibitors of apoptosis, suggesting that this form of cell death is causally involved in I/R-induced inflammation and subsequent tissue injury. Moreover, more apoptotic cells were detected in transplanted kidneys with early graft dysfunction when compared with grafts that function immediately^{7,8}. Understanding the apoptotic pathways activated in the course of renal cell death induced by I/R may provide a basis for therapeutic targeting of apoptosis in the course of acute or chronic renal injury. Therefore, initiation of the apoptotic cascade was studied in a human ex vivo model for warm ischemic injury (Chapter 3). In this model, consecutive biopsies were taken from kidneys nephrectomized because of renal cell carcinoma.

Warm ischemia resulted in upregulation of pro-apoptotic Bax and suppression of the anti-apoptotic proteins Bcl-2 and cFLIP in a time-dependent fashion. During ischemia, activated caspase-9 increased with ischemia time, whereas caspase-8 and caspase-3 were not activated.

These data indicate that during normothermic ischemia, conditions are created that facilitate execution of apoptosis upon reperfusion. The decrease of anti-apoptotic proteins Bcl-2 and cFLIP and activation of pro-apoptotic proteins Bax and caspase-9 during ischemia point to these proteins as therapeutic targets for the prevention of renal I/R injury. Interestingly, a caspase inhibitor IDN-6556 was shown to ameliorate early hepatic injury in an ex vivo rat model of warm and cold ischemia⁹. In addition, the applicability of this caspase inhibitor in the clinical setting was demonstrated¹⁰. In particular, administration of IDN-6556 in cold storage and flush solutions during liver transplantation offered local therapeutic protection against I/R-mediated apoptosis

and injury¹⁰. Although the potential of therapeutics that modulate activity of apoptotic proteins such as caspases seems evident, it is questionable whether apoptosis can be selectively modulated in one organ or cell type without adverse effects (e.g. carcinogenesis) on other vital functions.

Infectious inflammation

Microorganisms residing on mucosal surfaces are the most important source of infectious inflammation. Of the mucosal tissues, the gastrointestinal tract contains the highest number of microorganisms with a remarkable diversity. It is important to sequester microbiota in the gut lumen, since the immune system may be excessively activated following translocation of microorganisms across the intestinal epithelial barrier into the lamina propria, mesenteric lymph nodes and circulation. In patients undergoing major surgery, reduced splanchnic blood flow is considered to result in translocation of luminal gut microbiota across an impaired intestinal epithelial barrier into the circulation¹¹⁻¹³. These translocated bacteria are predominantly Gram-negative aerobic genera including *Escherichia*, *Proteus* and *Klebsiella* that can act as a source of sepsis¹⁴.

LPS, the principal inducer of inflammatory responses to Gram-negative bacteria is sensed by TLR4 and MD-2¹⁵⁻¹⁷. To gain more insight into the biology of MD-2 during systemic inflammation, the biochemical and functional properties of this molecule were studied during sepsis and experimental human endotoxemia (Chapter 4). In this study, strong suggestive evidence was provided that endothelial cells are an important source for release of soluble MD-2 (sMD-2) *in vivo*. It was shown that human sMD-2 circulate constitutively in the low ng/ml range as large inactive multimers. During endotoxemia and sepsis, the total amount of sMD-2 in plasma was strongly elevated and contained next to polymeric also a small amount of monomeric sMD-2. In line with this observation, in Chapter 4 and in previous publications, it was shown that septic but not normal plasma supported LPS activation of TLR4 positive MD-2 deficient epithelial cells. Based on these findings, it is tempting to speculate that active sMD-2 in septic plasma is a source for TLR4 expressing MD-2 negative cells *in vivo*, thereby acting as a mediator of organ damage during severe systemic inflammation. Consistent with this hypothesis, different studies suggest that sMD-2 can modulate LPS signaling during Gram-negative infections by supplementing cells that express TLR4 but lack mMD-2 such as pulmonary and intestinal epithelial cells^{18,19}. This is further extended by Ohnishi et al. who showed that LPS can induce the formation of CD14-TLR4 complexes on the cell surface in the absence of mMD-2 while subsequent binding of sMD-2 is essential for activation of these cells¹⁹. Furthermore, there is growing evidence that the presence of active sMD-2 such as seen during endotoxemia and sepsis plays an important role in the anti-bacterial defense. In particular, recent studies elegantly demonstrated that opsonization of

Gram-negative bacteria by sMD-2 accelerates bacterial internalization and enhances intracellular killing, predominantly by neutrophils²⁰.

The apparent immuno-modulating and anti-bacterial role of sMD-2 identifies the TLR4/MD-2 complex as an attractive pharmacological target for antisepsis therapy. Different investigators have aimed to dampen excessive immune activation in animal models of sepsis through targeting TLR4/MD-2. First, an antagonistic antibody directed against TLR4/MD-2 was shown to protect mice from an otherwise lethal dose of LPS²¹. In addition, administration of a single dose of this antibody protected mice against mortality in a peritonitis model of polymicrobial sepsis²¹. Second, an analog of the non-toxic lipid A from *Rhodobacter sphaeroides*, Eritoran, competes with LPS for binding to MD-2, and has been shown to be an effective inhibitor of the toxic effects of LPS in animal models of endotoxemia^{22,23}. Finally, TAK-242, which selectively suppresses TLR4-signaling by binding to its intracellular domain, protects mice from endotoxin-induced mortality^{24,25}.

Whether targeting of one particular factor such as LPS is sufficient during clinical acute infectious inflammatory disorders such as sepsis remains to be established. Since bacteria possess ligands for multiple TLR and other PRR, it is likely that individual PRR ligands have to be included in combination therapies to treat acute inflammatory disorders. Alternatively, interventions can be developed which inhibit the action of proteins involved in signal transduction such as MyD88, TRIF and MAL, in order to prevent the stimulatory effects of multiple PRR agonists. Multiplex approaches such as DNA chips and protein arrays are ideal tools to identify such (combined) pharmacological targets.

Gut microbiota are also suspected to play an important role in mucosal inflammation as seen in ulcerative colitis, Crohn's disease and NEC. Although the etiologies of these diseases remain largely unknown, an inappropriate intestinal inflammatory response directed against normal resident intestinal bacteria or their products is considered to play a crucial role in the pathophysiology of these disorders.

Hypothesizing that inappropriate recognition and sensing of microbiota derived products by TLR plays a role in the pathogenesis of IBD and NEC, the distribution of the LPS recognition complex was analyzed in the healthy and inflamed premature and adult gut (Chapter 5). In the healthy ileum of term born infants and adults, evidence was provided that Paneth cells are the predominant, if not sole epithelial cells that express the molecules of the LPS recognition complex. These findings that Paneth cells are equipped with molecules essential for detection and sensing of LPS, are consistent with work from Ayabe et al. who showed that Paneth cells secrete their antimicrobial peptides following exposure to microbial components, such as LPS²⁹. Finally, data described in Chapter 5 are in agreement with a recent report which showed that penetration of luminal commensal and pathogenic bacteria is controlled by Paneth cell intrinsic MyD88-signaling³⁰. Collectively, Paneth cells actively sense luminal bacterial products, potentially via microbial detector molecules such as TLR4/MD-2, resulting in the release of their antimicrobial peptides. On their turn, Paneth cells

derived antimicrobial peptides are considered to play an essential role in maintaining host-microbial homeostasis at the mucosal interface³⁰.

The strong staining for MD-2 in the cytoplasm of Paneth cells suggests that Paneth cells might have an “opsonophagocytic” function mediated by secretion of sMD-2. Such a role of Paneth cell derived sMD-2 would be in line with the antibacterial defence function of these cells.

Immunohistochemical analysis revealed that in IBD patients many MD-2-positive inflammatory cells were present in the lamina propria (Chapter 5). These data are partially supported by a report from Cario et al. In this study they showed abundant expression of MD-2 by lamina propria located inflammatory cells in the inflamed colon of IBD patients whereas a few MD-2 expressing cells were detected in the terminal ileum of patients with active IBD³¹.

Studies which investigated the distribution of TLR4 in the lamina propria in the healthy and inflamed gut are paralleled by the MD-2 data provided in Chapter 5: enhanced numbers of TLR4 positive inflammatory cells in the lamina propria during active IBD in man were detected when compared with healthy tissue^{32,33}.

Of interest, during active IBD, Paneth cells were still the only MD-2 expressing epithelial cells in the gut (Chapter 5). Although the debate regarding TLR4 and MD-2 expression by epithelial cells during active IBD is still open³¹⁻³³, data shown in this Chapter indicate that in the healthy and inflamed mature gut, apical epithelial cells facing the gut lumen are not directly involved in the response to LPS. Generation of inducible TLR-deficient mice with the Cre-LoxP system would be an important tool to further evaluate the cell-type-specific responses of intestinal TLR during health and disease.

In the healthy and even in the inflamed gut of premature infants, MD-2 expression is absent (Chapter 5), indicating that the premature intestine lacks crucial molecules required for detection and sensing of LPS. To explore the functional consequences of luminal endotoxin exposure of the premature and mature gut, endotoxin was administered intra-amniotically to fetal sheep (Chapter 6). As in man, TLR4 and MD-2 mRNA expression levels were reduced in the intestine of preterm lambs when compared to mRNA levels for both genes in the mature gut (Chapter 6). Consistent with the reduced or absent expression of TLR4 and MD-2, endotoxin exposure for two days did not induce an inflammatory response in the gut in preterm animals. Remarkably, despite the absence of an initial inflammatory response in these preterm animals, the already low intestinal TLR-4 and MD-2 mRNA levels were further down regulated within two days after induction of endotoxin-induced chorioamnionitis. The inhibition of intestinal TLR in the absence of local inflammation might be the consequence of a systemic rather than a local response of the fetal intestine. Additional studies such as esophageal occlusion experiments are warranted to further investigate this issue.

The data discussed in Chapter 5 and 6 suggest that besides prematurity, also inflammation in utero leads to a reduced capacity to sense bacterial components such

as LPS. Once such immuno-compromised neonates are born, an intact gut barrier is crucial in order to avoid bacterial translocation and subsequent overgrowth in the underlying intestinal layers.

Therefore, it was studied whether gestation in the presence or absence of intraamniotic endotoxin affects the distribution of ZO-1, a protein that plays a central role in the maintenance of the paracellular intestinal barrier (Chapter 6). The distribution of ZO-1 was shown to be disturbed at low GA whereas endotoxin induced chorioamnionitis prevented maturation of tight junctions during later gestation.

These data suggest that the immature gut cannot cope with the transition from the normally sterile intra-uterine situation into the foreign antigen-rich environment which is created upon colonization of the intestinal tract by microorganisms, thereby predisposing to postnatal intestinal pathologies such as NEC. Future therapeutic strategies are needed to accelerate maturation of the intestinal immune system and the gut barrier function in the premature neonate to prevent intestinal disorders.

References

1. Kim BS, Lim SW, Li C, Kim JS, Sun BK, Ahn KO, Han SW, Kim J, Yang CW. Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation* 2005;79:1370-1377.
2. Robson MG. Toll-Like Receptors and Renal Disease. *Nephron Exp Nephrol* 2009;113:e1-e7.
3. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der Poll T, Weening JJ, Florquin S. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest* 2005;115:2894-2903.
4. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, Alexander SI, Sharland AF, Chadban SJ. TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest* 2007;117:2847-2859.
5. Pulskens WP, Teske GJ, Butter LM, Roelofs JJ, van der Poll T, Florquin S, Leemans JC. Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. *PLoS ONE* 2008;3:e3596.
6. Kruger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, Lin M, Walsh L, Vella J, Fischereder M, Kramer BK, Colvin RB, Heeger PS, Murphy BT, Schroppel B. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc Natl Acad Sci U S A* 2009;106:3390-3395.
7. Schwarz C, Hauser P, Steininger R, Regele H, Heinze G, Mayer G, Oberbauer R. Failure of BCL-2 up-regulation in proximal tubular epithelial cells of donor kidney biopsy specimens is associated with apoptosis and delayed graft function. *Lab Invest* 2002;82:941-948.
8. Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, Feuerstein D, Kaskel FJ, Tellis V, Devarajan P. Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. *Transplantation* 2003;76:50-54.
9. Hoglen NC, Anselmo DM, Katori M, Kaldas M, Shen XD, Valentino KL, Lassman C, Busuttil RW, Kupiec-Weglinski JW, Farmer DG. A caspase inhibitor, IDN-6556, ameliorates early hepatic injury in an ex vivo rat model of warm and cold ischemia. *Liver Transpl* 2007;13:361-366.
10. Baskin-Bey ES, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, Burgart L, Garrity-Park M, van Vilsteren FG, Oliver LK, Rosen CB, Gores GJ. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. *Am J Transplant* 2007;7:218-225.
11. MacFie J, O'Boyle C, Mitchell CJ, Buckley PM, Johnstone D, Sudworth P. Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 1999;45:223-228.
12. Derikx JP, Poeze M, van Bijnen AA, Buurman WA, Heineman E. Evidence for intestinal and liver epithelial cell injury in the early phase of sepsis. *Shock* 2007;28:544-548.
13. Poeze M, Ramsay G, Buurman WA, Greve JW, Dentener M, Takala J. Increased hepatosplanchnic inflammation precedes the development of organ dysfunction after elective high-risk surgery. *Shock* 2002;17:451-458.
14. Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet* 2003;361:512-519.
15. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999;162:3749-3752.
16. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.
17. Qureshi ST, Gros P, Malo D. The Lps locus: genetic regulation of host responses to bacterial lipopolysaccharide. *Inflamm Res* 1999;48:613-620.
18. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB, Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L428-437.
19. Ohnishi T, Muroi M, Tanamoto K. The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. *FEMS Immunol Med Microbiol* 2007;51:84-91.
20. Jain V, Halle A, Halmen KA, Lien E, Charrel-Dennis M, Ram S, Golenbock DT, Visintin A. Phagocytosis and intracellular killing of MD-2 opsonized gram-negative bacteria depend on TLR4 signaling. *Blood* 2008;111:4637-4645.

21. Daubeuf B, Mathison J, Spiller S, Hugues S, Herren S, Ferlin W, Kosco-Vilbois M, Wagner H, Kirschning CJ, Ulevitch R, Elson G. TLR4/MD-2 monoclonal antibody therapy affords protection in experimental models of septic shock. *J Immunol* 2007;179:6107-6114.
22. Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, Kobayashi S, Przetak M, Chow J, Gusovsky F, Christ WJ, Rossignol DP. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. *J Pharmacol Exp Ther* 2003;304:1093-1102.
23. Hawkins LD, Christ WJ, Rossignol DP. Inhibition of endotoxin response by synthetic TLR4 antagonists. *Curr Top Med Chem* 2004;4:1147-1171.
24. Sha T, Sunamoto M, Kitazaki T, Sato J, Li M, Iizawa Y. Therapeutic effects of TAK-242, a novel selective Toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model. *Eur J Pharmacol* 2007;571:231-239.
25. Kawamoto T, Li M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol* 2008;584:40-48.
26. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol* 2008;8:776-787.
27. van der Poll T, Opal SM. Host-pathogen interactions in sepsis. *Lancet Infect Dis* 2008;8:32-43.
28. Wehkamp J, Stange EF. Paneth cells and the innate immune response. *Curr Opin Gastroenterol* 2006;22:644-650.
29. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 2000;1:113-118.
30. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 2008;105:20858-20863.
31. Cario E, Golenbock DT, Visintin A, Runzi M, Gerken G, Podolsky DK. Trypsin-sensitive modulation of intestinal epithelial MD-2 as mechanism of lipopolysaccharide tolerance. *J Immunol* 2006;176:4258-42566.
32. Hausmann M, Kiessling S, Mestermann S, Webb G, Spottl T, Andus T, Scholmerich J, Herfarth H, Ray K, Falk W, Rogler G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* 2002;122:1987-2000.
33. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 2000;68:7010-7017.

Samenvatting

Samenvatting

Micro-organismen worden voornamelijk herkend door speciale receptoren, de zogenaamde Pattern Recognition Receptors (PRR). Toll-like receptoren (TLR) vormen een belangrijke familie binnen deze PRR. TLR activeren ons aangeboren immuunsysteem na herkenning van geconserveerde microbiële structuren. Daarnaast is er toenemend bewijs dat lichaamseigen moleculen, die vrijkomen bij weefselschade als ligand voor TLR kunnen dienen. Deze endogene moleculen kunnen om die reden ook zorgen voor het activeren van ontstekingsreacties.

Een ontstekingsreactie, geactiveerd door TLR is belangrijk voor bestrijding van infectieuze micro-organismen, het opruimen van celresten en de initiatie van weefselregeneratie. Het is echter van cruciaal belang dat deze TLR gemedieerde ontstekingsreactie nauwkeurig gereguleerd wordt. Een te hevige reactie op bacteriële componenten of lichaamseigen moleculen kan namelijk leiden tot excessieve ontsteking met ernstige weefselschade, multi-orgaan falen en zelfs de dood tot gevolg. Onvoldoende gecontroleerde activatie van de aangeboren afweer wordt onder andere waargenomen na trauma, grote chirurgie zoals vaat-, hart- en transplantatiechirurgie, en bij ziekten zoals sepsis, 'inflammatory bowel disease' (IBD) en necrotiserende enterocolitis (NEC).

In dit proefschrift is de distributie en regulatie van TLR bestudeerd in de gezonde situatie, tijdens de foetale ontwikkeling en gedurende ontsteking. Een beter inzicht in de cellulaire lokalisatie en regulatie van TLR moleculen tijdens immuunactivatie is van belang voor de ontwikkeling van therapeutische interventies, die erop gericht zijn buitensporige activatie van het immuunsysteem te voorkomen.

Weefselschade na renale ischemie reperfusie

Bij niertransplantaties is weefselschade, veroorzaakt door I/R-geïnduceerde ontsteking geassocieerd met vertraagd functioneren van de getransplanteerde nier. Acute weefselschade ten gevolge van I/R kan de nierfunctie ook op lange termijn nadelig beïnvloeden. De signalen die de aangeboren afweer aanzetten tot de schadelijke ontstekingsreactie na I/R zijn afkomstig van beschadigde cellen. Moleculen die vrijkomen na celschade en producten die door beschadigde cellen actief gemaakt en uitgescheiden worden, activeren de afweerreactie. Verondersteld wordt dat deze lichaamseigen moleculen vrijgekomen na I/R, een belangrijke rol vervullen bij de activatie van de afweer via binding aan TLR.

In hoofdstuk 2 werd de cellulaire lokalisatie en regulatie van TLR onderzocht in een muizenmodel voor renale I/R schade. In gezonde nieren bleek de expressie van TLR2 en TLR4 mRNA voornamelijk in tubulair epitheel en in epitheliale cellen van het kapsel van Bowman gelokaliseerd te zijn. I/R resulteerde in een sterke toename van de TLR2 en TLR4 mRNA expressie in het distale tubulaire epitheel, het dunne segment van de lis van Henle en de verzamelbuisjes. Deze toename was volledig afhankelijk van de

cytokines TNF- α en IFN- γ . Samen met recente bevindingen van anderen laten deze bevindingen zien dat TLR een belangrijke rol spelen bij het schadelijke ontstekingsproces na I/R. Aanvullende studies zijn vereist om te onderzoeken via welke mechanismen TLR ons aangeboren immuun systeem activeren tijdens I/R. Het ontrafelen van deze activatieroutes is van belang voor de ontwikkeling van nieuwe therapieën ter reductie van I/R geïnduceerde schade in de nier en andere organen.

Eerdere studies uitgevoerd in ons laboratorium hebben aangetoond dat het ontstekingsproces dat optreedt na I/R, voorkomen kan worden met specifieke apoptose remmers, hetgeen suggereert dat er een causaal verband bestaat tussen deze vorm van celdood en I/R-geïnduceerde ontsteking. Bovendien werden in niet functionerende getransplanteerde nieren meer apoptotische cellen aangetoond dan in nieren met intacte functie. Meer inzicht in de apoptotische routes die geactiveerd worden na I/R, kan een mogelijke basis vormen voor anti-apoptotische therapie bij acute of chronische nierschade. In hoofdstuk 3 werd daarom het optreden van apoptotische celdood ten gevolge van warme ischemie onderzocht. Humane nieren, verwijderd vanwege een niercelcarcinoom, werden ex vivo bij 37°C bewaard. Tijdens de ischemische incubatie werden in de tijd opeenvolgende bipten genomen om apoptotische processen in kaart te brengen.

Warme ischemie resulteerde in een toename van het pro-apoptotische eiwit Bax en een tijdsafhankelijke afname van de anti-apoptotische eiwitten Bcl-2 en cFLIP. In overeenstemming hiermee werd gedurende warme ischemie een toename van de hoeveelheid actief caspase-9 aangetoond. Echter, activatie van caspase-8 en caspase-3 werd niet waargenomen tijdens warme ischemie. Deze gegevens laten zien dat tijdens ischemie de omstandigheden gecreëerd worden, die een snelle voltooiing van het apoptotisch proces ten tijde van reperfusie mogelijk maken. De afname van de anti-apoptotische eiwitten Bcl-2 en cFlip en de activatie van het pro-apoptische Bax en caspase-9 tijdens ischemie maken deze eiwitten interessante therapeutische targets ter voorkoming van I/R schade.

Infectieuze ontsteking

Micro-organismen aanwezig op slijmvliezen zijn de belangrijkste bron van infectieuze ontsteking. Het maag-darmstelsel bevat van alle mucosale weefsels het grootste aantal micro-organismen. Het is van belang dat deze microbiota in het darmlumen afgeschermd worden van de omringende weefsels door een intacte darmbarrière. Translocatie van microbiota naar de lamina propria, de mesenteriale lymfeknopen of de circulatie kan namelijk leiden tot hevige stimulatie van het aangeboren immuunsysteem door microbiële componenten. Verondersteld wordt dat tijdens grote operaties een verminderde doorbloeding van het splanchnicus gebied resulteert in darmschade en barrière verlies. Verstoring van de darmbarrière kan leiden tot translocatie van luminale darmmicrobiota naar de mucosa en de circulatie. Deze

getransloceerde bacteriën, die voornamelijk bestaan uit Gram-negatieve aerobe species zoals *Escherichia*, *Proteus* en *Klebsiella*, kunnen vervolgens leiden tot sepsis. Lipopolysaccharide (LPS) dat aanwezig is in de celwand van Gram-negatieve bacteriën, activeert het aangeboren immuunsysteem na binding aan TLR4 en de cruciale cofactor MD-2. Om meer inzicht te krijgen in de werking van MD-2 tijdens systemische ontsteking werden de biochemische en functionele eigenschappen van MD-2 bestudeerd in een experimenteel humaan endotoxine model en tijdens sepsis (hoofdstuk 4). In deze studie is indirect bewijs geleverd dat endotheel cellen een belangrijke bron zijn voor circulerend oplosbaar MD-2 (sMD-2) *in vivo*. De hoeveelheid sMD-2 in de circulatie van gezonde personen bedroeg enkele ng/ml. Dit circulerend sMD-2 bestond uitsluitend uit inactieve complexen (multimeren). Tijdens endotoxemie en sepsis bleek de totale hoeveelheid circulerend sMD-2 sterk verhoogd en werd naast inactieve multimeren ook een geringe hoeveelheid functioneel actief monomeer sMD-2 aangetoond. De aanwezigheid van dit actieve sMD-2 tijdens endotoxemie en sepsis is in overeenstemming met functionele *in vitro* MD-2 proeven. Deze functionele experimenten lieten zien dat septisch plasma, TLR4 positieve MD-2 negatieve cellen in staat stelde op endotoxine te reageren, dit in tegenstelling tot plasma van gezonde individuen. sMD-2 in septisch plasma zou dus een bron kunnen zijn voor cellen *in vivo* die wel TLR4, maar geen MD-2 tot expressie brengen.

Naast de beschreven rol in sepsis, worden darmmicrobiota ook in verband gebracht met de buitensporige ontsteking die bij IBD en NEC wordt gezien. Hoewel de etiologie van deze ziekten nog grotendeels onbekend is, wordt verondersteld dat een te hevige respons van de aangeboren afweer op producten van de normale darmflora, een cruciale rol speelt in de pathofysiologie van deze aandoeningen.

Zoals vermeld is LPS een belangrijke bacteriële component die in staat is ons aangeboren immuunsysteem te activeren. In deze context werd de distributie van het LPS herkenningcomplex bestudeerd in de gezonde en ontstoken darm van prematuren en volwassenen (hoofdstuk 5). In het gezonde ileum van aterm geboren kinderen en volwassen werd aangetoond dat de Paneth-cel de voornaamste, zo niet de enige epitheelcel is, die de moleculen van het LPS herkenningcomplex tot expressie brengt.

Immunohistochemische analyse van darmweefsel van IBD-patiënten liet zien dat er veel MD-2 positieve ontstekingscellen aanwezig zijn in de lamina propria. Net zoals in de gezonde situatie bleek de Paneth cel in de ontstoken darm van volwassenen de enige MD-2 positieve epitheelcel te zijn.

In de gezonde en ontstoken darm van te vroeg geboren kon daarentegen geen MD-2 worden aangetoond. Deze bevinding werd nader bestudeerd in een LPS-geïnduceerd chorioamnionitis model. Dit model stelde ons in staat de *in vivo* reactie van de premature darm op LPS te onderzoeken. Twee dagen na LPS blootstelling werd in de premature darm geen ontstekingsreactie gevonden, hetgeen in overeenstemming is met de geringe TLR4/MD-2 expressie in deze dieren. Ondanks de afwezigheid van een ontstekingsreactie in de darm, twee dagen na endotoxine

geïnduceerde chorioamnionitis, werd een vermindering van de toch al lage expressie van TLR4 en MD-2 in dit orgaan gevonden. Deze vermindering van de TLR4/MD-2 expressie in de darm wordt mogelijk veroorzaakt door een systemische ontstekingsreactie in de foetus.

De data in hoofdstuk 5 en 6 suggereren dat naast prematuriteit, ontsteking in utero verantwoordelijk kan zijn voor een beperkte capaciteit van de darm om op bacteriële componenten zoals LPS te reageren. Voor deze immuno-gecompromiteerde prematuren is het essentieel dat bij geboorte, de darmbarrière intact is om bacteriële translocatie te voorkomen. Deze darmbarrière wordt gevormd door epitheelcellen die verbonden zijn door tight-junctions. In hoofdstuk 6 werd tijdens de ontwikkeling in utero de histologische distributie van het tight-junction eiwit ZO-1 in de darm bestudeerd in de aan- of afwezigheid van LPS. De ZO-1 distributie in de darm van premature dieren bleek verstoord te zijn.

Verder werd gevonden dat LPS geïnduceerde chorioamnionitis maturatie van tight junctions tijdens ontwikkeling in utero verhinderde. Deze bevindingen suggereren dat de immature darm niet klaar is voor de overgang van de steriele intra-uterine situatie naar het antigeenrijke milieu dat gecreëerd wordt na kolonisatie van de darm door micro-organismen. De massale bacteriële invasie van de darm die na geboorte plaatsvindt, zou bij prematuren met een verstoorde darmbarrière en een immatuur immuunsysteem kunnen resulteren in intestinale aandoeningen waaronder de ziekte NEC. Toekomstige therapeutische strategieën moeten er op gericht zijn de ontwikkeling van het intestinale immuunsysteem en de darmbarrière functie te versnellen om intestinale aandoeningen in de premature neonat te voorkomen.

Dankwoord

Dankwoord

Mijn boekje is klaar, al is **mijn** absoluut niet op zijn plaats. Er zijn namelijk heel veel mensen die een rol gespeeld hebben in de totstandkoming van dit boekje.

Op de eerste plaats mijn promotor Prof. dr. Buurman. Beste Wim, heel veel dank voor alles wat ik bij je geleerd heb. De inspirerende discussies, het blijven hameren op structuur en je hulp bij het schrijven van mijn manuscripten zijn essentieel geweest voor dit proefschrift en mijn persoonlijke ontwikkeling.

De leden van de beoordelingscommissie ben ik zeer erkentelijk voor het lezen en beoordelen van dit proefschrift.

Mo, mijn goede vriend en eeuwige partner in crime. Vele markante personages (waaronder Fred Willems, Jac Thissen, El Funghi, Vladimir Edzak en vele anderen) hebben we het levenslicht geschonken. Nooit was één dag hetzelfde en vaak kwamen we nieuwe bekenden tegen. Je bent een heel goede vakman en ook op sociaal vlak ben je een kanjer. Nieuwe collega's onderkennen deze eigenschappen snel en vragen naast analytisch advies om raad als het AIO leven minder voor de wind gaat. Dit alles maakt jou onvervangbaar.

Francois, kamergenoot, de enige Quake initiator, Bossche bol, surfleraar, beroeps-ouwehoer maar bovenal een echte maat waar ik altijd op kan rekenen!

Bart, kamergenoot, betrouwbaar, nuchter en vriendelijk, meestal rustig maar af en toe ook wild zoals tijdens het knallen in Halle, belangrijk medeauteur van mijn eerste papers waaronder het inmiddels beroemde hoofdstuk 2. Ik ben blij met onze goede en blijvende vriendschap.

Joep, kameroad, kamergenoot, notoire deurenklopper, Carnaval in Euverhoove en Mestreech, Oktoberfeste, Solden, goede discussies over wetenschap maar ouch unne houb geawwehoor euver Sjengen die toch weer boven Zitterd geëindigd zien en andere onzin.

Robert (ik zal me voor het gemak beperken tot deze naam), kamergenoot, skileraar, geknutsel aan C5 neoepitopen, bieren in de Alla en andere feestjes. We blijven mekaar zeker zien!

Trudy, moeder overste, altijd rustig, behulpzaam en vriendelijk. Ik kon helaas maar één uur in jouw huid kruipen. Je vertrek op het lab laat zich nog altijd voelen. De andere belangrijke (voormalige) krachten op de afdeling: Annemarie, prettige collega en tijdelijke kamergenoot, Sarah (Rinsie) en Kim die het secretariaat lang en met veel succes bewaakt heeft.

Kees, kamergenoot, bourgondische Zeeuw (ze bestaan echt!), gevreesd aanvoerder van Lokomotief 2, en een fantastisch vakman. Ik heb heel veel van je geleerd. Ook na je overstap van Meerssen naar Maarssen is het contact gelukkig gebleven. Het 'eigen projectje' is bij deze afgerond.

Tim Lubbers, kamer DJ, photoshopheld en squashkoning. Ik zal ervoor zorgen dat we onze verjaardagen samen blijven vieren als jij nu eindelijk een keer regelt dat we de Toppers ook live zien.

Alle andere kamergenoten waaronder Mart-Jan, Mirjam, Yvonne, Joep Grootjans en Kimmetje, heel erg bedankt!

Boris, altijd even enthousiast, vernieuwend, constructief en vrolijk. Ik ben trots op het PLoS manuscript en ik verheug me enorm op het vervolg.

Misha, interessante discussies over wetenschap en het muzikaal opvoeden van mensen die toch hun eigen ding willen, super ingooier en wakeboard koning. Jammer dat het jaarlijkse Someren niet meer op de kalender staat.

Voor de 'humane studies' ben ik veel dank verschuldigd aan Prof. dr. van Heurn, Dr. van Gemert, Dr. Beets, Dr. Olde Damink, Dr. van Dam en Dr. Bemelmans van de chirurgie en Dr. Oosterhof van de Urologie voor de hulp en de altijd vriendelijke ontvangst op de operatiekamer. Ook bij de afdeling pathologie stond de deur altijd open. In het bijzonder wil ik Prof. dr. De Bruïne, Dr. Peutz, Dr. Van Suylen en Dr. Driessen van harte bedanken voor het verschaffen van de weefsels en het kritisch analyseren hiervan na kleuring in het lab.

Het lab tumorimmunologie en in het bijzonder Wilfred en Joris. Rasta wil jullie bedanken voor de leuke en vruchtbare samenwerking op de soms meest onmogelijke tijdstippen.

Een goede buur is beter dan een verre vriend! Alle pulmo's heel hartelijk dank voor het delen van kennis, apparatuur en momenten van ontspanning.

Dan de vele studenten waarmee ik met veel plezier heb samengewerkt. Dian, Tine, Caroline, Bas, Gilbert en Dennis (allen later collega's), Julia, Nona, Isabelle, Leon, Jasmin, Rob en Sandra: jullie werk heeft een heel belangrijke bijdrage geleverd aan dit proefschrift.

Verder dank aan alle mede-auteurs op de stukken.

Naast de al vermelde collega's wil ik alle anderen die mijn tijd op het lab zo bijzonder hebben gemaakt bedanken, waaronder: Marc Daemen, Anita, Joris, Juanita, Mieke, Patricia, Marloes, Anneke, Corine, Nicole, Marcel, Jacco, Johanne, Sander (dank voor je constructieve opmerkingen tijdens het schrijven van mijn proefschrift), Maarten, Eva, Ruben, Maartje, Kirsten, Kaatje, Nina, Mark, Hans, Nina, Sedi, Yanti, Froukje en Charlotte.

Zonder goede vrienden en familie zou de last van de onvermijdelijke tegenslagen veel zwaarder zijn en zou succes geen enkele betekenis hebben.

Lieve Sas, dank voor je begrip, steun, geduld en je blijvende interesse. Verder wil ik Jeroen en Saskia, Iris en Thijs, Luuk en Petra bedanken voor de gezelligheid, de nodige ontspanning en de oprechte belangstelling.

Lieve pap en mam. Eeuwig dankbaar ben ik voor jullie engelengeduld (met dat menneke dee meh neet wou liere), de onvoorwaardelijke steun voor mijn keuzes (ook als jullie er in eerste instantie niet blij mee waren), de gezelligheid, aandacht en liefde. Betere ouders kan ik me niet wensen.

Lieve Geertje, zomaar een zondag in het zuiden werd “unne super sjoenen daag”. Jouw liefde en vrolijkheid maken mijn leven compleet. Ik verheug me enorm op al die mooie toekomstplannen.

Publications

List of publications

1. **Wolfs TG**, Derikx JPM, Hodin CM, Driessen A, van Gemert WG, Heineman E, Bevins CL, Buurman WA. The localization of the LPS-recognition complex in the human healthy and inflamed gut. *Inflamm Bowel Dis*. 2010;16:68-75.
2. Debats IB, **Wolfs TG**, Gotoh T, Cleutjens JP, Peutz-Kootstra CJ, van der Hulst RR. Role of arginine in superficial wound healing in man. *Nitric Oxide*. 2009;21:175-183.
3. **Wolfs TG**, Buurman WA, Zoer B, Moonen RM, Derikx JP, Thuijls G, Villamor E, Gantert M, Garnier Y, Zimmermann LJ, Kramer BW. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. *PLoS One*. 2009;4:e5837
4. **Wolfs TG**, Dunn-Siegrist I, van't Veer C, Hodin CM, Germeraad WT, van Zoelen MA, van Suylen RJ, Peutz-Kootstra CJ, Elson G, Pugin J, Buurman WA. Increased secretion of MD-2 during human endotoxemia and sepsis; a crucial role for endothelial cells. *Mol Immunol*. 2008;45:3268-3277.
5. Brown HJ, Lock HR, **Wolfs TG**, Buurman WA, Sacks SH, Robson MG. Toll-like receptor 4 ligation on intrinsic renal cells contributes to the induction of antibody mediated glomerulonephritis via CXCL1 and CXCL2. *J Am Soc Nephrol*. 2007;18:1732-1739.
6. Luyer MD, Buurman WA, Hadfoune M, **Wolfs T**, van't Veer C, Jacobs JA, Dejong CH, Greve JW. Exposure to bacterial DNA before hemorrhagic shock strongly aggravates systemic inflammation and gut barrier loss via an IFN- γ dependent route. *Ann Surg*. 2007;245:795-802.
7. Vernooy JH, Reynaert N, **Wolfs TG**, Cloots RH, Haegens A, de Vries B, Dentener MA, Buurman WA, Wouters EM. Rapid pulmonary expression of acute-phase reactants after local lipopolysaccharide exposure in mice is followed by an interleukin-6 mediated systemic acute-phase response. *Exp Lung Res*. 2005;31:855-871.
8. **Wolfs TG**, de Vries B, Walter SJ, Peutz-Kootstra CJ, van Heurn LW, Oosterhof GO, Buurman WA. Apoptotic cell death is initiated during normothermic ischemia in human kidneys. *Am J Transplant*. 2005;5:68-75.
9. de Vries B, Walter SJ, Peutz-Kootstra CJ, **Wolfs TG**, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol*. 2004;165:1677-1688.
10. de Vries B, Walter SJ, **Wolfs TG**, Hocheppied T, Rabina J, Heeringa P, Parkkinen J, Libert C, Buurman WA. Exogenous alpha-1-acid glycoprotein protects against renal ischemia-reperfusion injury by inhibition of inflammation and apoptosis. *Transplantation*. 2004;78:1116-1124.
11. de Vries B, Walter SJ, von Bonsdorff L, **Wolfs TG**, van Heurn LW, Parkkinen J, Buurman WA. Reduction of circulating redox-active iron by apotransferrin protects against renal ischemia-reperfusion injury. *Transplantation*. 2004;77:669-675.
12. de Vries B, Matthijsen RA, van Bijnen AA, **Wolfs TG**, Buurman WA. Lysophosphatidic acid prevents renal ischemia-reperfusion injury by inhibition of apoptosis and complement activation. *Am J Pathol*. 2003;163:47-56.
13. de Vries B, Kohl J, Leclercq WK, **Wolfs TG**, van Bijnen AA, Heeringa P, Buurman WA. Complement factor C5a mediates renal ischemia-reperfusion injury independent from neutrophils. *J Immunol*. 2003;170:3883-3889.
14. de Vries B, Matthijsen RA, **Wolfs TG**, Van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation*. 2003;75:375-382.
15. **Wolfs TG**, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, van 't Veer C. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN- γ and TNF- α mediated up-regulation during inflammation. *J Immunol*. 2002;168:1286-1293.
16. Daemen MA, de Vries B, van't Veer C, **Wolfs TG**, Buurman WA. Apoptosis and chemokine induction after renal ischemia-reperfusion. *Transplantation*. 2001;71:1007-1011.
17. Daemen MA, Denecker G, van't Veer C, **Wolfs TG**, Vandenabeele P, Buurman WA. Activated caspase-1 is not a central mediator of inflammation in the course of ischemia-reperfusion. *Transplantation*. 2001;71:778-784.

18. Daemen MA, Heemskerk VH, van't Veer C, Denecker G, **Wolfs TG**, Vandenabeele P, Buurman WA. Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. *Circulation*. 2000;102:1420-1426.
19. Daemen MA, van 't Veer C, Denecker G, Heemskerk VH, **Wolfs TG**, Clauss M, Vandenabeele P, Buurman WA. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest*. 1999;104:541-549.
20. Daemen MA, van't Veer C, **Wolfs TG**, Buurman WA. Ischemia/reperfusion-induced IFN-gamma up-regulation: involvement of IL-12 and IL-18. *J Immunol*. 1999;162:5506-5510.

Curriculum Vitae

Curriculum Vitae

Tim Wolfs was born in Maastricht on the 2nd of August 1974. In June 1993, he finished high school at the Trichter College (currently Bonnefanten College) in Maastricht. From August 1993 until January 1998 he attended Applied Science in Microbiology at the Hogeschool Eindhoven (currently Fontys Hogescholen). In February 1998 he joined the Department of Surgery at the University of Maastricht (headed by Prof. dr. W.A. Buurman) as a research technician. Besides his work as research technician, he started his PhD project supervised by Prof. dr. W.A. Buurman in 2004.

In December 2009 he received a Postdoc Grant of the School of Oncology and Developmental Biology (GROW) for his research proposal entitled “mechanisms underlying the developmental alterations of the preterm gut during chorioamnionitis”. In January 2010 he started as a post-doctoral fellow with dr. B.W. Kramer at the department of Neonatology (headed by Prof. dr. L.I.J. Zimmermann).

Colour figures

Figure 2.2 Localization of constitutive and inflammation induced renal TLR2 mRNA by in situ hybridization. Renal TLR2 localization is shown in medulla (A) and cortex (B) of healthy kidneys, and in mice subjected to renal ischemia followed by 1 day (C,D) and 5 days (E,F) of reperfusion respectively. Constitutive expression for TLR2 was moderate and diffuse in cortex (B) and relatively low in the medulla (A). One day post-ischemia staining for TLR2 mRNA was reduced (C,D). Five days post-ischemia TLR2 expression is strongly enhanced (E,F) and predominantly located in collecting ducts, distal tubular epithelium and Henle's loop. The arrow indicates a macrophage like cell.

Figure 2.3 Localization of constitutive and inflammation induced renal TLR4 mRNA by in situ hybridization. TLR4 localization is shown by incubation with the anti-sense riboprobe in medulla (A) and cortex (B) in healthy kidneys, and in mice subjected to renal ischemia followed by 1 day (C,D) and 5 days (E,F) of reperfusion respectively. Control incubations using TLR4 sense riboprobe were negative (G). Constitutive expression for TLR4 was moderate and diffuse in cortex, mainly by the tubular epithelial cells (B), and relatively low in the medulla (A). One day after ischemia TLR4 expression was primarily observed in collecting ducts, distal tubules and the thin limb of Henle's loop (C,D). Five days after ischemia TLR4 mRNA staining was strongly enhanced in collecting ducts, the distal tubules and the thin limb of the loops of Henle (E,F). A small number of macrophage like cells was observed, which stained all positive. Arrows indicate macrophage like cells. Distal tubules were identified by Tamm Horsfall protein staining (H).

Figure 3.2 Activated Bax protein is predominantly expressed by distal tubular epithelial cells. While hardly detectable in control kidneys (**A**), Bax protein was increasingly expressed (red) in distal tubules in kidneys exposed to 30 min (**B**) or 80 min (**C**) of ischemia. In the ischemic sections also some focal staining of proximal tubules was observed (**B,C**). Proximal tubules were identified by FITC Lotus Lectin staining (green). Red staining, active Bax protein (Texas Red); blue staining, nuclei (4,6-diamidino(2)phenylindole). Magnification: x200.

Figure 3.4 Immunofluorescence staining of Bcl-2 during renal normothermic ischemia. Renal Bcl-2 staining was most abundant (red) in proximal tubules of control tissue (A) and in kidneys rendered ischemic for 35 min (B) and 80 min respectively (C). Less staining was detected in distal tubules in both control and ischemic tissue. Proximal tubules were identified by FITC Lotus Lectin staining (green). Red staining, Bcl-2 protein (Texas Red); green staining, Lotus Lectin (fluorescein isothiocyanate); blue staining, nuclei (4,6-diamidino(2)phenylindole). Magnification: x200

Figure 4.3 *In vivo* MD-2 expression in lung and liver. Paraffin sections of autopsy lung and liver tissue from patients that died of bronchopneumonia related sepsis (n=4) were immunostained with anti MD-2. A) In septic lungs MD-2 was clearly detected in endothelial cells (indicated by arrows) and pericytes (arrow heads). Asterix denote MD-2 positive inflammatory cells. B) In healthy lung (n=4), some endothelial (arrows) and inflammatory cells (arrow heads) stained weakly positive for MD-2. C) No AEC reaction product in septic lung tissue was detected using a mouse isotype control. D) In the healthy liver (n=4), MD-2 positive cells were hardly detected. E) MD-2 positive staining in the septic liver is represented by endothelial cells (arrow), Kupffer cells (arrow head) and inflammatory cells (inset) (original magnification 200x).

Figure 5.1 Characterization of monoclonal antibodies to human MD-2. Paraffin sections of MD-2 transfected CHO cells, stained for the presence of human MD-2, using AEC as a substrate (red) showed that MD-2 positive staining was observed in transfected cells, being 50% of the cells (A). No positive cells were detectable in mock-transfected cells (B).

Figure 5.2 MD-2 expression in the healthy and inflamed intestine of preterm and term infants and adults.

MD-2 positive cells, visualized with AEC (red), were not detected in paraffin sections of the immature intestine (n=10) (A1). In the healthy ileum of term infants and adults (n=10), MD-2 expression is mainly located at the base of the crypts of Lieberkühn (B1+C1). Sporadically, MD-2 positive inflammatory cells were detected in the lamina propria (indicated by arrows) (B1+C1). No MD-2 staining was detected in epithelial cells in paraffin sections of the mature healthy colon (n=10) (D1). Occasionally, MD-2 expressing inflammatory cells were observed in the lamina propria (indicated by arrows) (D1). No staining could be observed when an isotype-matched primary control antibody was used (E1). MD-2 expressing cells were not detected in paraffin sections of the immature intestine of NEC patients (n=10) (A2). During intestinal inflammation of the mature ileum, expression of MD-2 is observed in Paneth cells, but most is expressed by a subset of characteristic inflammatory cells in the lamina propria (C2). In the inflamed mature colon (n=20), large numbers of MD-2 positive cells were detectable in the lamina propria whereas epithelial staining for MD-2 was absent (D2). A representative section is shown for the outcome of three separate immunohistochemical experiments on the indicated samples.

Figure 5.3 Sequential double-antigen localization of MD-2 and alpha-defensin-5. For antigen colocalisation, MD-2 positive cells were visualized with DAB (brown) and a classical Paneth cell marker alpha-defensin-5 with HistoGreen (green). Immunoreactivity for alpha-defensin-5 but not for MD-2 was seen in the ileum of preterm infants (A). In the ileum of term infants and adults (B+C), Alpha-defensin-5 colocalized with all MD-2 positive epithelial cells (n=10). Representative examples of the immunohistochemical staining patterns are shown.

Figure 5.5 Identification of cells expressing MD-2 in the inflamed mucosa of the adult gut. Many inflammatory cells, positive for MD-2 (brown), co-express the marker for plasma cells, CD138 (green).

Figure 6.2 Immunolocalisation of ZO-1 (red) in the fetal intestine. At 125d GA, a fragmented staining pattern for ZO-1 was seen in control animals (A) or lambs exposed to endotoxin for 2 (B) or 14d (C). At 140d GA, a normal ZO-1 distribution was detected in control animals (D) that became disrupted upon endotoxin exposure for 30d (E). Magnification 200x. For inset, 1000x magnification was used.

Figure 6.3 At 14 and 30d after intraamniotic endotoxin injection, massive influx of PMNs was detected (A+B). At 140d GA, some MPO positive cells were detected in control sections (C). For each experimental group mean cell counts of MPO positive cells are depicted per high-power field (D).

Figure 6.4 Intraamniotic endotoxin results in increased ileal T-cell density. Significant increase of CD-3 immunoreactivity was seen at 14 and 30d after endotoxin exposure (A+B). For each experimental group mean cell counts of CD-3 positive cells are depicted per high-power field (C).

Figure 6.5 Endotoxin induced chorioamnionitis results in migration of gammadelta T-cells to sites of mucosal damage at late GA. After 2d or 14d of endotoxin exposure, hardly any gammadelta T-cells were detected in the upper mucosa of the preterm intestine (A+B). At 140d GA, gammadelta T-cells were not detected in the lamina propria whereas lymphoid follicles in the basal mucosa became populated with gamma delta T-cells (C+D). 30d after endotoxin injection, increased numbers of gammadelta T-cells were found within areas of mucosal damage (E).

Figure 6.8 Intraamniotic endotoxin induces eNOS expression. Weak eNOS staining was seen at 2d post endotoxin treatment (A) while expression increased in animals exposed to endotoxin for 14 or days 30d (B-C).