CHARLES UNIVERSITY IN PRAGUE
1st Faculty of Medicine

THE ROLE OF GUT MICROBIOTA AND LIPOPOLYSACCHARIDE CONTENT OF THE DIET IN THE DEVELOPMENT, MATURATION AND FUNCTION OF THE IMMUNE SYSTEM

By

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ABSTRACT

Mammals are essentially born germ-free but the epithelial surfaces are promptly colonized by astounding numbers of bacteria soon after birth. The most extensive microbial community is harboured by the distal intestine. The gut microbiota outnumbers ~10 times the total number of our somatic and germ cells. The host-microbiota relationship has evolved to become mutually beneficial. Studies in germ-free mice have shown that gut microbiota is essential for the proper development of the immune system. The pivotal role of the innate immune system in the complex and dynamic host-microbiota interactions has become increasingly evident.

The principal aims of the present study were: firstly, to determine whether LPS-rich sterile diet can promote maturation of the immune system in germ-free mice, secondly, to elucidate whether gut microbiota and LPS-rich sterile diet influence the LPS susceptibility, and finally, to investigate a role of the adaptive immunity in endotoxin shock. We have shown that the adaptive immunity plays a key role in the control of hyper-inflammatory immune reaction in endotoxin shock. We have also demonstrated that the presence of live gut microbiota and/or LPS-rich sterile diet increases susceptibility to LPS challenge in both immunocompetent and immunodeficient mice. Further, we have found that both live gut microbiota and LPS-rich sterile diet drive the expansion of B and T cells in Peyer's patches and mesenteric lymph nodes. The most prominent was the expansion of CD4+ T cells including Foxp3-expressing Tregs in mesenteric lymph nodes. We also investigated the effect of gut microbiota and LPS-rich sterile diet on the T_H1/T_H2 cytokine response and interleukin-10 production. We have shown that gut microbiota and

LPS-rich sterile diet shift the "default" germ-free T_H2-skewed cytokine profile towards T_H1-mediated response and stimulate interleukin-10 production.

To conclude, our data clearly show that both live gut microbiota and LPS-rich sterile diet increase susceptibility to endotoxin shock. Further, we demonstrate that immunodeficient SCID mice, which lack mature B and T cells, are more sensitive to endotoxin shock than immunocompetent Balb/c mice. In addition, we show that not only live gut microbiota but also LPS-rich sterile diet stimulates the development, expansion and function of the immune system. Our results are consistent with, and further expand the "hygiene hypothesis" by confirming that not only live organisms but also sterile microbiota-derived antigens drive the maturation of the immune system. Finally, we would like to emphasize that the quality of diet should be regularly tested, especially in all gnotobiotic models, as the LPS content of the diet may significantly alter the outcomes of experiments.

DEDICATION

This PhD thesis is dedicated to my entire family who always supported me in my academic undertakings. I dedicate this thesis to my dear wife Lucia and our sweet children Adam and Eva who have patiently supported my research over the years in spite of long hours of laboratory work. I also dedicate this thesis to my parents and my brother who have always shown support and interest in my work. Finally, I would also like to dedicate this thesis to my mother-in-law who was always ready to substitute my parental liabilities.

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BACKGROUND

Gut Microbiota
Definition, Localization, Composition and Functions

The gut microbiota is a vast and complex community of microorganisms, which normally lives in the gastrointestinal tract. The microbiota comprises mainly bacteria, but viruses, fungi and protozoa's are also present. The human intestinal microflora is estimated to contain 500 to 1000 species and the size of the population is ~10 times greater than the total number of our somatic and germ cells [1]. However, it is highly probable that 99% of the bacteria come from about 30 or 40 species.

The greatest numbers of bacteria and the most different species colonize colon. The activity of these bacteria makes the colon the most metabolically active organ in the body. The colonic bacteria make up about 60% of the mass of feces. Most of the bacteria in the colon are Gram-negative, while those in the small intestine are Gram-positive. The gut microbiota consists mainly of anaerobic bacteria that are difficult to analyze by conventional culturing techniques. Populations of species vary widely among different individuals but stay fairly constant within an individual over time. Most bacteria come from the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium*. Other genera such as *Escherichia* and *Lactobacillus* are present to a lesser extent. Species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that that genus is especially important in the functioning of the host.

The host-microbiota relationship is based mainly on mutualism, which is defined as a biological interaction in which two or more species benefit each other. The host provides an attractive niche with a regular supply of nutrients and stable temperature. The gut microbiota provides a host some useful functions, including fermentation of the undigested carbohydrates to short-chain fatty acids (SCFA), stimulation of the growth of intestinal epithelial cells, prevention of the growth of pathogenic bacteria by competing for nutrients and adhesion sites, producing vitamins, and regulating fatty acid metabolism [2-13].

Gut Microbiota
Effects on the Innate Immune System

One of the main functions of the immune system is to maintain a balance between the protection of barrier surfaces from pathogens and the establishment of a beneficial relationship with commensal bacteria. The fact that commensal bacteria do not trigger (in contrast to pathogens) inflammatory responses in mucosal tissues of the normal, healthy host is referred to as “commensal paradox” [14]. Multiple mechanisms have been identified by which tolerance to commensal organisms is induced and maintained. In addition to direct exclusion of bacteria and bacterial products via the physical barrier created by tight junction proteins, epithelial cells themselves produce factors that actively exclude bacteria from the intestinal lamina propria. Two of these are mucus, which is produced by goblet cells, and small antibacterial peptides, known as defensins. Mucus is composed of mucin glycoproteins, which are highly hydrophilic molecules that can bind to complex carbohydrates attached to the surface of absorptive epithelial cells (the glycocalyx). This

layer of mucus and surface carbohydrates is an effective barrier to bacterial attachment, and is abnormal in patients with ulcerative colitis.

The release of microbicidal molecules by barrier cells constitutes a crucial mechanism for maintaining the integrity of barrier epithelia by protecting the host against commensal organisms as well as potentially harmful microbes. The mammalian microbicidal repertoire includes several classes of antimicrobial peptides such as secretory leukocyte protease inhibitor (SLPI) [15], defensins [16], murine cryptidin-related sequence (CRS) peptides [17], and cathelicidins [18]. Although several anti-microbial peptides are constitutively expressed, depending on the cell type and peptide studied, their expression and release can be enhanced and/or induced by mediators such as retinoic acid, vitamin D3, butyrate, proinflammatory cytokines (e.g. TNF-α, IL-1, IL-6, and IFN-γ), as well as whole bacteria or MAMPs known to activate TLRs [16-18], or Nod1 and Nod2 [19-21].

The intestinal homeostasis is regulated by microbial-detection mechanisms of the innate immune system. Innate detection mechanisms involve the recognition of specific microbe-associated molecular patterns (MAMPs) by various families of germ line-encoded receptors, including transmembrane Toll-like receptors (TLRs) [22] and cytosolic nucleotide oligomerisation domain (NOD) proteins [23], containing leucine-rich repeats (NLRs). Investigation of the importance of pathogen recognition through PRRs by specialized antigen-presenting cells (APCs) such as dendritic cells (DCs) has become a major focus of study, because MAMP-PRR interaction followed by intracellular signaling and gene expression together with antigen processing and presentation have

been shown to play a central role in the initiation of T- and B-cell immune responses [22].

ECs possess microbial-detection mechanisms including a tightly regulated and specifically localized set of PRRs and their signaling components [24, 25]. ECs express a whole range of TLRs including TLR2, TLR4, TLR9 and TLR5, but their polarity and responsiveness serves to dampen positive signals. For example, TLR4 is poorly expressed, and after birth intestinal epithelial cells become tolerant to TLR4 signaling that may be mediated by IL-4 and IL-1 [26, 27]. Furthermore, TLR5 is more highly expressed at the basolateral surfaces, and the outcome of TLR9 signaling depends on the site of ligand exposure. Thus, basolateral TLR9 mediates NF-κB activation, while apical TLR9 appears to inhibit NF-κB activation [28], Furthermore, *TLR9*$^{-/-}$ mice are more susceptible to DSS colitis than wild-type mice [28]. Consistent with an overall protective role for TLR signaling in the epithelium, in DSS colitis, *MyD88*$^{-/-}$ mice are more susceptible to disease, which results from the repositioning of PGE2-producing stromal cells to intestinal crypts [29, 30]. *In vitro* or *in vivo* studies also indicate that TLR2 signaling in epithelial cells promoted PI3K/Akt-mediated cell survival via MyD88 [31].

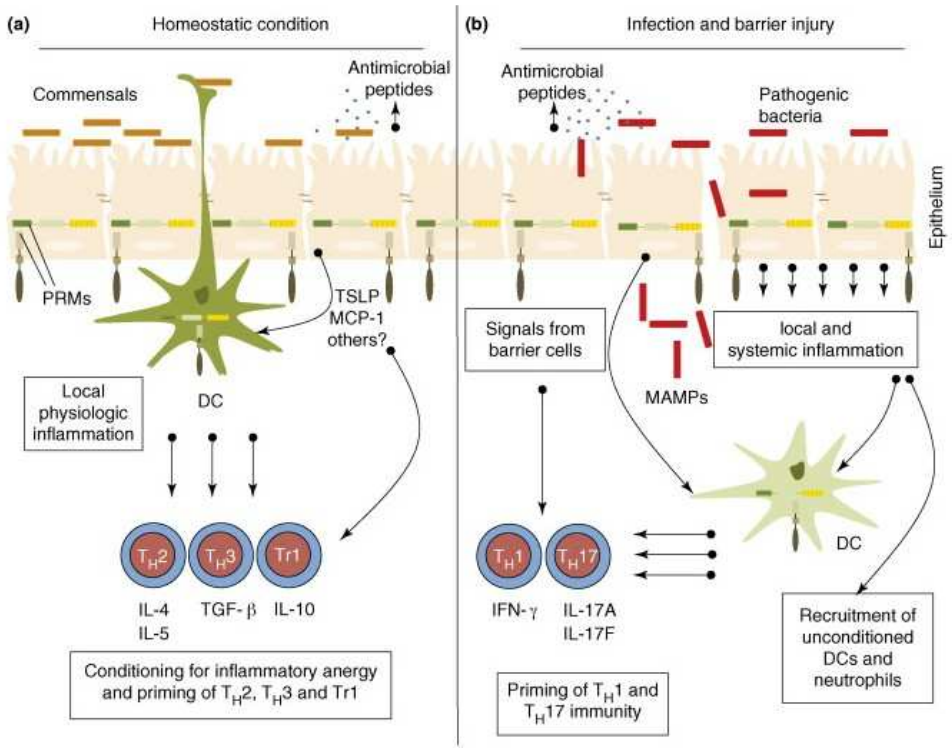
The enteric bacterial flora appears to be the major stimulus for the development of the mucosal immune system, as demonstrated by the improper development of mucosal lymphoid tissue in germ-free animals [32]. Moreover, microbial-detection mechanisms by the innate immune system have been thought to contribute to the establishment of mucosal lymphoid tissues as well as to the maintenance of barrier integrity, which involves the modulation of cell turnover and tissue repair functions, innate inflammatory

responses, antimicrobial protein expression, and the induction of adaptive immune responses. Collectively, these responses are thought to maintain a balance between protection of mucosal surfaces from pathogens on one hand, and the establishment of a beneficial relationship with enteric bacteria on the other.

Figure 1
Consequences of innate immune recognition by barrier cells: homeostasis versus infection and barrier injury

(a) *Under noninfectious, homeostatic conditions, exposure of ECs to commensal microbes and microbially derived constituents activates membrane spanning as well as intracellular PRRs to initiate the production of TSLP, MCP-1, and probably other mediators, which confer a local 'physiologic inflammatory' state for the maintenance of barrier integrity and baseline defense mechanisms, in the form of direct antimicrobial mediators. In parallel, these EC-derived factors condition antigen-sampling DCs for a state of 'inflammatory anergy,' thereby favoring the priming of T_H2, T_H3, and Tr1 cell responses (characteristic effector T-cell cytokines are depicted; IL-4, IL-5, TGF-β, and IL-10) and maintaining homeostasis.* ***(b)*** *Upon infection by pathogenic bacteria, or in situations of barrier injury, DCs sense two signals: the MAMPs of translocated microbes and signals emanating from the infected epithelial cells. The concerted action of these signals results in local and systemic inflammation. These events induce the release of cytokines and chemokines that recruit monocytes and neutrophils. Newly arriving monocytes give rise to unconditioned DCs, which then become fully activated. These de novo DCs are characterized by their ability to prime T_H1 and T_H17 immune responses for microbial clear-*

ance (characteristic effector T-cell cytokines are depicted; IFN-γ, IL-17A, and IL-17F). DC, dendritic cell; EC, epithelial cell; MAMP, microbe-associated molecular pattern; MCP-1, monocyte chemotactic protein-1; PRM, pattern-recognition molecule; T_H, T helper cell; Tr1, type-1 regulatory T cell; TSLP, thymic stromal-derived lymphopoietin (from Philpott et al., Trends in Immunology, 2007).



Microbe-associated Molecular Patterns
Definition, Function and Examples

Microbe-associated molecular patterns, or MAMPs, are small conservative molecular motifs consistently found on microbes. MAMPs are ligands for host receptors of

the innate immune system called pattern recognition receptors, or PRRs, which include the transmembrane Toll-like receptors (TLRs) [22] and the cytoplasmic NLR proteins (NLRs) [33]. They activate innate immune responses by identifying exogenous or endogenous ligands and their main function is to protect host from infection and signal a tissue damage. Bacterial lipopolysaccharide (LPS) is considered to be the prototypical MAMP. Other MAMPs include bacterial flagellin, lipoteichoic acid from Gram-positive bacteria, peptidoglycan, and nucleic acid variants normally associated with viruses, such as double-stranded RNA (dsRNA) or unmethylated CpG motifs.

Lipopolysaccharide (LPS)

LPS is a major structural component of the outer membrane of Gram-negative bacteria, consisting of a hydrophobic domain known as lipid A, a non-repeating core polysaccharide, and polysaccharide (O) side chains. Lipid A is embedded in the outer membrane while the core polysaccharide with polysaccharide side chains projects from the surface. The lipid portion of LPS is highly conserved, and extraction of lipid from bacteria that cannot synthesize polysaccharide proved the lipid A to be the source of toxicity. The polysaccharide side chains, also called O-chains or O-antigens, are easily recognized by the antibodies of the host; however, the O-antigens are very often modified by Gram-negative bacteria to avoid detection.

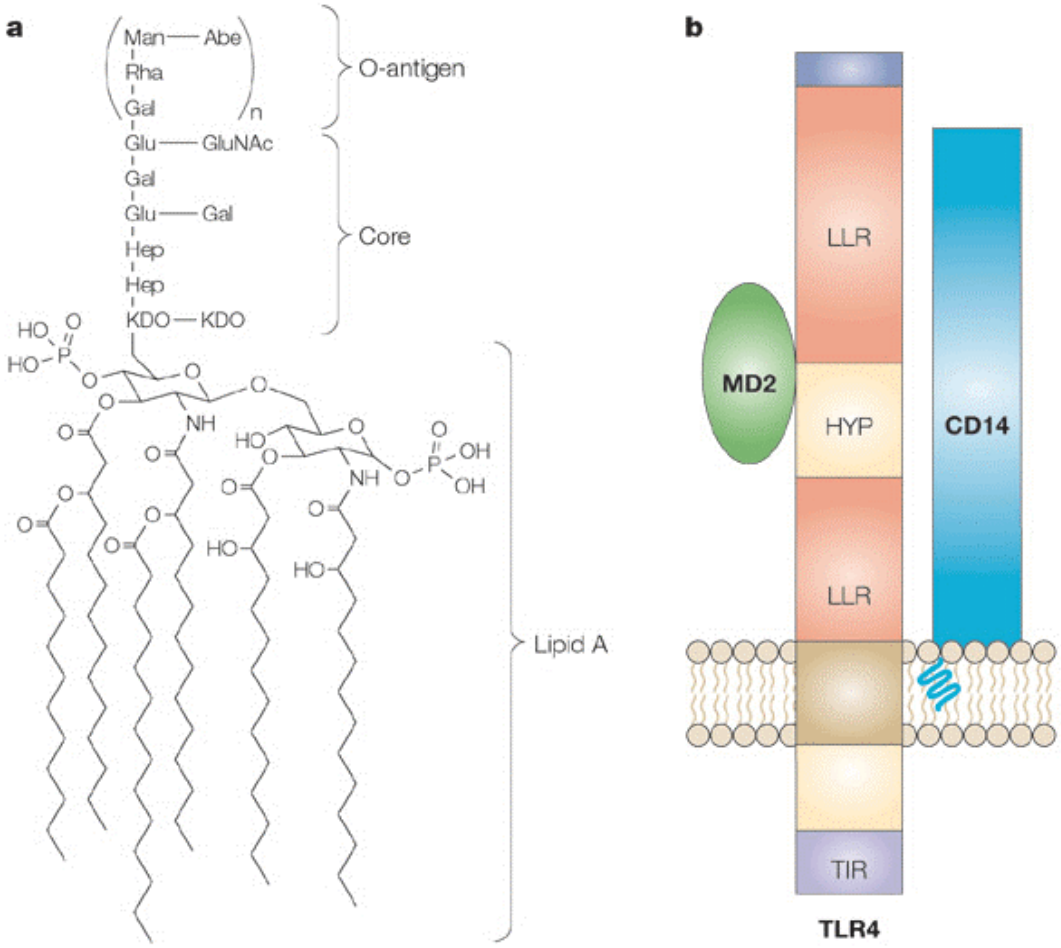
LPS is an endotoxin that induces an extremely strong inflammatory response of the innate immune system. LPS binds to the LPS binding protein (LBP) in the serum, which transfers it to CD14 on the cell membrane, which in turn transfers it to another

non-anchored protein, MD2, which associates with Toll-like receptor-4 (TLR4).The receptor complex of CD14/TLR4/MD2 promotes the secretion of nitric oxide and pro-inflammatory cytokines such as tumor necrosis alpha (TNFα) and interleukin-1β (IL-1β) in macrophages and monocytes, and may lead to sepsis or even septic shock.

However, it should be mentioned that several recent observations doubt the classical model of sepsis. Some scientists support the notion that the sepsis syndrome may be caused by endogenous substances such as heparan sulfate. Heparan sulfate is a biologically active sacharide, which is released from cell surfaces and extracellular matrices by almost every type of inflammation. It has been shown that heparan sulfate and oligosaccharides of hyaluronan activate dendritic cells via TLR4 [34, 35].

Figure 2
LPS structure and TLR receptor complex

(a) *LPS is composed of lipid A (endotoxin), core oligosaccharide and O-antigen.* ***(b)*** *Components of the TLR4–MD2–CD14 receptor complex. Different TLR4 regions are shown: leucine-rich repeats (LRR), a hypervariable region (HYP) and the intracellular TIR domain (from Bader et al., Nature Reviews, 2005).*



Pattern Recognition Receptors
Definition, Function and Examples

Pattern recognition receptors, or PRRs, are proteins expressed by cells of the immune system. Their main function is to detect molecules associated with microbial pathogens or cellular stress. The microbe-specific molecules that are recognized by a given PRR include bacterial carbohydrates such as LPS and mannose, bacterial or viral nucleic

acids, peptidoglycans and lipotechoic acids of Gram-positive bacteria, *N*-formylmethionine, lipoproteins and fungal glucans. Endogenous stress signals are called danger-associated molecular patterns (DAMPs) and include heparan sulfate, hyaluronic acid, fibrinogen, heat-shock proteins, uric acid and others. PRRs are classified according to their ligand specificity, function, localization and/or evolutionary relationships. On the basis of function, PRRs may be divided into endocytic PRRs or signaling PRRs. Signaling PRRs include the large families of membrane-bound Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding domain, leucine rich repeat containing (NLR) proteins. Endocytic PRRs promote the attachment, engulfment and destruction of microorganisms by phagocytes and are not involved in intracellular signaling. These PRRs recognize carbohydrates and include mannose receptors, glucan receptors and scavenger receptors of macrophages.

Membrane-bound PRRs

Toll-like Receptors and the Mannose Receptor

TLR are type I membrane glycoproteins, characterized by a cytoplasmic Toll/interleukin-1 receptor homology (TIR) signaling domain and an external antigen recognition domain comprising 19–25 tandem leucine-rich repeat (LRR) motifs. In humans, the ten functional TLR (TLR1–10) can be subdivided according to their subcellular localization. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface and migrate to phagosomes after activation, whereas TLR3, 7, 8 and 9 are expressed in intracellular compartments in nearly all cell types, principally in the endosomes and the endoplasmic reticulum, with the ligand-binding domains sampling the lumen of the

vesicle [28, 36, 37]. TLR tend to form noncovalent dimers in the absence of ligand. TLR2 preferentially forms heterodimers with either TLR1 or TLR6, whereas the other TLR appear to associate as homodimers [38].

Innate immune responses begin with TLR (or other PRR) recognition of specific microbial components widely expressed by bacteria, fungi, protozoa and viruses. Pathogen-encoded TLR ligands fall into three broad categories: lipids and lipopeptides (TLR2/TLR1; TLR2/TLR6; TLR4), proteins (TLR5) and nucleic acids (TLR3, 7, 8, 9) [38, 39]. TLR recognize groups of structurally similar and widely distributed molecules, in contrast to the highly selective molecular-level recognition of T- and B-cell receptors. The current view is that the cell surface TLR receptors recognize bacterial, fungal and protozoan pathogens by recognizing external molecules on these organisms, whereas viral infection of cells is recognized by the appearance of nucleic acids in intracellular compartments.More recently, endogenous ligands have also been identified for most TLR [38].

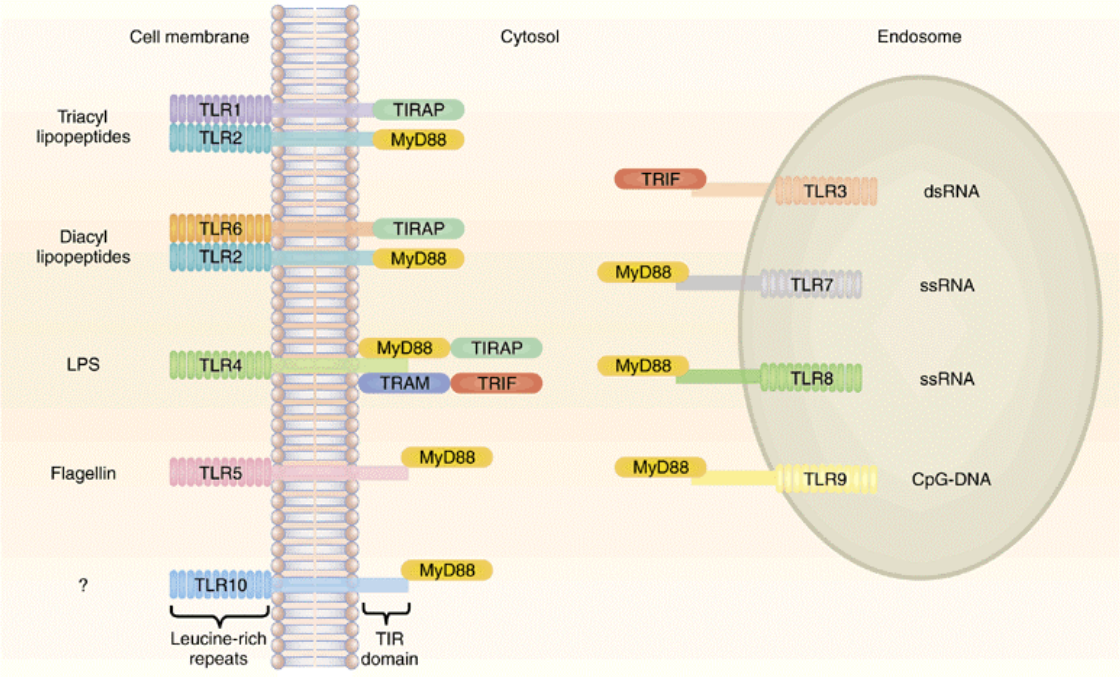
Pathogen recognition through TLR and other PRR serves three distinct functions [40-42]: sensing the presence and type of the pathogen, provoking an immediate antimicrobial response and stimulating the development of long-lasting adaptive response with effector functions appropriate to the type of pathogen. Thus, TLRs are key elements of the innate and adaptive immunity.

The mannose receptor recognizes a range of carbohydrates present on the surface and cell walls of microorganisms. It is primarily expressed on macrophages and dendritic cells and and its activation triggers endocytosis and phagocytosis of the microbe. The

mannose receptor belongs to the multilectin receptor protein group and, like the TLRs, provides a link between innate and adaptive immunity [43].

Figure 3
Toll-like receptors and their ligands

Schematic diagram of human Toll-like receptors showing adaptors, cellular orientation and examples of ligands (from Coffman et al., Nature Medicine, 2007).



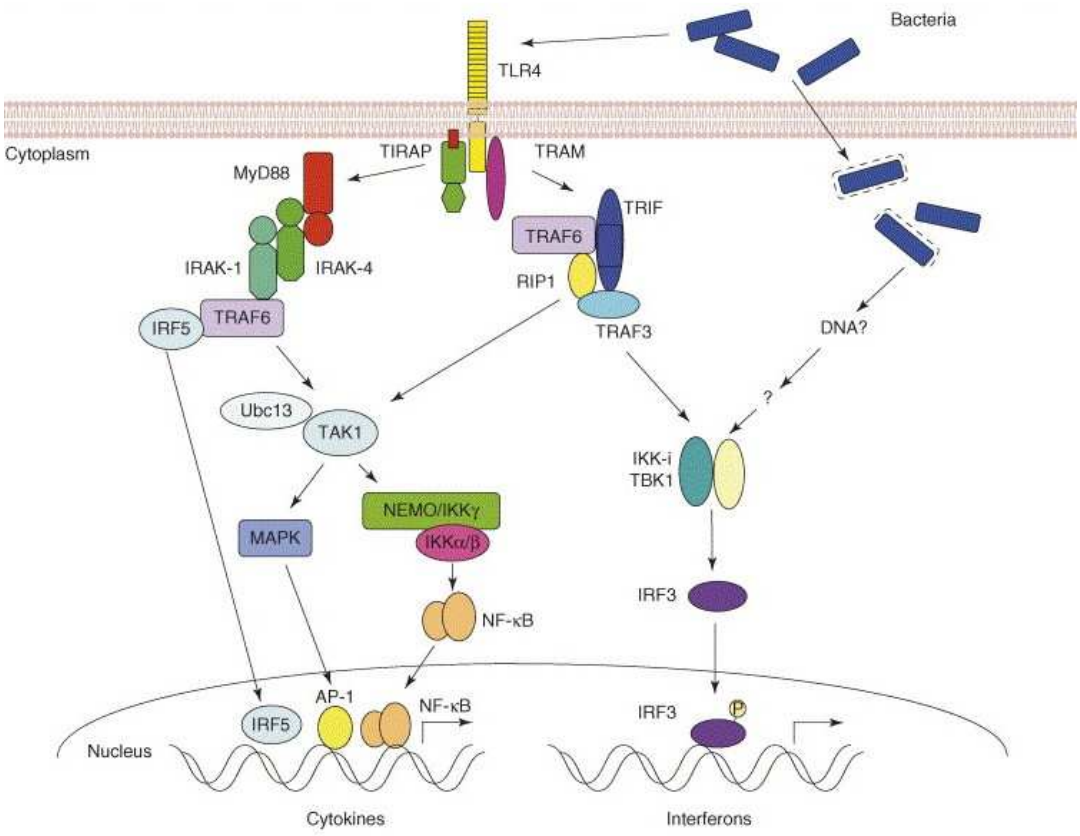
Signaling via TLRs

TLRs function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires MD-2. CD14 and LPS Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2. When activated, TLRs recruit adaptor molecules within the cytoplasm of cells in order to propagate a signal. Four adaptor molecules are known to be involved in signaling. These proteins are known as MyD88, MAL, Trif, and Tram. The adapters activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1, and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, thousands of genes are activated by TLR signaling, and collectively, the TLRs constitutes one of the most powerful and important gateways for gene modulation.

Figure 4
Signaling via TLR

Bacteria-mediated signaling pathways. TLR4 signaling pathways are shown as representative. Stimulation with TLR ligands recruits TIR domain-containing adaptors including MyD88 and TIRAP to the receptor, and sequential activation of IRAK-4, IRAK-1 and TRAF6 occurs. TRAF6- and UBC13-mediated ubiquitination activates the TAK1 complex, resulting in the activation of the IKK complex and nuclear translocation of NF-κB that initiates the expression of proinflammatory cytokine genes. TAK1 also activates

MAP kinases, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 also recruits other TIR containing adaptors, TRAM and TRIF, which trigger induction of type I interferons via TBK1/IKK-i. On the other hand, intracellular bacteria such as Listeria are recognized in the cytoplasm by unidentified receptor(s), and type I interferon is induced via a TBK1/IKK-i-dependent mechanism (from Takeuchi et Akira, Current Opinion in Cell Biology, 2007).



TLR4 Signaling
MyD88-dependent Pathway

The recognition of bacterial LPS by TLR4 leads to the activation of a signaling pathway resulting in the production of pro-inflammatory cytokines via adaptor molecule MyD88. MyD88 is shared by IL-1R family members and TLRs for the signaling, with the exception of TLR3. In TLR2 and TLR4 signaling, another adaptor, TIRAP (TIR domain-containing adaptor protein), is required for the recruitment of MyD88 to TLRs. TIRAP localizes to the plasma membrane by binding to phosphatidylinositol 4, 5-bisphosphate (PIP2), and this binding recruits MyD88 to the TLR [44]. MyD88 triggers sequential activation of IRAK-4, IRAK-1 and TRAF6. Together with an ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, TRAF6 catalyzes the formation of a K63-linked polyubiquitin chain on itself and on IKK-γ/NF-κB essential modulator (NEMO) [45]. TRAF6 also activates TGF-β-activated kinase 1 (TAK1), which phosphorylates IKK-β and MAP kinase kinase 6; these in turn modulate the activation of NF-κB and MAP kinases, which induce genes involved in inflammatory responses. Deletion of TAK1 and UBC13 in mice revealed that these molecules play a critical role in TLR-mediated cytokine production, in addition to their role in embryonic development [46-48]. TAK1 is essential for both NF-κB and MAP kinases, whereas UBC13 was dispensable for NF-κB activation. It was shown that IRF-5 associates with MyD88, and regulates the expression of cytokine genes, but not interferon genes, in response to stimulation with TLR ligands [49]. However, another report showed that IRF-5 was responsible for TLR7-mediated type I interferon induction [50]. Therefore, further studies are required to determine the role of IRF5 in the regulation of the interferon pathway.

TLR4 Signaling
TRIF-dependent Pathway

Detection of bacterial LPS via TLR4 also induces production of type I interferons via the TRIF-dependent pathway. However, infection of macrophages with intracellular bacteria, such as *Listeria monocytogenes* and *Legionella pneumophila*, appeared to induce type I interferons independent of the TLR system [51]. The induction requires a hemolysin, Lysteriolysin O (LLO), suggesting that the escape of bacteria into the cytosol of infected cells is required for the induction of type I interferons. *Listeria*-induced interferon-β production is dependent on the presence of TBK1 and IRF3 [52, 53]. It was proposed that genomic DNA from *Listeria* and *Legionella* was responsible for the production of type I interferons [51]. DNA- and *Listeria*-mediated interferon responses were not profoundly impaired in IPS1$^{-/-}$ mice (IPS1; an adaptor molecule linking retinoic acid-inducible gene I (RIG-I) and TBK1). Thus, the receptor and signaling pathways responsible for the induction of interferons by bacteria are yet to be identified.

Cytoplasmic PRRs
NLR Proteins (Nod Proteins, Ipaf and Naip, and Nalp Proteins)

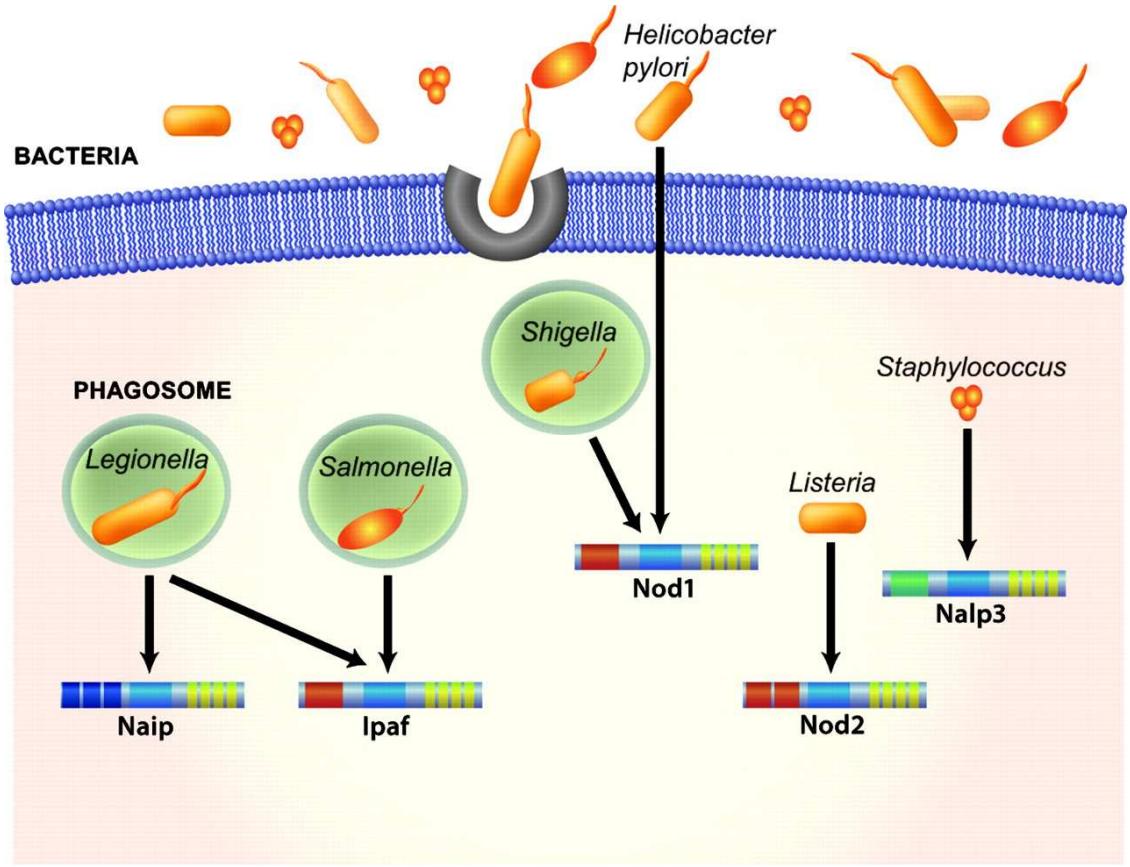
The nucleotide-binding domain, leucine rich repeat containing (NLR) proteins are a diverse family of cytoplasmic PRRs. These proteins are characterized by a central oligomerization domain, termed nucleotide-binding domain (NBD) and a protein interaction domain, leucine-rich repeats (LRRs) at the C terminus. The critical role of NLR proteins in host-pathogen interactions was first discovered in plants [54]. These molecules, which respond to microbial virulence factors, are referred to as disease-

resistance genes (R genes) [54]. NLR molecules in plants are a diverse protein family and mediate some of the most important mechanisms of host defense against infection in plants. It has been shown recently that some of the R gene-virulence factor interactions are not direct and the R genes seem to recognize enzymatic activities of the virulence proteins on an intermediate host protein [55, 56]. The NLR protein family in animals is a newly emerging class of innate immune molecules. Their similarity to plant R genes was first suggested by the cloning of cytoplasmic caspase-recruiting domain 4 (CARD4)/NOD1 [57, 58], and it was postulated early on that CARD4/NOD1 may act as a cytoplasmic sensor of microbial products [57].

To date, it is known that Nod1 and Nod2 detect bacterial cell wall components, whereas Ipaf and Naip detect bacterial flagellin, and NACHT/LRR/Pyrin 1 (Nalp1) has been shown to detect anthrax lethal toxin. NLR proteins comprise a diverse protein family indicating that NLRs have evolved to acquire specificity to various pathogenic microorganisms, thereby controlling host-pathogen interactions. Activation of NLR proteins results in inflammatory responses mediated by NF-κB, MAPK, or Caspase-1 activation, accompanied by subsequent secretion of proinflammatory cytokines. Mutations in several members of the NLR protein family have been linked to inflammatory diseases, suggesting these molecules play important roles in maintaining host-pathogen interactions and inflammatory responses.

Figure 5
NLR proteins and their ligands

Bacteria are internalized into the host cell by phagocytosis. Some bacteria survive in the phagosomes by suppressing host defenses and phagolysosomal fusion, and others have pathogenic mechanisms, which allow them to escape into the cytosol. Bacteria, which remain in the phagosomes, may use their pathogenic secretion system-generated pores to leak MAMPs into the cytosol for recognition by NLRs. Such a mechanism has been suggested for flagellin perception by Ipaf and Naip5. In addition, peptidoglycan moiety perception by Nod1, conferring resistance to extracellular H. pylori, has been suggested to be mediated by that bacterium's type IV secretion system. NLRs such as Nod1, Nod2, and Nalp3 have been shown to confer resistance to other bacteria (from Kobayashi et al., Journal of Leukocyte Biology, 2008).



Nod1 and Nod2

Nod1 and Nod2 were the first NLRs reported to function as intracellular microbial recognition molecules. These two proteins were shown to recognize moieties of the bacterial cell wall component, peptidoglycan. Many bacteria are classified by their cell wall structure and thickness of the peptidoglycan layer as Gram-positive, having thick peptidoglycan, or Gram-negative, having a thin layer of peptidoglycan. Nod2 has been shown to respond to MDP, consisting of NAM-L-Ala-D-Glu [59, 60]. This moiety is

conserved in Gram-positive and Gram-negative organisms, suggesting that Nod2 may confer resistance to a wide variety of bacteria. Indeed, Nod2 has been proposed to sense peptidoglycan from a variety of Gram-positive and Gram-negative heat-killed bacteria and bacterial extracts [61]. However, it is interesting that not all bacterial species containing large amounts of peptidoglycan have strong Nod2 stimulatory activity [61]. This may suggest that there is a specificity of Nod2 toward certain organisms, but the nature of this specificity, whether it is the ligand itself or the mode of ligand presentation, is unclear. Nod1, conversely, has been shown to detect GM-triDAP (GlcNAc-MurNAc tripeptide muropeptide) as well as simpler peptide versions containing *meso*-DAP [62, 63]. The sensing of peptidoglycan moieties with *meso*-DAP, found predominantly in Gram-negative bacteria, suggests that Nod1 restricts the growth of certain types of bacteria. Studies with heat-killed bacteria and their extracts also indicated that there is vast variation in Nod1 stimulatory activity from organisms, which contain the minimal iEDAP (isoglutamyldiaminopimelic acid) structure [61]. It is interesting that in those studies, *Bacillus* species had the greatest Nod1 stimulatory activity and a strong Nod2 stimulatory activity. Nod1 and Nod2 have been implicated in restricting growth of a number of specific bacteria, such as *L. monocytogenes* and *S. flexneri* [54, 63-65]. Although Hasegawa et al. [61] show that live *Listeria* infection and infection with the listeriolysin O mutant, which does not allow *Listeria* to escape into the cytosol, are capable of activating NF-κB in a Nod1-dependent manner, the same study shows that heat-killed bacterial cells and cell supernatants from overnight cultures failed to induce Nod1-dependent NF-κB activation. This suggests that the putative extracellular Nod1

stimulatory molecules are linked to *Listeria* viability. It is then possible that Nod1 may have additional stimulatory molecules, which may not be peptidoglycan moieties, or that bacterial and not host enzymes are required to generate Nod1 ligands. Overall, whether the nature of the specific growth restrictions is a direct result of sensing those specific bacteria or a secondary result of regulation of antimicrobial peptide production as a result of broad-range sensing remains to be determined.

Ipaf and Naip

Another set of proteins of the NLR family, which have been shown to recognize microbial structures, is Ipaf and Naip. Both proteins respond to flagellin, the main component of the bacterial flagellum [66, 67]. Experimental evidence using flagellin mutants in bacteria such as *Salmonella* and *Legionella* has indicated that those strains were unable to signal through Ipaf or Naip5/Birc1e. This showed that both proteins detect flagellin from *Salmonella* and *Legionella*, suggesting redundancy between Ipaf and Naip5.

Nalp3

Unlike the NLRs mentioned above, Nalp3 has been shown to respond to several ligands such as bacterial RNA, uric acid crystals, ATP, and pore-forming toxins [68-70]. Studies showing that Nalp3 may sense bacterial RNA and uric acid crystals independently or partially independently of TLRs, suggest that there may be two distinct pathways leading to the release of IL-1β [68, 70]. The active cytokine is generated by cleavage of its pro-IL-1β form by Caspase-1, which is produced as an inactive zymogen,

also needing to be cleaved into an active form. NLR family proteins such as Ipaf and Nalp3 have been implicated in regulating Caspase-1 activation. However, additional studies have shown that IL-1β release from macrophages requires two stimuli, one from TLR signaling, where pro-IL-1β is generated, and the other from a stimulus such as ATP, which presumably induces oligomerization and inflammasome assembly [69, 71, 72]. Recent studies showed that heat-killed bacteria and TLR ligands such as LPS can induce Caspase-1 activation in the presence of ATP in macrophages deficient in TLR4, MyD88, or TRIF, suggesting that bacterial products may activate Caspase-1 together with ATP in a TLR-independent manner [71]. In addition, studies suggest that Nalp3 does not sense ATP directly but rather, intracellular potassium depletion resulting from ATP signaling [69, 73]. Pathogen toxins, which insert themselves into host membranes, have been proposed to alter intracellular potassium in a Nalp3-dependent manner [69]. Such toxins include: nigericin, a potassium ionophore; maitotoxin, primarily a Ca^{2+} channel but also a transporter of other cations; and listeriolysin O. Therefore, it is likely that exogenous ATP may serve as a „danger signal“ leading to potassium depletion. This suggests that some NLRs may also sense nonmicrobial signals and molecules involved in cell defense.

Nalp1

The ligands for Nalp1 have been suggested to be MDP and the anthrax lethal factor of the *Bacillus anthracis* toxin [74, 75]. Studies using MDP included considerable biochemical and kinetic analysis between MDP and Nalp1 [75]. In vitro reconstitution experiments with those molecules showed that Nalp1 was activated and signaled

efficiently for IL-1β production [75]. The activation of purified Nalp1 with pure MDP in controlled reconstitution experiments raises the possibility that Nalp1 may not need the aid of another protein to recognize MDP, suggesting that interaction may be direct, although further studies are necessary to confirm this. One potential problem of the Nalp1 reconstitution experiments is that they were performed in vitro using purified protein. An in vivo study with a Nalp1 knockout is necessary to confirm MDP as a true ligand. As mentioned previously, Nalp1 was also shown to respond to anthrax lethal factor [74], which is a Zn^{2+}-dependent endoprotease, cleaving the N-terminus of MAPK kinases, altering signaling pathways, and leading to apoptosis. It is not known precisely how Nalp1 responds to the lethal factor, but this could be the first NLR shown to respond directly to a disease causing virulence factor secreted by a bacterium. In plant systems, the resistance genes recognize specific microbial virulence factors delivered into the host, but this recognition has been shown in some cases to be indirect, where the R genes actually recognize the virulence protein's enzymatic activities on another host protein [55, 56]. It is possible that the recognition of the lethal factor by Nalp1 may be through a similar mechanism.

Signaling via NLR Proteins

As a sensor of microorganisms, NLR proteins are programmed to activate host defense mechanisms. Two major signaling pathways have been described. One is NF-κB and MAPK activation through a serine/threonine kinase Rip2 [76-78]. A typical example is signaling through Nod1 or Nod2, which detect active moieties of bacterial

peptidoglycan. The other is activation of Caspase-1 through the activation of several NLRs, including Nalp1, Nalp3, Ipaf, and Naip. This pathway leads to IL-1β secretion and programmed cell death .

The latest proposed model of NLR activation postulates that inactive NLR proteins may rest in an autoinhibited conformation through intramolecular inhibition of the NACHT domain by LRRs [79]. The ligand recognition first may cause a conformational change of NLR proteins. Nucleotide triphosphate binding to the P-loop of the NACHT (NBD) domain changes its conformation further, which leads to oligomerization of the molecules. Oligomerization of NLRs subsequently recruits downstream effector molecules. In the case of Nod1 and Nod2, they recruit the downstream kinase, Rip2, which may cause autophosphorylation [77]. Rip2 activates the downstream signaling cascades including MAPKs and NF-κB . In the case of Nalp3, it recruits pro-Caspase-1 via the adaptor ASC [80]. The pro-Caspase-1 proteins cause autocleavage and activate Caspase-1 itself [81]. It is still unknown how many NLR molecules are involved in the final oligomeric protein complex.

Figure 6
Cellular signaling of NLR proteins

There are two major NLR signaling pathways, receptor-interacting protein 2 (Rip2)- and ASC-dependent pathways. Nod1 and Nod2 detect active moieties in bacterial peptidolycan, GM-triDAP, and MDP, respectively. Nod1 and Nod2 signal through a kinase, Rip2, which activates NF-κB and MAPKs, leading to the activation of immune response genes. In contrast to the Rip2-dependent pathway, the ASC-dependent pathway results in the activation of Caspase-1. NLRs, such as Nalp3, Nalp1, Ipaf, and Naip, activate Caspase-1 upon ligand recognition. Active Caspase-1 has dual roles: production of mature IL-1β by cleavages of pro-IL-1β and induction of programmed cell death of host cells, which may act as a host defense mechanism against pathogenic organisms (from Kobayashi et al., Journal of Leukocyte Biology, 2008).

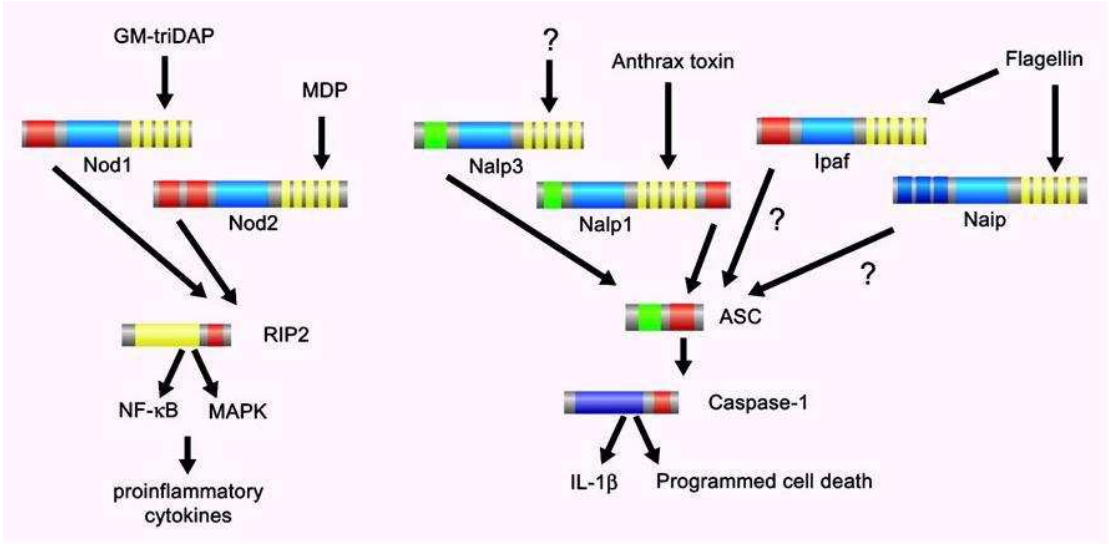
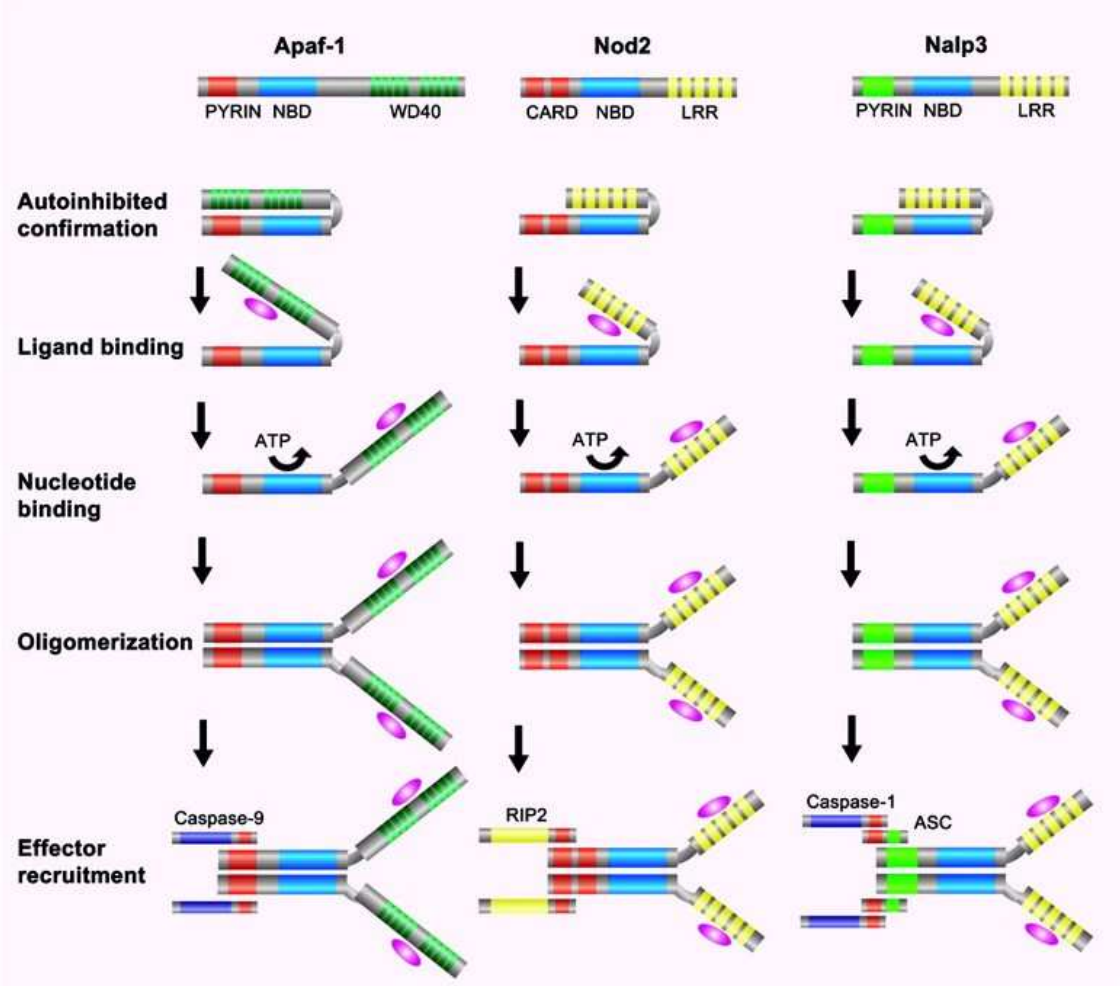


Figure 7
Activation of NLR proteins

The current, proposed model of NLR activation is analogous to the activation of Apaf-1, a critical mediator of apoptosis by the mitochondrial pathway. Without apoptotic stimuli, Apaf-1 holds an auto-inhibited conformation by binding of WD40 domains to CARD. When Cytochrome-c released from mitochondria binds to the WD40 domain (ligand binding), this event changes the conformation of Apaf-1, allowing access of ATP to the NBD. ATP binding to the NBD of Apaf-1 changes the confirmation of Apaf-1 (nucleotide binding) further, generating "active" Apaf-1, and Apaf-1 oligomerizes as a heptamer protein complex using NBD as an oligomerization domain (oligomerization). The oligomerized Apaf-1 recruits the downstream effector, Caspase-9, which causes autocleavage. By analogy, ligand binding of Nod2 and Nalp3 may result in a conformational change, subsequent oligomerization, and recruitment of downstream effectors. Nod2 may recruit the Rip2 kinase, which autophosphorylates and activates downstream signaling cascades. Nalp3 may recruit ASC adaptor and pro-Caspase-1, which activates self and cleaves downstream substrates such as pro-IL-1β (from Kobayashi et al., Journal of Leukocyte Biology, 2008).



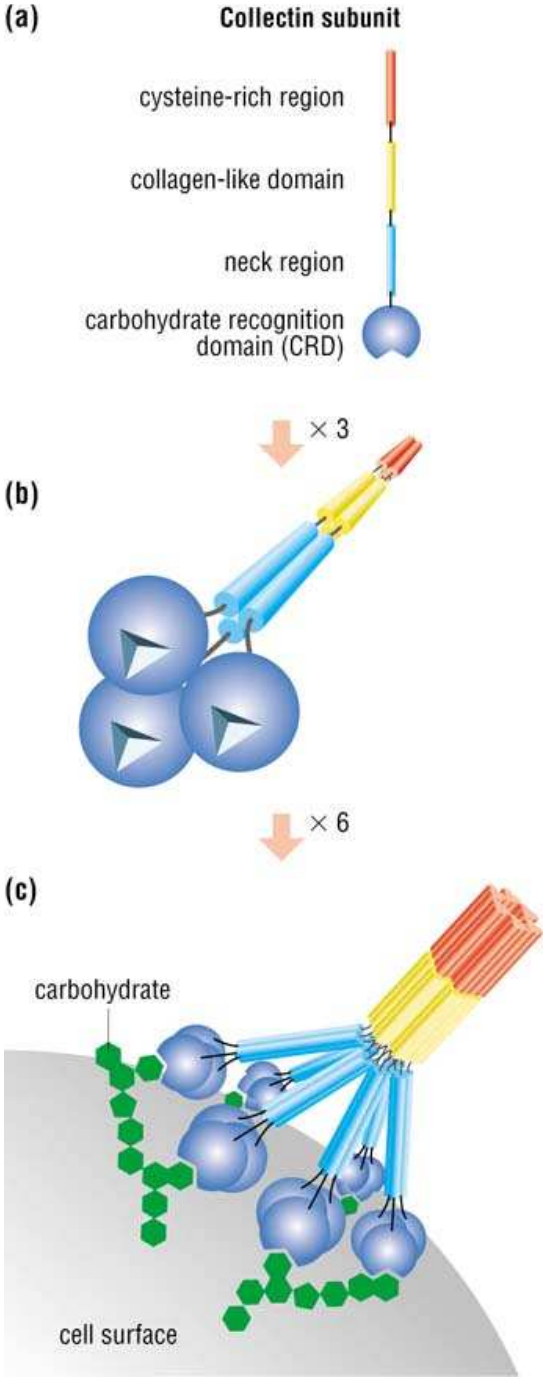
Secreted PRRs
Collectins, Pentraxins and Lysozyme

A number of PRRs do not remain associated with the cell that produces them. The most important examples of secreted PRRs are complement receptors including collectins, such as mannose-binding lectin and ficolins, and pentraxin proteins including serum

amyloid and C-reactive protein. Lysozymes, lipid transferases and peptidoglycan recognition proteins are other soluble molecule with a pattern recognition capacity.

Figure 8
Structures of the collectins

(a) *Each polypeptide chain of the collectin family of proteins is composed of an amino-terminal cysteine-rich region followed by a collagen-like region, an alpha-helical neck region and a carboxy-terminal globular domain that is a C-type lectin and is referred to as the carbohydrate recognition domain (CRD).* ***(b)*** *The polypeptide chains trimerize by coiled-coil interactions of the alpha-helical neck regions to form the basic subunit of structure.* ***(c)*** *Both mannose-binding lectin (MBL) and surfactant protein A (SP-A) are hexameric structures in which six of the basic subunits are linked together via the amino-terminal cysteine-rich regions and collagen-like regions to give hexameric units with 18 polypeptide chains. The resulting multiple arrays of CRDs give high-avidity binding to the repetitive macropattern of polysaccharide ligands in microbial cell walls. Surfactant protein D (SP-D) forms a tetramer of the trimeric basic units (12 polypeptide chains). Ficolins are also hexamers or tetramers of a similar structure, the main difference being the type of domain that comprises the carbohydrate recognition domain. (from Robertson et al., Immunity: The Immune Response in Infectious and Inflammatory Disease, New Science Press Ltd, 1999-2007).*


(a)
Collectin subunit
cysteine-rich region
collagen-like domain
neck region
carbohydrate recognition domain (CRD)
× 3
(b)
× 6
(c)
carbohydrate
cell surface

Mannose-binding Lectin

One very important collectin is mannose-binding lectin (MBL), a major PRR of the innate immune system that binds to a wide range of organisms. MBL has been shown to bind to yeasts such as *Candida albicans*, viruses such as *HIV* and *influenza A*, many bacteria including *Salmonella* and *Streptococci*, and parasites like *Leishmania*. MBL predominantly recognizes certain sugar groups on the surface of pathogens but also binds phospholipids, nucleic acids and non-glycosylated proteins. Serum MBL is bound to another protein, a serine protease called MASP-2 (MBL-associated serine protease-2). When MBL binds to its target the MASP protein activates the complement cascade by cleaving the blood protein C4 into C4a and C4b fragment. The C4b fragments then bind to the surface of the pathogen, and initiate the formation of a C3 convertase. The subsequent complement cascade catalyzed by C3 convertase results in creating a membrane attack complex, which causes lysis of the pathogen [82, 83].

Ficolins

Ficolins are also members of the collectin family of proteins which are able to recognize MAMPs on microbial surfaces. Upon binding to their specific MAMP, ficolins may trigger activation of the immune system by either binding to cellular receptors for collectins or by initiating activation of complement via the lectin pathway. The human ficolins including L-ficolin, H-ficolin and M-ficolin and murine ficolin-A were shown to associate with the lectin pathway-specific serine protease MASP-2 and catalyse its activation which in turn activates C4 and C4b-bound C2 to generate the C3 convertase

C4b2a. There is mounting evidence underlining the lectin nature of ficolins with a wide range of carbohydrate moieties recognized on microbial surfaces. However, not all members of the ficolin family appear to act as lectin pathway recognition components. For example, murine ficolin-B does not associate with MASP-2 and appears to be absent in plasma and other humoral fluids. Its stringent cellular localization points to other functions within the immune response, possibly acting as an intracellular scavenger to target and facilitate clearance of MAMP-bearing debris [83-85].

Figure 9
MASPs couple MBL and ficolins to the complement pathway

Mannose-binding lectin-associated serine proteases (MASPs) couple some collectins and some ficolins to the complement pathway. MBL, H-ficolin and L-ficolin bind MASPs. Before ligand binding, these MASPs lack significant protease activity. Binding of multiple carbohydrate recognition domains of the collectin or ficolin to a microbial cell surface leads to conformational changes in the molecule, perhaps due to the geometry of the ligand, which promotes proteolytic activation of the MASPs, which then can initiate the complement cascade (from Robertson et al., Immunity: The Immune Response in Infectious and Inflammatory Disease, New Science Press Ltd, 1999-2007).

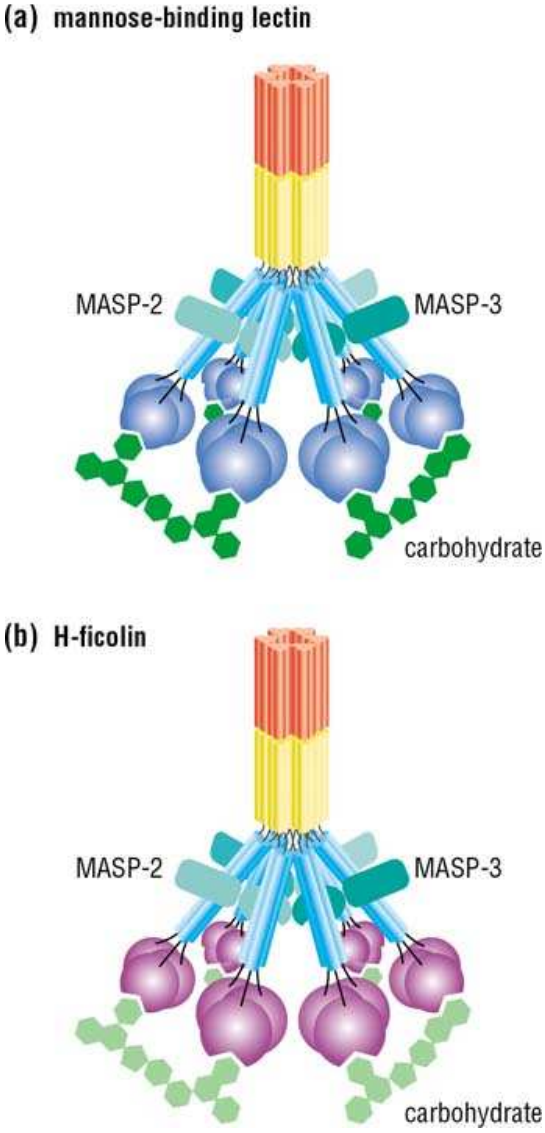
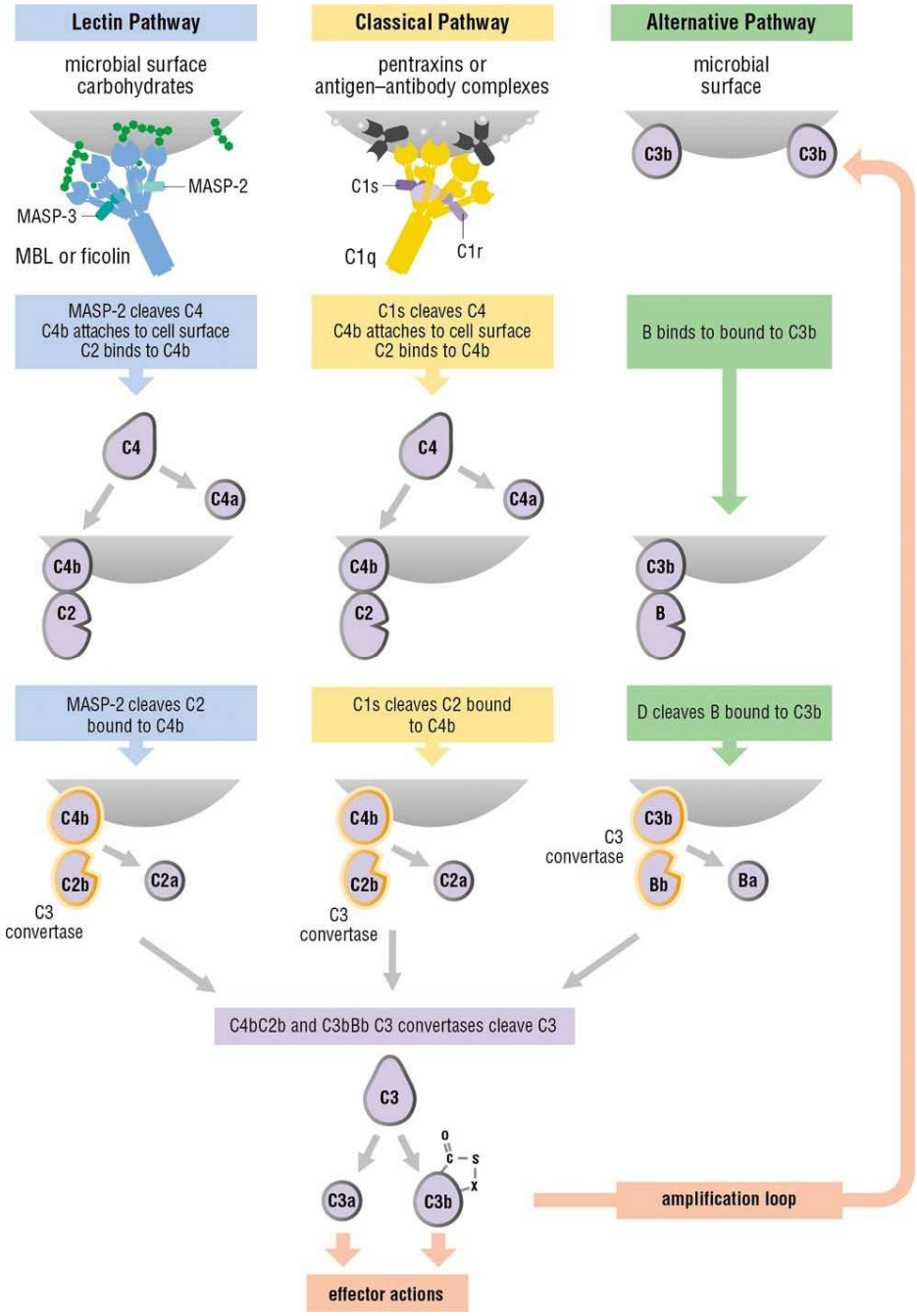

(a) mannose-binding lectin
MASP-2
MASP-3
carbohydrate
(b) H-ficolin
MASP-2
MASP-3
carbohydrate

Figure 10
Activation of the complement cascade

The lectin pathway, the classical pathway, and the alternative pathway all lead to the production of C3 convertases, proteolytic enzymes that cleave C3. In the lectin pathway and the classical pathway, the convertase is generated by cleavage of C4, binding of C2 to the newly generated C4b and then cleavage of C2, resulting in the C4bC2b complex, which is the C3 convertase of these two pathways. These cleavages are conducted primarily by MBL-associated serine protease 2 (MASP-2) in the lectin pathway and the C1q-associated protease C1s in the classical pathway. C1q and its associated proteases are closely related to MBL and the ficolins and their associated proteases. In the alternative pathway, C3b or the water-reacted form of C3 recruits factor B, which then becomes a substrate for the protease factor D. This generates the alternative pathway C3 convertase, which is a complex between C3b and the large fragment of factor B (Bb). C3 is cleaved by the C3 convertases into its two major fragments, C3a, which promotes inflammation by triggering mast cells, and C3b, which is central to the other effector functions of complement (from Robertson et al., Immunity: The Immune Response in Infectious and Inflammatory Disease, New Science Press Ltd, 1999-2007).


Lectin Pathway
Classical Pathway
Alternative Pathway
microbial surface carbohydrates
pentraxins or antigen–antibody complexes
microbial surface
MASP-2
MASP-3
MBL or ficolin
C1s
C1q
C1r
C3b
C3b
MASP-2 cleaves C4
C4b attaches to cell surface
C2 binds to C4b
C1s cleaves C4
C4b attaches to cell surface
C2 binds to C4b
B binds to bound to C3b
C4
C4a
C4b
C2
C4
C4a
C4b
C2
C3b
B
MASP-2 cleaves C2 bound to C4b
C1s cleaves C2 bound to C4b
D cleaves B bound to C3b
C4b
C2b
C2a
C3 convertase
C4b
C2b
C2a
C3 convertase
C3b
Bb
Ba
C3 convertase
C4bC2b and C3bBb C3 convertases cleave C3
C3
C3a
C3b
amplification loop
effector actions

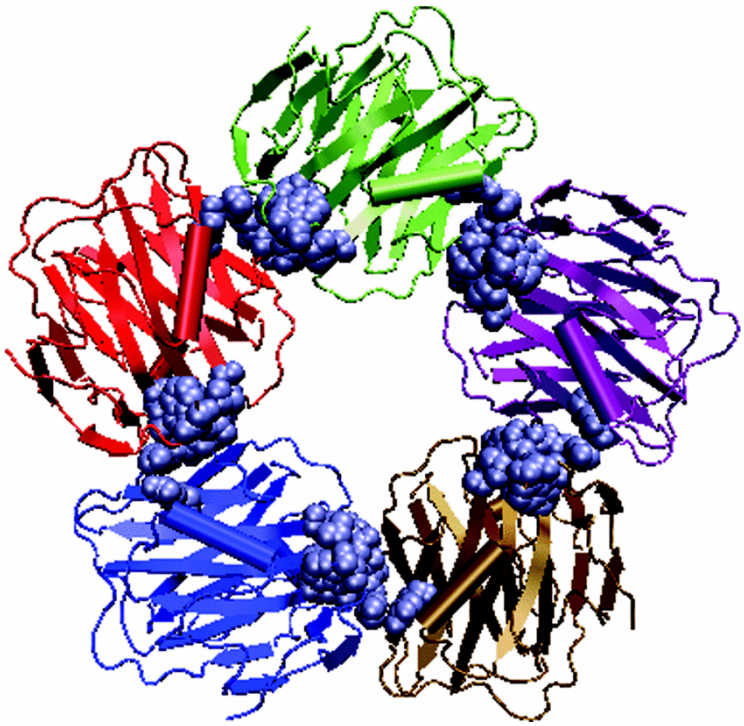
Pentraxins

Pentraxins are a family of multimeric PRRs, which are highly conserved in evolution. Based on the primary structure of the subunit, the pentraxins are divided into two groups: short pentraxins and long pentraxins. C-reactive protein and serum amyloid P-component are classic short pentraxins produced in the liver, whereas the prototype of the long pentraxin family is PTX3. Innate immunity cells and vascular cells produce PTX3 in response to proinflammatory signals and Toll-like receptor engagement. PTX3 interacts with several ligands, including growth factors, extracellular matrix components, and selected pathogens, playing a role in complement activation, facilitating pathogen recognition, and acting as a predecessor of antibodies. In addition, PTX3 is essential in female fertility acting on the assembly of the cumulus oophorus extracellular matrix. Thus, PTX3 is a multifunctional soluble PRR acting as a nonredundant humoural component of of innate immunity and is involved in tuning inflammation, in matrix deposition and female fertility. Evidence suggests that PTX3 is a useful new serological marker, rapidly reflecting tissue inflammation and damage [86-88].

Figure 11
Structure of pentameric pentraxin

The pentraxins are a phylogenetically ancient family of oligomeric plasma proteins, all of which bind Ca^{2+} ions. The binding of Ca^{2+} is necessary for the expression of ligand-binding activities. These proteins have evolved very little and hence they are highly conserved. They exhibit remarkable conservation of structure and binding specificities. All of the pentraxins are oligomers arranged in a discoid-like pentagonal (rarely hex-

agonal) cyclic symmetry. These proteins were named pentraxins because of their cyclic configuration of five non-covalently bound identical subunits. (from Vishveshwara et al., The Biochemical Journal, 2005).



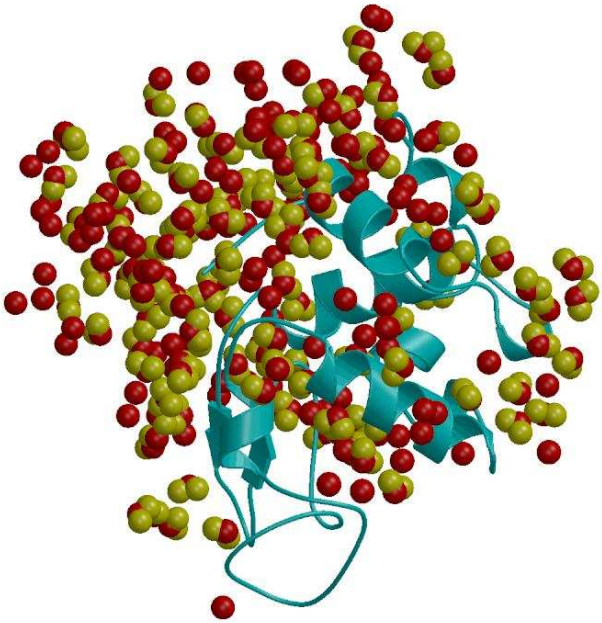
Lysozyme

Lysozyme is a family of enzymes which damage bacterial cell walls by catalyzing hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. It is abundant in a number of secretions, such as tears, saliva, and mucus. Lysozyme is also present in cytoplasmic granules of the polymorphonuclear neutrophils.

Large amounts of lysozyme can be found in egg whites. Lysozyme serves as a non-specific innate opsonin by binding to the bacterial surface, reducing the negative charge and facilitating phagocytosis of the bacterium. In addition, it can act also as an lytic enzyme attacking bacterial cell wall peptidoglycans and hydrolyzing the glycosidic bond that connects *N*-acetylmuramic acid with the fourth carbon atom of N-acetylglucosamine [89, 90].

Figure 12
Structure of lysozyme

Neutron crystallographic structure of hen egg-white lysozyme obtained at 1.7 resolution using the Laue neutron diffractometer. Orthogonal views are shown with the alpha-carbon backbone coloured cyan, oxygen atoms red and deuterium atoms yellow (from Lehman et Wilkinson, Acta Crystallographica, 1999).



Induction of the Innate Immune Responses

Recognition of ligands by TLRs leads to a series of signaling events resulting in the induction of acute responses necessary to kill the pathogen [91, 92]. These tasks include activation of the complement pathway and phagocytosis. In addition, anti-microbial proteins and peptides are induced by TLRs in various cell types, especially those of myeloid origin and also in Paneth cells of the gut epithelium [93]. TLRs also induce activation of cytokines such as IL-1β, IL-6, TNF and chemokines (for example, KC-1 and MCP-1) that collectively induce acute inflammatory responses to pathogens. TLRs also induce enhanced phagocytosis and killing by macrophages. Induction of type I interferons by TLRs in response to viral DNA or RNA leads to transcription of several anti-viral

proteins necessary to overcome viral infections. Several TLRs are known to participate in viral recognition, and the list includes TLR3, TLR7, TLR8 and TLR9 [94-96]. The importance of innate immune responses to microbial infection can be stressed by the fact that such a response is sufficient to protect the vast majority of existing animal species. Vertebrate hosts, however, have evolved an additional, more sophisticated form of defense system possessing properties of clonal expansion and memory. Accumulating evidence suggests that functional activation of the adaptive immune system depends on signals from TLRs of the innate immune system.

Induction of the Adaptive Immune Responses

TLRs are also responsible for the induction of dendritic cell maturation, which is responsible and necessary for initiation of adaptive immune responses. An optimal activation of naïve T cells requires signals through both TCR and CD28 molecules [97, 98]. These requirements are met only by a fully mature DC exposed to TLR ligands either in the secondary lymphoid organ or the peripheral tissues. Engagement of TLRs on DCs by TLR ligands leads to up-regulation of both MHC and co-stimulatory molecules [99]. Migration of this professional APC to the draining lymph node and subsequent interaction with naïve T cells ensures that immune responses are mounted only to pathogen-derived antigens. The interaction of immature DCs and naïve T cells leads to tolerance or induction of suppressor T cells [100].

In addition to controlling the co-stimulatory pathway, DCs seem to contribute to T cell activation by overcoming suppression mediated by Treg cells. Experiments with

MyD88-deficient mice revealed that LPS can induce DC maturation even in the absence of MyD88. However, MyD88-independent signaling induces up-regulation of MHC and co-stimulatory molecules but cannot induce secretion of cytokines. Further experiments showed that cytokines secreted in response to TLR ligands are necessary to overcome the suppressive effect of Treg cells [101]. The cytokine that is essential for overcoming suppression mediated by Treg cells is IL-6. This cytokine along with others secreted by DCs in response to TLR ligands acts by making responder T cells refractory to suppression and not by inactivating the suppressors. The advantage of this mechanism is that continued functioning of Treg cells prevents activation of self-reactive T cells during an immune response to infection. Cytokines secreted by DCs in response to TLR ligands are insufficient to induce T cell activation in the absence of induction of co-stimulatory molecules.

DC maturation induced by TLRs also involves secretion of cytokines and chemokines that control the choice of the effector class of adaptive immune responses. TLR signaling in macrophages and DCs leads to secretion of IL-12 that skews the resultant T cell response towards Th1 phenotype. Th1 cells secrete IFN-γ, which has multiple effector functions. It activates macrophages and enhances their phagocytic and microbicidal ability. It is also responsible for induction of class switching to IgG2a isotype in B cells [102]. TLR-mediated responses also induce IgG1 class of antibody, which is important for opsonization of microbes to facilitate uptake by phagocytes and also for activation of the complement cascade. An important issue that remains to be resolved is whether different DC subsets control T_H cell differentiation into effector cells.

Beneficial and Harmful Effects of LPS Activity

Antimicrobial Defense

Activation of the immune system by MAMPs, such as LPS, is beneficial, and helps the organism to recruit all humoral and cellular immune components in order to fight the invading pathogens. However, TLR mediated innate and/or adaptive immune responses play an important role in a variety of diseases, including sepsis, atherosclerosis, kidney failure, liver disease or inflammatory bowel diseases [103, 104].

LPS, TLR4 and Sepsis Syndrome

"Sepsis syndrome" is a condition characterized by fever, tachycardia, tachypnea and shock, regardless of the cause. The term "sepsis" refers to the sepsis syndrome associated with bacteria in the blood or large focus of infection [105]. The term "systemic inflammatory response syndrome" (SIRS) refers to the sepsis syndrome without detectable infection [106]. Sepsis, the sepsis syndrome and SIRS account for greater use of critical health care and more deaths than any condition other than coronary artery disease [107].

The classical model of sepsis states that injury or infection of tissues leads to entry of microorganisms such as bacteria. Penetrating microorganisms, their components and other microbial antigens trigger the production and release of pro-inflammatory mediators, such as TNFα, IL-1β, IL-6 or IL-8, which act to stimulate immune responses resulting in decreased tissue perfusion, multiple organ failure and eventually death.

The role of Gram-negative bacteria in the pathogenesis of sepsis has to be still elucidated. Although LPS can induce the sepsis syndrome when injected into animals [108] and humans [109, 110], it has been difficult to demonstrate that LPS causes the syndrome in those infected with Gram-negative bacteria. The level of LPS in the blood of septic patients does not predict the manifestations of sepsis or its outcome [111], and agents that block LPS do not decrease the manifestations or improve the outcome of sepsis [112] and [113]. For example, McCloskey *et al.* [114] showed that 32% of patients with Gram-negative sepsis treated using anti-LPS antibodies and 33% of control subjects died. Furthermore, the outcome of sepsis is not improved by LPS-neutralizing agents such as taurolidine or polymyxin B [112]. Of course, Gram-negative bacteria might have agonists other than LPS for TLR4, or for other TLR. However, this would cast doubt on the role of LPS and TLR4 in the classical model of sepsis.

The classical model of Gram-negative sepsis places TLR4 in a pivotal position: when stimulated by LPS or other MAMPs from microorganisms, TLR4 causes fever, shock and death in sepsis. This concept is supported by the fact that Sultzer's mutant mice (C3H/HeJ), which have non-functional TLR4, and mice lacking TLR4 [115] do not develop shock and do not die when given LPS. However, when C3H/HeJ mice are infected with Gram-negative bacteria, the manifestations of sepsis worsen and the rate of death increases. Thus, the LD50 for virulent *Escherichia coli* in C3H/HeJ mice is less than ten organisms, whereas the LD50 for wild-type mice is 10 000 organisms [116]. Thus, TLR4 appears to protect against rather than cause shock in sepsis.

TLR4 also paradoxically protects humans from Gram-negative infection. Smirnova *et al.* [117] found that TLR4 mutations, which are rare and probably disadvantageous in humans [118], are over-represented in patients with meningococcal meningitis or septicemia. Similarly, Lorenz *et al.* [119] genotyped 91 patients with septic shock and found that some of those with the most severe manifestations had TLR4 mutations (Asp299Gly and/or Thr399Ile) that impaired responses to LPS [120]. Thus, contrary to the predictions of the classical model of sepsis, TLR4 function might prevent the sepsis syndrome.

To address some of the most difficult challenges to the classical model of sepsis a new model of sepsis was suggested. This model of sepsis states that TLR4, and perhaps other TLR, are not ready to respond to MAMPs such as LPS, but must first be released from constitutive suppression. Injury or infection incites inflammation, which activates one or more proteases. Protease activation releases TLR4 from constitutive inhibition, and also liberates endogenous agonists of the receptor. Once released, TLR responds to endogenous and/or exogenous activators to amplify inflammation and initiate the sepsis syndrome.

LPS, TLR4 and Other Diseases

Inflammatory process plays a fundamental role in ischemic coronary artery disease (CAD) in terms of both the etiology of atherosclerosis and the pathophysiology of CAD. In particular, chronic inflammation plays a key role in coronary artery plaque instability and subsequent occlusive thrombosis. In recent studies, TLR4 signaling has been shown to be involved in the pathogenesis of CAD, establishing a key link between the

progression of coronary atherosclerosis and immune response to both foreign pathogens and endogenously generated inflammatory ligands. Guha and Mackman [121] have shown that activated TLR4 elicits the production of inflammatory cytokines and chemokines. Edfeldt *et al.* [104] have also found that TLR4 is prominently expressed in endothelial cells of human atherosclerotic lesion, but poorly expressed in normal human arteries. In the early atherosclerotic lesion, LPS and other ligands can stimulate the TLR4 expression on macrophages. The activated receptors can then initiate the signaling cascade that induces the expression of inflammatory cytokines, proteases, and cytotoxic oxygen and nitrogen radicals. These entities further speed up the progression of the atherosclerotic lesion [122]. In advanced atherosclerotic lesion, LPS can induce the proliferation of vascular smooth muscle cells, as well as the expression of elastin-degrading enzyme via TLR4 [123]. Besides that, in response to chemokines, more smooth muscle cells will also migrate to the sites of the lesions [124]. These predominant changes cause the accumulation of cells, extracellular matrix components, thickening of the intima, as well as the deformity of the arterial wall. Furthermore, TLR4 signaling might also be involved in atherosclerotic plaque destabilization. Grenier and Grignon have demonstrated that LPS induces the expression of matrix metalloproteinase-9 (MMP-9) by TLR4 in macrophages; MMP-9 has been shown to degrade collagen fibrous cap, thus predisposing plaque to rupture [125].

In many forms of liver diseases such as alcoholic or non-alcoholic liver disease, liver failure and inflammation are the result of a cascade of insults, which result in hyperactivation of inflammatory pathways and liver injury [126, 127]. Velayudham et al. [126]

have shown that there is an up-regulation of TLR4 receptors in liver granulomas and LPS induced liver injury. Pathogen-induced TLR4 activation also activates reactive oxygen species (ROS), which is a major source of acute hepatocyte injury and death in the liver. Up-regulation of peripheral blood monocyte expression of TLR4 also occurs in patients with chronic hepatitis C [128]. In addition, endogenous gut-derived bacterial LPS have also been implicated as important cofactors in the pathogenesis of liver injury. Within the liver, LPS binds to LPS-binding protein (LBP), which then facilitates its transfer to membrane CD14 on the surface of Kupffer cells in the liver [129]. Moreover, TLR4 can also interact with a protein ligand released from damaged hepatocytes to extend an existing injury in the liver [130].

In other studies, there is evidence that high-mobility group box 1 (HMGB1) can interact with both TLR2 and TLR4 to induce an inflammatory response during liver ischemia/reperfusion (IR) injury similar to that initiated by LPS [131, 132]. HMGB1 is an intracellular protein present in many species that functions in regulation and modulation of gene transcription [132]. HMGB1 is released readily from necrotic or damaged cells, which may signal through TLR4 the presence of advancing tissue injury, initiating an inflammatory response that further damages viable cells [132].

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the large intestine and, in some cases, the small intestine. The main forms of IBD are Crohn's disease and ulcerative colitis. The main difference between Crohn's disease and ulcerative colitis is the location and nature of the inflammatory changes. Crohn's disease can affect any part of the gastrointestinal tract, from mouth to anus, although a majority of the cases

start in the terminal ileum. Ulcerative colitis, in contrast, is restricted to the colon and the rectum. Microscopically, ulcerative colitis is restricted to the mucosa, while Crohn's disease affects the whole bowel wall. Finally, Crohn's disease and ulcerative colitis present with extra-intestinal manifestations (such as liver problems, arthritis, skin manifestations and eye problems) in different proportions.

The intestinal flora has long been thought to play a role either in initiating or in exacerbating the inflammatory bowel diseases (IBD). Host defenses, such as those mediated by the Toll-like receptors (TLR), are critical to the host/pathogen interaction and have been implicated in IBD pathophysiology. De Jager *et al*. showed that TLR4 and its signaling molecule TIRAP affect susceptibility to IBD [133]. Recent studies have shown that $TLR4^{-/-}$ and $MyD88^{-/-}$ knockout mice tend to be more prone to severe dextran sulfate sodium-induced colitis than their wild-type littermates [134]. Interestingly, CRX-526 a TLR4 antagonist has been shown to prevent an inflammatory disease in the dextran sulfate sodium and $mdr1a^{-/-}/1b^{-/-}$ deficient mice models [135]. To explain these contradictory results we have to consider that constitutive signaling through TLR4 may result in the production of tissue protective factors such as IL-6 and TNF-α [133]. This is the scenario in the $MyD88^{-/-}$ knockout mice, while in the case of the CRX-526 we may have selective downregulation of one of the TLR-4/LPS signaling pathways.

A recent hypothesis claims that some IBD cases are caused by an overactive immune system attacking various tissues of the digestive tract because of the lack of traditional targets such as parasites and worms. The number of people being diagnosed with IBD has increased as the number of infections by parasites, such as roundworm, hook-

worm and human whipworms, has fallen, and the condition is still rare in countries where parasitic infections are common. This is similar to the hygiene hypothesis applied to allergies.

Initial reports suggest that "helminthic therapy" may not only prevent but even cure IBD: a drink with roughly 2,500 ova of the *Trichuris suis* helminth taken twice monthly decreased symptoms markedly in many patients. It is even speculated that an effective "immunization" procedure could be developed—by ingesting the cocktail at an early age [136]. Prebiotics and probiotics are showing increasing promise as treatments for IBD [137] and in some studies have proven to be as effective as prescription drugs [138].

Gut Microbiota
Effects on the Adaptive Immune System

The Effect on GALT

Studies in germ-free (GF) mice have shown that gut microbiota plays a crucial role in the development and maturation of the immune system [14, 139-146]. There are many differences between GF animals and conventional (CV) animals. It was demonstrated that the gut-associated lymphoid tissue, which is the largest immune organ, is immature in GF mice. The content of the lamina propria CD4+ T cells, IgA producing B cells and intraepithelial T cells is reduced in GF mice [6, 147-154]. Peyer’s patches are hypoplastic with few germinal centers [155]. Comparative experiments have also shown that the gene expression profiles of the intestinal epithelial cells is shaped by the presence

of gut microbiota and that upregulated genes contribute to secretion of antibacterial molecules at the intestinal surface and the regulation of intestinal angiogenesis [156-158].

The Effect on Systemic Immunity

The effects of gut microbiota are not only limited to the short-range interactions on the gut-associated lymphoid tissue. Secondary lymphoid tissues and systemic immunity are also affected. GF mice have lower serum immunoglobulin levels and their mesenteric lymph nodes are smaller, less cellular and do not have germinal centers [6, 143, 144, 159, 160]. Spleens of GF mice are also smaller and the content of CD4+ T cells is reduced [141]. CD4+ T cells are a major cellular component of the adaptive immune system. CD4+ T cells are involved in activating and directing other immune cells. They are essential in determining B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages.

The Effect on T_H1/T_H2 Balance and T_H17 Cells

Activated CD4+ T cells could be functionally divided into four major subsets, designated T_H1, T_H2, T_H17 and Treg populations. These subsets are generally distinguished by their actions, including their production of specific cytokines and involvement in different types of immune reactions. T_H1 cells produce IFNγ, IL-2, TNFα, and lymphotoxin and participate in cell-mediated responses to intracellular pathogens. T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are involved in responses to large

extracellular pathogens such as parasites. The proper balance between T_H1 and T_H2 immunological responses is critical to overall human and animal health [161, 162]. A role of gut microbiota in establishing this equilibrium has been postulated [141, 163-165]. T_H17 cells produce IL-17A and IL-17F and were initially described as a pathogenic population implicated in autoimmunity; they are now thought to have their own distinct effector and regulatory functions [166, 167].

The Effect on Regulatory T Cells (Tregs)

Regulatory T cells are a specialized subpopulation of T cells, which suppresses activation of other immune cells and thus maintain immune system homeostasis. Depletion or functional abrogation of these cells can be a cause of autoimmune diseases and allergies [168-172].

The latest research suggests that Tregs are best defined by the expression of the transcription factor Foxp3 [170-172]. The large majority of Foxp3-expressing Tregs is found within CD4+ helper T cell population and expresses high levels of the interleukin-2 receptor alpha chain (CD25). Regulatory T cells comprise about 5-10% of the mature CD4+ helper T cell subpopulation in mice. Mutations in the gene encoding Foxp3 result in the development of overwhelming systemic autoimmunity in the first year of life in both humans and mice.

It is still controversial whether gut microbiota and microbiota-derived antigens play a role in the development and maturation of Tregs. In the transfer model of colitis developing in CD4+CD45RB^{high} T cell reconstituted immune-deficient SCID mice we

have shown that the presence of normal gut microbiota enhances a functional potency of the Treg population. The inhibitory activity of CD4+CD45RB^{low} T cells from GF mice was significantly impaired compared to the population isolated from specific-pathogen free mice [173]. It has been recently reported that gut microbiota is crucial for the generation and expansion of Tregs [174]. Ostman et al. reported that CD25+ Tregs from GF mice are less effective in suppressing proliferation of responder CD4+CD25- T cells. However, they did not find any difference in the proportion of CD4+Foxp3+ T cells between CV and GF mice. The only deficit of CD4+Foxp3+ T cells in GF mice was detected in the liver-draining celiac lymph nodes [175]. Paradoxically, it was reported that CD25+ Tregs from GF mice are as suppressive and protective as those from CV mice [176, 177] and Booki et al. recently reported that peptide antigens derived from intestinal microorganisms are not essential for the generation, in vivo proliferation or suppressive activity of Tregs [178].

Consequences of Insufficient Microbial Stimulation of the Immune System "Hygiene Hypothesis"

The incidence of several chronic inflammatory disorders has been increasing markedly in developed countries. These comprise allergic disorders including asthma and hay fever, some autoimmune diseases including type 1 diabetes and multiple sclerosis and inflammatory bowel diseases. The "hygiene hypothesis", which was firstly proposed by Strachan in 1989, suggests that increases in chronic inflammatory disorders seen in Westernized populations might be partially attributable to insufficient exposure to organ-

isms including symbiotic microorganisms and parasites that were always part of mammalian evolution. The diminished exposure to microorganisms, parasites and their components leads to a failure to adequately develop immunoregulatory mechanisms. The mechanism by which these microorganisms promote the maturation of immunoregulatory pathways has to be still elucidated. However recent studies suggest that microbiota and helminthes, recognized by PRRs, promote the maturation of DCs into regulatory DCs that drive regulatory T cell responses to the antigens of these organisms. The permanent presence of these antigens in the gut leads to continuous background production of anti-inflammatory cytokines, such as IL-10 and TGFβ, by Tregs. The resulting bystander suppression of other immune cells may thus help to downregulate autoimmune diseases, inflammatory bowel diseases or asthma.

CHAPTER TWO

SIGNIFICANCE OF THE STUDY

The intestinal mucosa is exposed to an enormous load of bacterial and food antigens. A single layer of epithelial cells separates host tissues from the gut luminal content. The peaceful coexistence of the host and gut microbiota has evolved eons and its impacts on the development, maturation and function of the immune system are not well understood.

It is well documented that a lack of early childhood exposure to infectious agents, gut microbiota, and parasites increases susceptibility not only to allergic diseases and asthma, but also to T_H1-driven diseases including type I diabetes and inflammatory bowel diseases (IBD). This theory was first published by David P. Strachan in 1989 and called "hygiene hypothesis" [179]. The major proposed mechanism is that the developing immune system must receive sufficient stimuli to adequately develop regulatory T cells. What are the effects and mechanisms of the microbial stimulation on the development of regulatory T cells and what is the role of innate immunity has to be still elucidated.

Thus it is obvious that the deeper understanding of the interactions between host immune system and gut microbiota and/or microbial antigens might have an impact on the development of new strategies in the prevention and therapy of allergic diseases, inflammatory bowel diseases or even colorectal carcinoma [14].

CHAPTER THREE

RESEARCH DESIGN

It has been well documented that the gut microbiota is essential for the development, maturation and function of the immune system. The aim of the study was to investigate the effects of live gut microbiota and LPS content of the sterile diet on the susceptibility to endotoxin shock and to determine their role in the development and function of the immune system.

To address this issue we have established three experimental groups different in terms of stimulation with live gut microbiota and LPS. A group of germ-free mice fed low LPS diet (AIN-93G), a group of germ-free mice fed LPS-rich diet (ST1) and a fully stimulated group of conventional mice fed LPS-rich diet (ST1). Our preliminary data have shown that the effects of decreased stimulation in conventional mice fed low LPS diet (AIN-93G) are negligible thus the group of conventional mice fed low LPS diet was excluded from further analysis. What concerns genetic background of experimental mice, most of the presented data have been generated using immunocompetent Balb/c mice. Immunocompromised SCID mice, which lack components of adaptive immunity, were used to determine the role of adaptive immunity in the pathogenesis of endotoxin shock.

However, to reveal the mechanisms by which gut microbiota and their antigens stimulate the adaptive immune system we have to focus on components of the innate immunity, which serve as a bridge between gut microbiota and the adaptive immunity. In future projects we plan to use animal models of mice deficient for IRAK-M or Rip2 proteins, which have increased and decreased signaling via TLR. By comparing their re-

sponse to LPS under conventional and germ-free conditions while simultaneously considering the level of LPS content of the diet, we would like to reveal the mechanisms by which gut microbiota interacts with the immune system.

CHAPTER FOUR

SPECIFIC AIMS OF THE STUDY

The principal aims of the present study were to investigate the effects of live gut microbiota and LPS content of the sterile diet on the development and maturation of components of the immune system and to assess the effects of live gut microbiota and LPS content of the sterile diet on the susceptibility to LPS. To address these aims, we have established three groups of mice different in terms of stimulation with gut microbiota and their antigens: a group of GF mice fed LPS low diet, a group of GF mice fed LPS-rich diet and a group of CV mice fed LPS-rich diet. To determine the role of adaptive immunity in the regulation of LPS susceptibility we have used immunocompetent Balb/c and immunodeficient SCID mice, which lack mature B and T cells.

Specific Aim 1: Rearing of Germ-free Balb/c and SCID Mice

The long-term colonies of germ-free Balb/c and SCID mice have been established by Dr Stepankova. Briefly, the mice have been delivered into germ-free conditions by hysterectomy. Hysterectomy was performed in gestational day 19-20. The delivered fetuses have been transferred to germ-free SCID foster mothers to ensure breast feeding of the newborns. After weaning, mice have been fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1). The sterility of germ-free mice has been tested every week using swabs, which were cultivated both for aerobes and anaerobes.

Specific Aim 2: To Determine the Concentration of LPS in Mouse Pelleted Diets.

For the purpose of the study it was essential to analyze the load of microbiota-derived antigens in mouse pelleted diets. The concentration of LPS was set as a measure of overall bacterial presence in diets. To study the effect of LPS content of the sterile diet on the development, maturation and function of immune system we had to first identify both the diet with very low and very high LPS content. The concentration of LPS was measured by chromogenic LAL test and expressed in endotoxin units (EU) per µg of pellet. The diet with the lowest LPS level was purified diet AIN-93G supplied by Harlan-Teklad and the diet with the highest LPS concentration was a grain-based diet called ST1 supplied by VELAZ. These two diets, selected on the basis of the maximum difference in the level of LPS concentration, were used in all experiments.

Specific Aim 3: What Is the Influence of Gut Microbiota and LPS Content of the Sterile Diet on the Weight and Cellularity of Lymphoid Organs?

To address this issue the weight of spleen and thymus isolated from conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) was recorded. In addition the cellularity of spleen, thymus, mesenteric lymph nodes, Peyer's patches and a number of cells isolated from peritoneal cavity of conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been determined.

Specific Aim 4: Are Animals Susceptible to LPS in the Absence of Gut Microbiota?

In Vivo Susceptibility to LPS

To address this aim, the conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diets (ST1) have been challenged with LPS intraperitoneally and

the levels of pro-inflammatory cytokines in sera have been measured. The determination of a survival curve was not possible due to capacity limitations.

Specific Aim 5: What Is the Influence of LPS Content of the Sterile Diet on the Susceptibility to LPS under Germ-free Conditions?

In Vitro Susceptibility of Spleen Cells to LPS

To address this issue the spleen cells isolated from conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been stimulated with different concentrations of LPS and ConA for 48 h. The levels of pro-inflammatory cytokines in culture supernatants have been determined using the Luminex multiplex analyzer.

In Vitro Proliferative Response of Spleen Cells to Stimulation with LPS

To investigate the effect of live gut microbiota and LPS content of the sterile diet on *in vitro* proliferative response. The spleen cells from conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been isolated and stained with CFSE. CFSE-labeled cells were stimulated with different concentrations of LPS and ConA for 48 h, and then CFSE dilution was analyzed by flow cytometry.

Specific Aim 6: Do the Live Gut Microbiota and LPS Content of the Sterile Diet Modify the Composition of Main Lymphocyte Subpopulations?

To address this aim the single cell suspensions isolated from Peyer's patches, mesenteric lymph nodes, spleen, peritoneal cavity and thymus of conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been surface stained with FITC and PE conjugated anti-mouse CD4 mAb, PE conjugated anti-mouse

CD8 mAb and PE conjugated anti-mouse CD19 mAb. To prevent non-specific staining the cells have been blocked with anti-mouse CD16/CD32 mAb prior to staining. The data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, USA) and analyzed with WinMDI software (Joseph Trotter).

Specific Aim 7: Do Gut Microbiota or LPS Content of the Sterile Diet Influence the Proportion of Foxp3-expressing Regulatory T Cells?

To analyze the effect of live gut microbiota and LPS content of the sterile diet on the frequency of Foxp3-expressing regulatory T cells the cell suspensions from Peyer's patches, mesenteric lymph nodes, spleen, peritoneal cavity and thymus of conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been isolated. The single cell suspensions were surface stained with FITC conjugated anti-mouse CD4 mAb or FITC conjugated anti-mouse CD8 mAb and intracellularly stained with PE conjugated anti-mouse Foxp3 mAb. The proportions of CD4+Foxp3+ and CD8+Foxp3+ T cells have been analyzed by flow cytometry.

Specific Aim 8: Do Gut Microbiota and LPS Content of the Sterile Diet Shift the T_H1/T_H2 Balance?

To determine whether the live gut microbiota and LPS content of the sterile diet correct T_H1/T_H2 imbalance present in germ-free mice I have isolated spleen cells from conventional and germ-free mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1). The cells have been stimulated with LPS and ConA for 48 h and the production of IFNγ, IL-4, IL-10 and IL-12 was determined by Luminex analyzer.

CHAPTER FIVE

MATERIAL AND METHODS

Experimental Mice

All mice were bred at the animal facility of the Institute of Microbiology of the AS CR (Novy Hradek, Czech Republic) and were used at the age of 8-10 weeks. Germ-free (GF) Balb/c and SCID mice were caesarean-derived and maintained in flexible plastic isolators. Faecal samples from GF mice were cultured under both aerobic and anaerobic conditions on a weekly basis and prior to the experiment to validate continued sterility of the colony. The conventional (CV) mice were regularly checked for the absence of potential pathogens according to an internationally established standard (FELASA). All the experiments were performed in the Department of Immunology and Gnotobiology of the Institute of Microbiology of the AS CR. All the researchers who handled the animals have been certified by the Central Committee for Animal Welfare. The Institute of Microbiology is authorized by the Central Committee for Animal Welfare to carry out experiments on laboratory animals. The local ethical guidelines are in compliance with Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes and Recommendation 2007/526/EC of the European Commission.

Determination of LPS Content of Mouse Feed Pellets

We have tested all the pelleted diets, which are commonly used in our animal facility. Namely AIN-93G diet (Harlan Winkelmann GmbH, Germany), 1430 diet with gluten-free modification (Altromin, Germany), Charles River's standard diet (Charles

River, USA), rodent NIH-07 22.5-5 diet (Ziegler, USA) and ST1 diet (Velaz, Czech Republic). To determine LPS content of mouse diets, the pellets were ground, sonicated in non-pyrogenic water and filtered. LPS concentration in the filtrate was measured using the Chromogenic Limulus Amebocyte Lysate (LAL) Test (Cambrex, USA) and expressed as endotoxin units (EU) per 1 µg of a diet.

Experimental Diets

Mice were fed ad libitum with either a purified diet (AIN-93G, Harlan) or a grain-based diet (ST1, Velaz). Both diets were sterilized by irradiation. The AIN-93G diet is the growth diet for rodents recommended by the American Institute of Nutrition. It is based mainly on purified ingredients, such as corn starch, vitamin free casein, maltodextrin, sucrose, soybean oil and powdered cellulose supplemented with mineral and vitamin mix. The ST1 diet is a grain-based diet, which is based mainly on wheat, oat, corn, wheat flour, snail clover fodder, soya pollard, bone meal and scrap cake. The AIN-93G has almost 100 times lower content of lipopolysaccharide (LPS) than ST1 diet.

Preparation of Cell Suspensions

The organs were cut with scissors, squeezed with a syringe plunger and filtered through a 70 µm cell strainer (BD Falcon, USA). Red blood cells in spleen cell suspensions were lysed with ACK lysing buffer (ph 7.3) for 5 min at room temperature. All the cells were washed twice in complete RPMI-5 medium (Sigma-Aldrich, USA) and resuspended in appropriate buffer (i.e. RPMI medium, FACS buffer). To harvest resident peri-

toneal cells, 10 ml RPMI-5 medium per mouse was injected into the peritoneal cavity. Collected peritoneal lavage fluid was centrifuged and then resuspended in harvest medium. The cells were counted and adjusted to appropriate cell concentration.

In Vivo Challenge with LPS

LPS was injected intraperitoneally (Ultrapure LPS, Invivogen, 10 µg/20 g of body weight) and 90 min later the levels of pro-inflammatory cytokines (TNFα and IL-6) in sera were measured.

In Vitro Stimulation

Spleen cells were cultured at 2 x 10^6/ml in a 96-well flat-bottom culture plate in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 µm 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin sulphate. The cells were stimulated with 100 ng/ml LPS (Ultra-Pure LPS, Invivogen) and 1.5 µg/ml ConA (Sigma-Aldrich, USA) and incubated in a 5%CO_2 at 37°C for 48 h. Culture supernatants were collected after 48 h and stored at -20°C. Cytokine profiles were determined using a multiplex cytokine analyzer (Luminex).

CFSE Proliferation Assay

Freshly isolated lymphocytes were resuspended in CFSE solution (5 µM final concentration) and mixed rapidly. After 5 min at room temperature, the cells were washed three times with 10 volumes of PBS containing 5% FBS. CFSE-labeled cells

were stimulated with 1.5 μg/ml ConA (Sigma-Aldrich, USA) in 96-well plates for 48 h, and then CFSE dilution was analyzed by flow cytometry.

Staining of Surface and Intracellular Antigens

Phenotypic analysis of cells isolated from spleen, thymus, MLNs, PPs and peritoneal cavity was performed by flow cytometry. The following mAb with matching isotype controls were used: FITC-conjugated anti-mouse CD3e (BD Pharmingen, USA), PE-conjugated anti-mouse CD19 (BD Pharmingen, USA), FITC-conjugated anti-mouse CD4 (BD Pharmingen, USA), PE-conjugated anti-mouse CD8a (BD Pharmingen, USA), PE-Cy5-conjugated anti-mouse CD25 (eBioscience, USA), FITC-conjugated mouse IgG2b, κ (BD Pharmingen, USA), FITC-conjugated rat IgG2a, κ (eBioscience, USA), PE-conjugated rat IgG2a, κ (eBioscience, USA), PE-conjugated mouse IgG1, κ (BD Pharmingen, USA). Cells were resuspended in FACS buffer (PBS containing 0.1% NaN_3 and 0.5% FBS) to a concentration of 2 x 10^7/ml and pre-incubated with 1μg of anti-mouse CD16/CD32 (eBioscience, USA) per million cells for 5 min on ice prior to staining. Primary antibodies were diluted to predetermined optimal concentrations in 50 μl of FACS buffer and dispensed into each well of a 96-well microtiter plate. 50 μl of cell suspension was added to each well and incubated for 20 min at 4°C in the dark. After staining, cells were washed twice and resuspended in 100 μl FACS buffer. Intracellular staining of mouse Foxp3 was performed using PE anti-mouse Foxp3 Staining Set (eBioscience) according to the manufacturer's protocol. The sample data were acquired on a FACSCali-

bur flow cytometer (Becton Dickinson, USA) and analyzed with WinMDI software (Joseph Trotter).

Multiplex Cytokine Determination

To determine cytokine profiles in culture supernatants we have used the Antibody Bead Kits (BioSource, USA), which are designed to be analyzed with the Luminex® 200™ System (Luminex Corporation, USA). The samples were stained and analyzed according to the manufacturer's recommendations. Briefly, beads of defined spectral properties conjugated to analyte-specific capture antibodies and samples were pipetted into the wells of a filter bottom microplate (Millipore, USA) and incubated for 2 h. After washing the beads, analyte-specific biotinylated detector antibodies were added and incubated with the beads for 1 h. After removal of excess biotinylated detector antibodies, streptavidin conjugated to R-Phycoerythrin was added for 30 min. After washing, the beads were analyzed with the Luminex 200™ instrument. By monitoring the spectral properties of the beads and the amount of R-Phycoerythrin fluorescence, the concentrations of analytes were determined.

Data Analysis

The Student's t-test (unpaired *t*-test) and one-way analysis of variance (ANOVA) were used to determine significant differences between the control and experimental groups. Values of $p < 0.05$ were regarded as significant and are denoted in the figures. * indicates $p<0.05$ and ** indicates $p<0.01$.

CHAPTER SIX

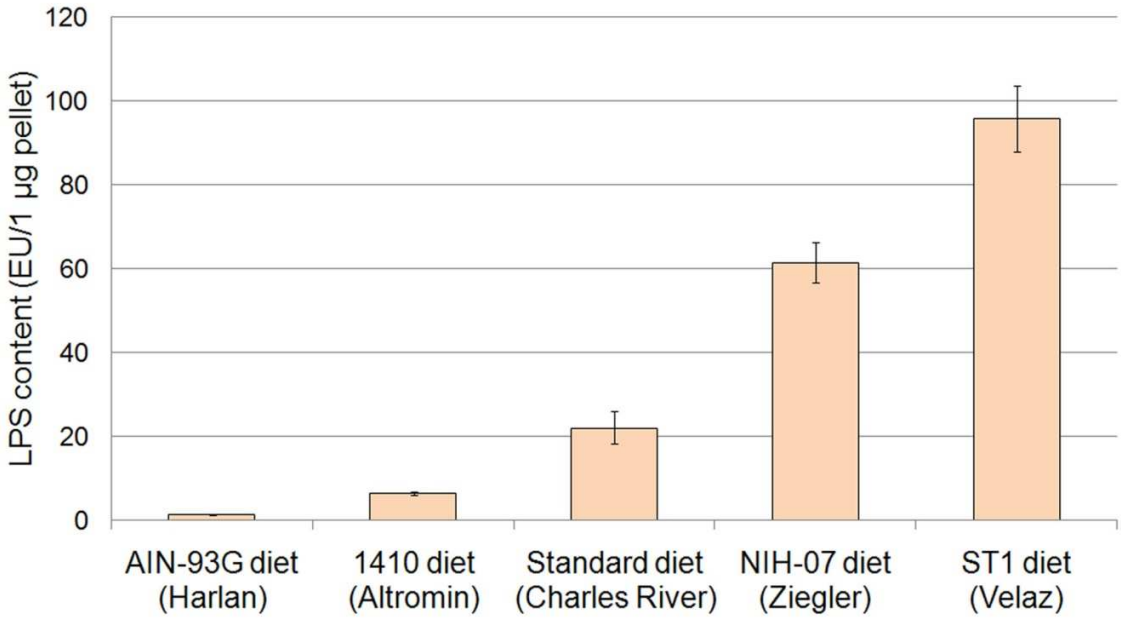
RESULTS

Purified Diet May Have Almost 100 Times Lower LPS Content than a Grain-based Diet

We have decided to use LPS concentration as a measure of overall bacterial contamination in diets. To study the effect of LPS content of the sterile diet on the development of the immune system we had to first identify both the diets with very low and very high LPS content. The concentration of LPS was measured by chromogenic LAL test and expressed in endotoxin units (EU) per µg of pellet. The diets sorted by LPS concentration from the lowest to the highest were AIN-93G diet (Harlan, USA), 1410 diet (Altromin, Germany), Standard diet (Charles River, USA), NIH-07 diet (Zeigler, USA) and ST1 diet (Velaz, Czech Republic). In our experiments we have used AIN-93G diet, which is a purified diet, and ST1 diet, which is a grain-based diet. These diets have been selected on the basis of maximum difference in the level of LPS content (Fig. 1).

Figure 1
Comparison of LPS content of mouse pelleted diets

To determine the load of microbiota-derived antigens in mouse diets the concentration of LPS was measured. The pellets were ground, sonicated in non-pyrogenic water and filtered. LPS concentration in the filtrate was analyzed using the Chromogenic Limulus Amebocyte Lysate (LAL) Test (Cambrex, USA) and is expressed as endotoxin units (EU) per 1µg of a diet. Results represent the mean (±SE) of four measurements.

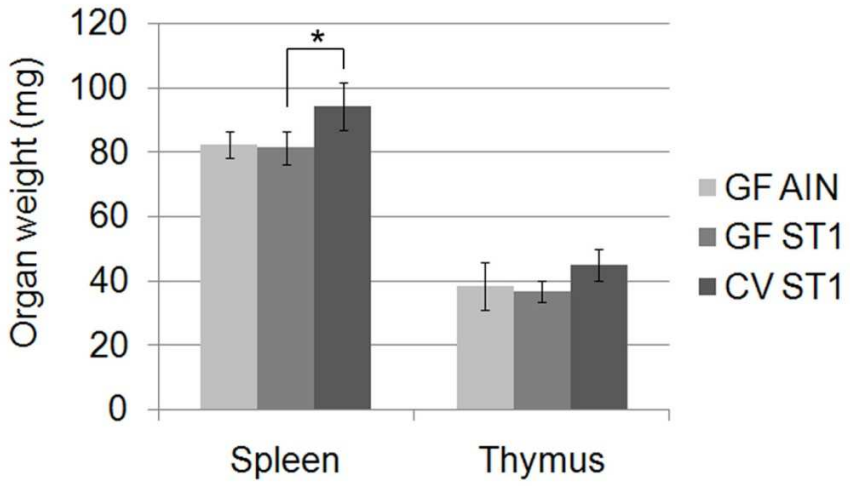


The Effect of Gut Microbiota and LPS-rich Sterile Diet on the Weight of Spleen and Thymus

We have not detected any significant difference in the weight of thymus isolated from CV or GF mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1). However we have observed an increase in the weight of CV spleen compared to GF spleen fed either diet (Fig. 2).

Figure 2
Germ-free mice have smaller spleens compared to conventional mice

We have found that the weight of spleen was significantly lower in the absence of gut microbiota. In contrast, the weight of thymus was not affected by gut microbiota or LPS content of the sterile diet. Results represent the mean (±SE) of at least 20 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA).



Gut Microbiota and LPS-rich Sterile Diet Increase the Cellularity of Mesenteric Lymph Nodes, Peyer's Patches and Spleen

Spleens isolated from GF mice fed either diet have lower total splenic lymphocyte count compared to CV spleens. The cellularity of thymus and peritoneal cell number is not affected by the LPS content of the sterile diet nor gut microbiota (Fig. 3). In contrast, the overall MLN and PP cell numbers increased in GF mice fed the LPS-rich diet (ST1)

compared to GF mice fed the low LPS diet (AIN-93G). In addition, the cell numbers further increased in the group of CV mice (Fig. 4).

Figure 3
Germ-free mice have lower total splenic lymphocyte count

*Spleens isolated from CV mice have significantly higher cellularity than spleens isolated from GF. Both groups of GF mice had similar splenic cell counts irrespective of the LPS content of the diet. All groups of mice had similar total thymocyte counts. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$.*

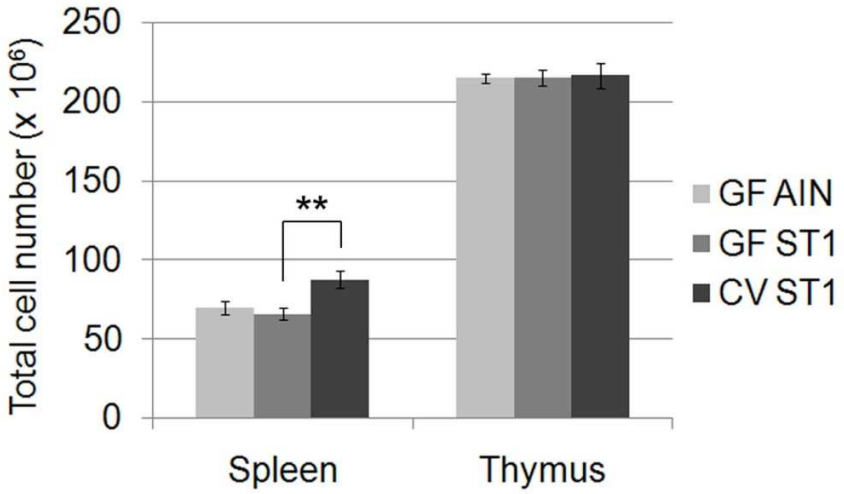
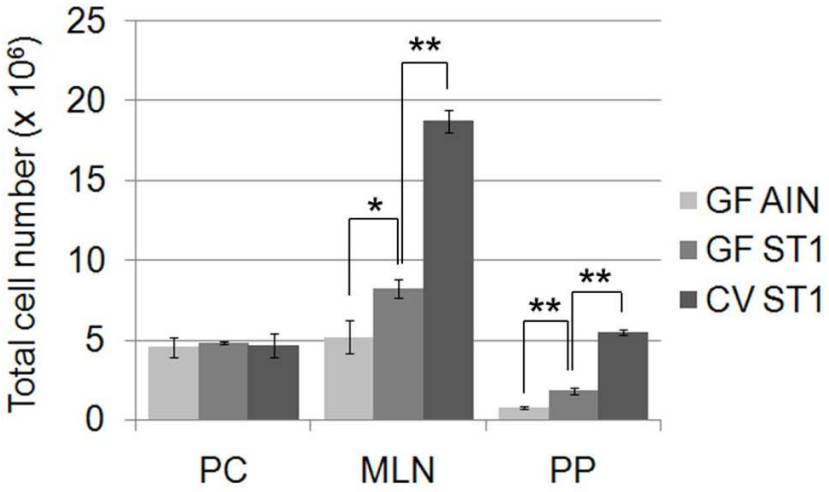


Figure 4

Gut microbiota and LPS-rich diet drive the cellular expansion in MLNs and PPs

*MLNs and PPs isolated from GF mice were reduced in size, number (not shown) and cellularity compared to those from CV mice. The size, number and cellularity of MLNs and PPs were even lower in GF mice fed the low LPS diet (AIN-93G). We have not observed any significant difference in the number of peritoneal cells isolated from peritoneal cavity of CV and GF mice regardless of LPS content of the diet. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$*



Immunodeficient SCID Mice Are More Susceptible to *In Vivo* LPS Challenge than Immunocompetent Balb/c Mice

We observed that immunodeficient SCID mice, which lack mature B and T cells, are significantly more susceptible to intraperitoneal LPS challenge than immunocompetent Balb/c mice. The levels of pro-inflammatory cytokines TNFα and IL-6 detected 90 min after LPS challenge were significantly higher in SCID mice compared to Balb/c mice. The increased susceptibility of SCID mice compared to Balb/c mice might be explained by the absence of regulatory components of adaptive immunity. However, we emphasize that the difference in LPS susceptibility *in vivo* between SCID and Balb/c mice was detected only under conventional conditions or under germ-free conditions when the mice were fed LPS-rich diet (ST1). Thus, GF mice fed the low LPS diet (AIN-93G) show similar LPS susceptibility irrespective of the presence of adaptive immunity (Fig. 5 and 6).

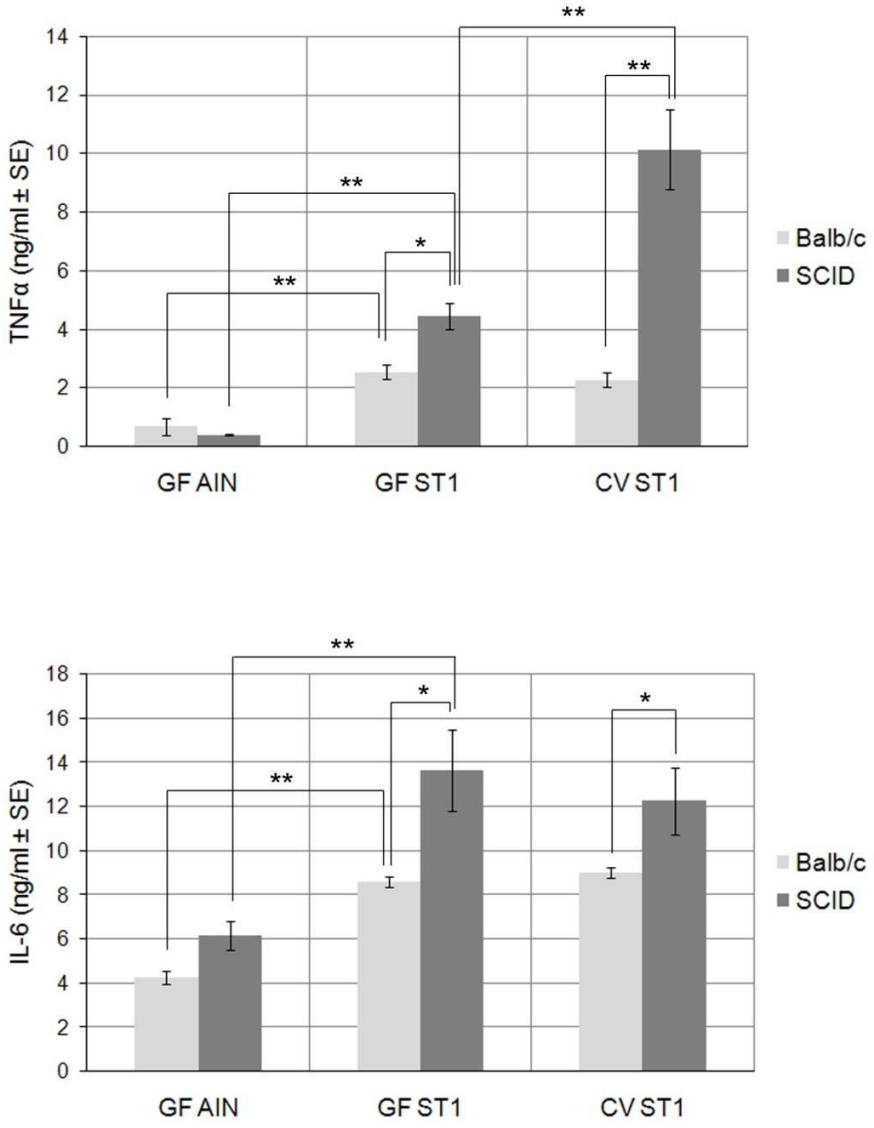
LPS-rich Sterile Diet Increases *In Vivo* Susceptibility of Germ-free Mice to LPS

We observed that both germ-free Balb/c mice and germ-free SCID mice fed the LPS-rich diet (ST1) are more susceptible to intraperitoneal challenge with LPS compared to germ-free mice fed the low LPS diet (AIN-93G) (Fig. 5 and 6).

Figure 5 and 6
Immunodeficient SCID mice are more susceptible to in vivo LPS challenge than immunocompetent Balb/c mice and LPS-rich sterile diet increases in vivo susceptibility of germ-free mice to LPS stimulation

The conventional or germ-free Balb/c and SCID mice fed either a LPS low diet (AIN-93G) or a LPS-rich diet (ST1) were challenged intraperitoneally with 10 µg LPS

*and after 90 min serum concentrations of pro-inflammatory cytokines were detected. Results represent the mean (±SE) of at least 8 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). ** indicates p<0.01*



Spleen Cells from Immunodeficient SCID Mice Show Increased Susceptibility to *In Vitro* Stimulation with LPS Compared to Immunocompetent Balb/c Mice

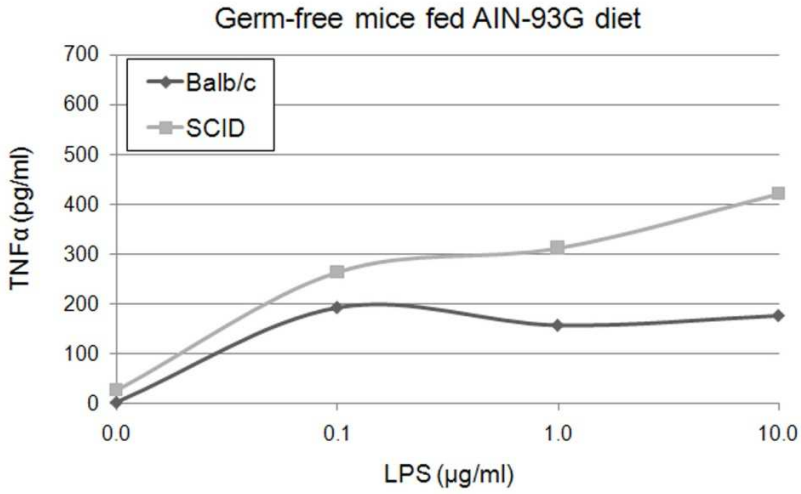
We found that spleen cells isolated from immunodeficient SCID mice, which lack mature B and T lymphocytes, are more susceptible to *in vitro* stimulation with LPS than spleen cells from immunocompetent Balb/c mice. Spleen cells from SCID mice showed an increased *in vivo* susceptibility, as measured by the production of pro-inflammatory cytokines TNFα and IL-6, in the full range of LPS concentrations (Fig. 7 and 12).

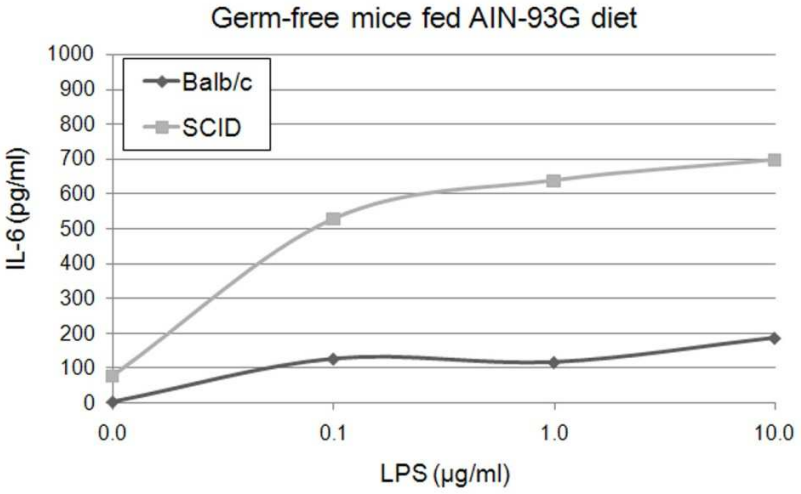
Neither Live Gut Microbiota nor LPS-rich Sterile Diet Influence *In Vitro* Susceptibility of Spleen Cells to LPS Stimulation

We observed that *in vitro* susceptibility of spleen cells isolated from Balb/c mice or SCID mice is not affected by the absence of gut microbiota or LPS content of a sterile diet (Fig. 7 and 12).

Figure 7 and 12
In vitro susceptibility of spleen cells from Balb/c and SCID mice to LPS stimulation is not influenced by the absence of gut microbiota or LPS content of the diet

Spleen cells isolated from conventional or germ-free Balb/c and SCID mice fed either a LPS low diet (AIN-93G) or a LPS-rich diet were stimulated with different concentrations of LPS for 48 h. The concentrations of pro-inflammatory cytokines in supernatants were determined by ELISA or a multiplex analyzer (Luminex®). Results represent the mean of at least 6 mice/group.


Germ-free mice fed AIN-93G diet
TNFα (pg/ml)
700
600
500
400
300
200
100
0
Balb/c
SCID
0.0
0.1
1.0
10.0
LPS (μg/ml)


Germ-free mice fed AIN-93G diet
IL-6 (pg/ml)
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900
800
700
600
500
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0
Balb/c
SCID
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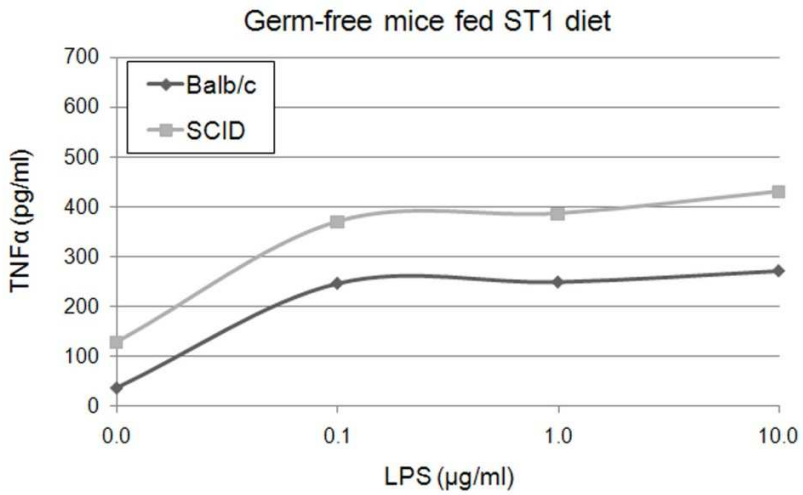
0.1
1.0
10.0
LPS (μg/ml)


Germ-free mice fed ST1 diet
Balb/c
SCID
TNFα (pg/ml)
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600
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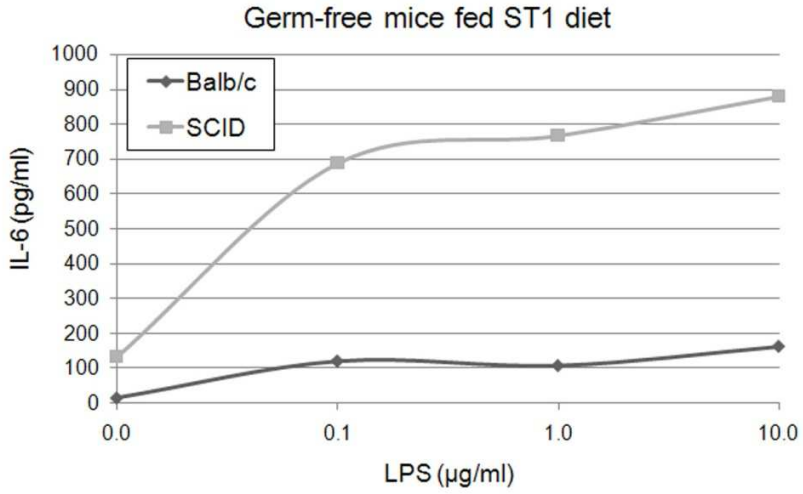
0.1
1.0
10.0
LPS (µg/ml)


Germ-free mice fed ST1 diet
Balb/c
SCID
IL-6 (pg/ml)
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900
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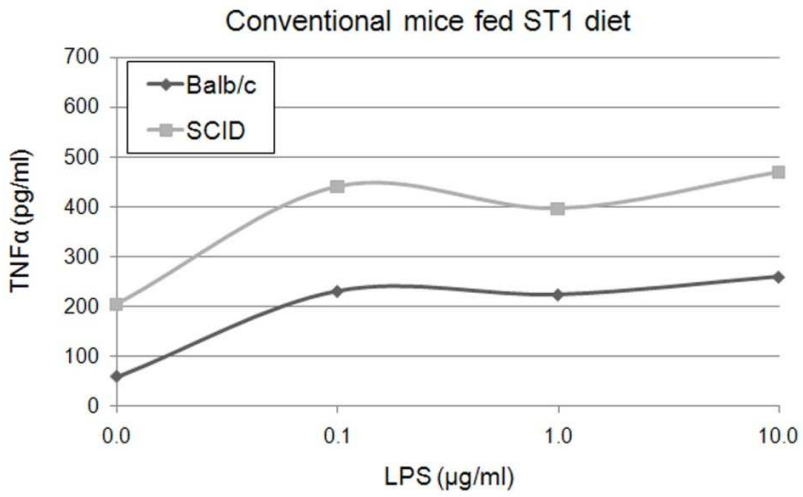
LPS (µg/ml)


Conventional mice fed ST1 diet
Balb/c
SCID
TNFα (pg/ml)
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600
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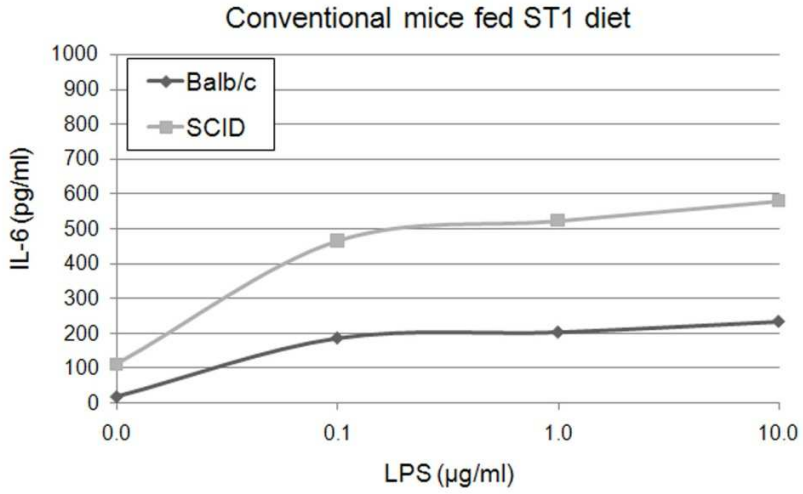
0.1
1.0
10.0
LPS (µg/ml)


Conventional mice fed ST1 diet
Balb/c
SCID
IL-6 (pg/ml)
1000
900
800
700
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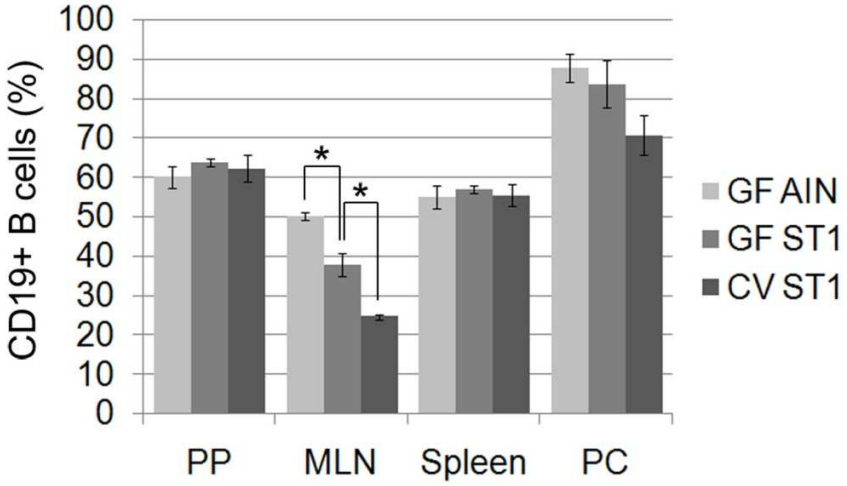
100
0
0.0
0.1
1.0
10.0
LPS (µg/ml)

Insufficient Microbial Stimulation Results in the Relative Expansion of CD19+ B Cells in Mesenteric Lymph Nodes

We have found that MLNs of GF mice fed the low LPS diet (AIN-93G) have higher proportion of CD19+ B cells than GF mice fed the LPS-rich diet (ST1). Accordingly, the group of GF mice fed the LPS-rich diet (ST1) had a higher proportion of CD19+ B cells in MLNs than the group of CV mice (Fig. 13). However we would like to emphasize that the absolute numbers of CD19+ B cells in MLNs were lower in the absence of sufficient microbial stimulation (Table 1). The proportion of CD19+ B cells in Peyer's patches, spleen and peritoneal cells remains constant irrespective of the degree of microbial stimulation.

Figure 13
Insufficient microbial stimulation leads to the relative expansion of CD19+ B cells in MLNs

*The absence of stimulation by gut microbiota and LPS-rich diet (ST1) results in the relative expansion of CD19+ B cells in MLNs. However the absolute cell numbers of all major lymphocyte subpopulations including CD19+ B cells, CD4+ and CD8+ T cells were significantly lower (Table 1). Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$.*



LPS-rich Diet Stimulates the Expansion of CD4+ T Cells in Mesenteric Lymph Nodes and Spleen

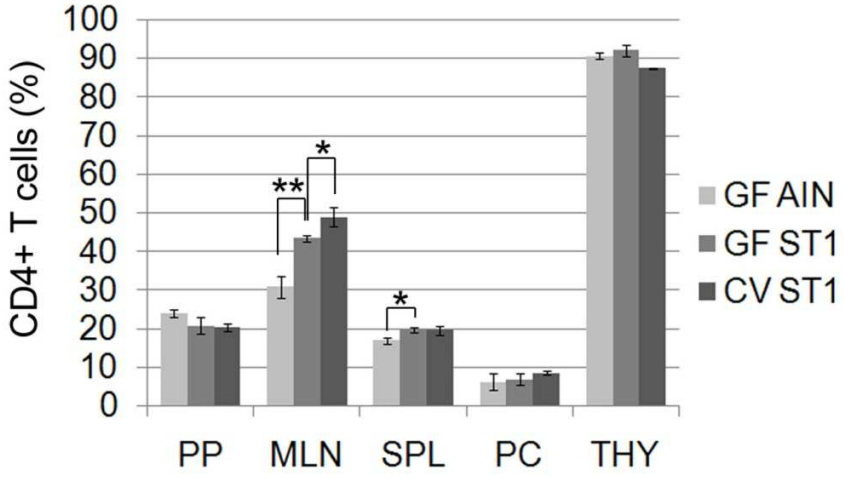
To determine the role of gut microbiota and LPS content of the diet in the development of major T cell subpopulations, including Tregs, we have isolated cells from Peyer's patches, MLNs, spleen, thymus and peritoneum of CV and GF mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1). The distinct cell surface and intracellular markers have been analyzed by flow cytometry. We have observed that the stimulating effect of high LPS content of the sterile diet leads to the expansion of CD4+ T cells in MLNs and spleen. The presence of gut microbiota further increased the proportion of CD4+ T cells in MLNs. In contrast to published data [141], we have not observed a significant increase in the proportion of CD4+ T cells of CV spleen compared to GF spleen. This discrepancy could be explained by the fact that the group of Kasper compared germ-free outbred Swiss Webster mice to conventional inbred Balb/c mice thus the stimulating effect of gut microbiota was determined in two groups of mice with different genetic

background. In addition, the stimulating effect of the LPS-rich diet (ST1) might be responsible for the complete restoration of CD4+ T cells in GF spleen to proportions found in CV mice. The proportion of CD4+ T cells in Peyer's patches, thymus and peritoneal cells remains constant regardless of the level of microbial stimulation (Fig. 14).

Figure 14

LPS-rich diet induces the expansion of CD4+ T cells in MLNs and spleen

*We have observed that LPS-rich diet (ST1) drives the expansion of CD4+ T cells in GF MLNs and spleen. The stimulating effect of gut microbiota further increased the proportion of CD4+ T cells in MLNs. The proportion of CD4+ T cells in PPs, thymus and peritoneal cells remained constant. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$.*

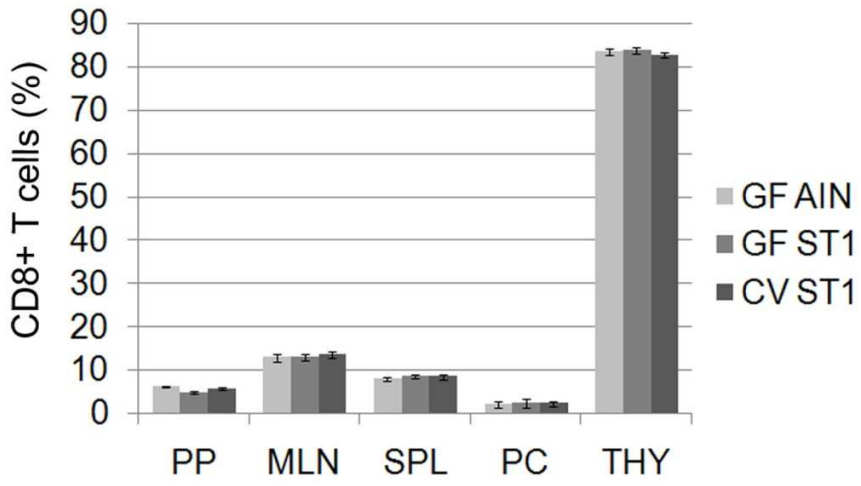


The Proportion of CD8+ T Cells Remains Constant in All Lymphoid Organs Irrespective of LPS Content of the Diet or Gut Colonization

Gut colonization has no effect on the proportion of CD8+ T cell in spleen, as previously described [180]. In addition, we show that the proportion of CD8+ T cells is independent of gut colonization also in other lymphoid organs including MLNs, Peyer's patches, thymus and peritoneal cells. In accordance with these findings we have not observed any effect of LPS content of the diet on the proportion of CD8+ T cells in lymphoid organs of GF mice (Fig. 15).

Figure 15
Microbial stimulation does not affect the proportion of CD8+ T cells

FACS analysis showed that the proportion of CD8+ T cells remains constant in all lymphoid organs regardless of both gut colonization and LPS content of the diet. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA).



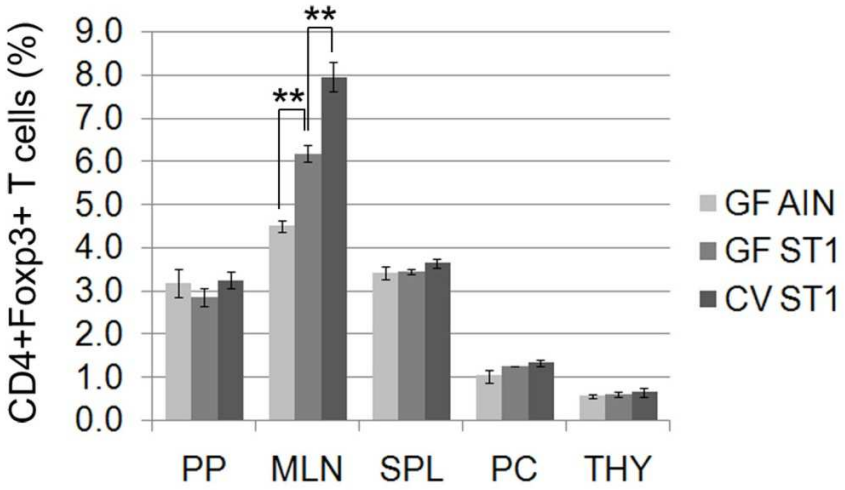
Gut Microbiota and the LPS-rich Diet Drive the Expansion of Foxp3-expressing CD4+ Tregs in Mesenteric Lymph Nodes

To investigate the effect of gut colonization and LPS content of the sterile diet on the development of Tregs we have analyzed the proportion of Foxp3-expressing CD4+ Tregs in Peyer's patches, MLNs, spleen, thymus and peritoneal cells in CV mice and GF mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1). We have found that both gut microbiota and the LPS-rich diet drive the expansion of CD4+Foxp3+ Tregs in MLNs (Fig. 16). We have not detected any significant difference in the proportion of Tregs in other lymphoid organs.

Figure 16
Both gut microbiota and LPS-rich sterile diet induce the expansion of Foxp3-expressing CD4+ Tregs in MLNs

To study the effect of gut microbiota and LPS-rich sterile diet on the proportion of CD4+Foxp3+ Tregs we have analyzed PPs, MLNs, spleen, thymus and peritoneal cells

*of CV and GF mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1). We have found that both gut microbiota and LPS-rich diet drive the expansion of CD4+Foxp3+ Tregs in MLNs. Our data show that microbiota-derived antigens, such as LPS, increase the proportion of CD4+Foxp3+ Tregs in GF MLNs. However the stimulating effect of gut microbiota is essential for the full development of CD4+Foxp3+ Tregs. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$.*



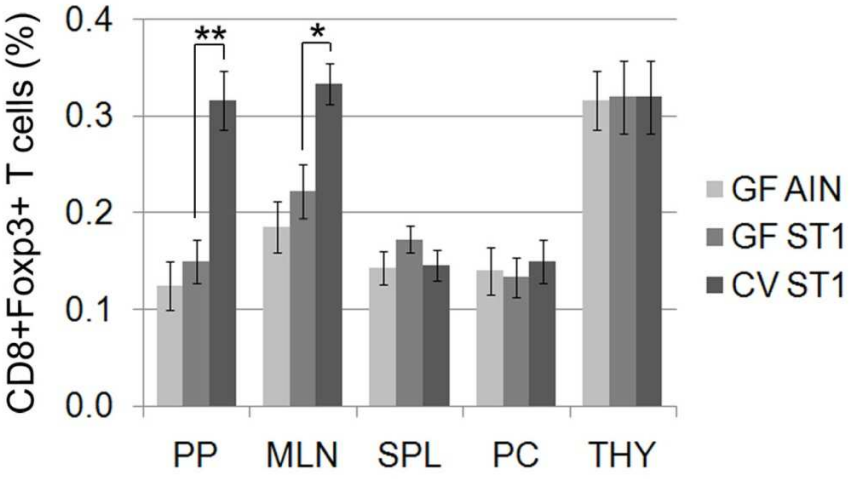
Gut Microbiota Stimulates the Expansion of Foxp3-expressing CD8+ Tregs in Peyer's Patches and Mesenteric Lymph Nodes

We have detected a stimulating effect of gut microbiota on CD8+Foxp3+ Tregs. The proportion of CD8+Foxp3+ Tregs increased in Peyer's patches and MLNs. The ef-

fect of LPS-rich diet on the expansion of CD8+Foxp3+ Tregs in all lymphoid organs of GF mice was not significant (Fig. 17).

Figure 17
Gut microbiota stimulates the expansion of Foxp3-expressing CD8+ T cells in PPs and MLNs

*FACS analysis revealed an increase in the proportion of CD8+Foxp3+ Tregs in CV PPs and MLNs compared to GF PPs and MLNs. The effect of LPS-rich sterile diet on the expansion of CD8+Foxp3+ Tregs in all lymphoid organs was not significant. Results represent the mean (±SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates p<0.05 and ** indicates p<0.01*



The Ratio of CD4+Foxp3- T Cells (non Tregs) to CD4+Foxp3+ T Cells (Tregs) Remains Unchanged in All Lymphoid Organs

We would like to emphasize that the non Tregs/Tregs ratio remains constant in all lymphoid organs and is not influenced during the lymphocyte expansion driven by gut microbiota or LPS (Fig. 18).

Figure 18
The ratio of CD4+Foxp3- T cells (non Tregs) to CD4+Foxp3+ T cells (Tregs) remains constant in all lymphoid organs

We have observed that the ratio of CD4+Foxp3- T cells to CD4+Foxp3+ T cells remains unchanged in all lymphoid organs regardless of both gut colonization and LPS content of the diet. Results represent the mean (± SE) of at least 10 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA).

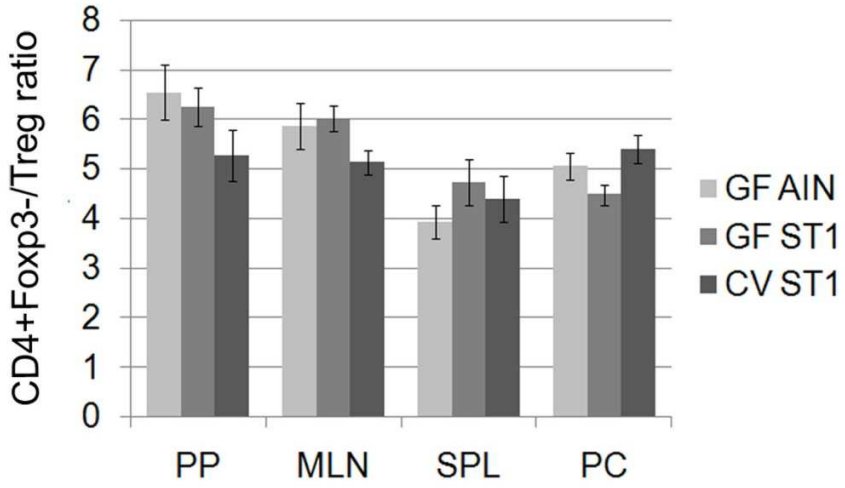


Table 1

FACS analysis of lymphocyte subpopulations in conventional and germ-free Balb/c mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1)

The single cell suspensions were stained with the monoclonal antibodies as described under "Methods" and analyzed by FACS. PP and MLN cell numbers represent the overall cell pool isolated from one mouse. Data is a summary of at least four independent experiments and values represent the mean ± SE.

		Number of positive lymphocytes (x 10^6)								
Lymphoid organ/tissue	Subpopulation	CV ST1			GF ST1			GF AIN		
Peyer's patches	Total	4.69	±	0.16	1.57	±	0.19	0.67	±	0.09
	CD19+	2.93	±	0.10	1.00	±	0.12	0.40	±	0.05
	CD3+	1.38	±	0.05	0.44	±	0.05	0.21	±	0.03
	CD4+	0.95	±	0.03	0.33	±	0.04	0.16	±	0.02
	CD8+	0.27	±	0.01	0.08	±	0.01	0.04	±	0.01
	CD4+Foxp3+	0.15	±	0.01	0.04	±	0.01	0.02	±	0.00
	CD8+Foxp3+	0.02	±	0.00	0.00	±	0.00	0.00	±	0.00
Mesenteric lymph nodes	Total	17.81	±	0.69	7.82	±	0.57	4.96	±	0.96
	CD19+	4.39	±	0.17	2.96	±	0.21	2.48	±	0.48
	CD3+	12.10	±	0.47	4.70	±	0.34	2.36	±	0.46
	CD4+	8.70	±	0.34	3.40	±	0.25	1.53	±	0.30
	CD8+	2.41	±	0.09	1.03	±	0.07	0.64	±	0.12
	CD4+Foxp3+	1.42	±	0.05	0.48	±	0.04	0.22	±	0.04
	CD8+Foxp3+	0.06	±	0.00	0.02	±	0.00	0.01	±	0.00
Spleen	Total	83.14	±	5.28	62.48	±	3.46	66.21	±	4.12
	CD19+	46.14	±	2.93	35.59	±	1.97	36.44	±	2.27
	CD3+	24.74	±	1.57	20.54	±	1.14	16.93	±	1.05
	CD4+	16.32	±	1.04	12.35	±	0.68	11.18	±	0.70
	CD8+	7.00	±	0.44	5.36	±	0.30	5.31	±	0.33
	CD4+Foxp3+	3.03	±	0.19	2.16	±	0.12	2.27	±	0.14
	CD8+Foxp3+	0.12	±	0.01	0.12	±	0.01	0.11	±	0.01
Peritoneal cells	Total	2.55	±	0.41	2.65	±	0.06	2.50	±	0.35
	CD19+	1.80	±	0.29	2.22	±	0.05	2.19	±	0.30
	CD3+	0.11	±	0.02	0.08	±	0.00	0.07	±	0.01
	CD4+	0.22	±	0.04	0.18	±	0.00	0.16	±	0.02
	CD8+	0.06	±	0.01	0.06	±	0.00	0.05	±	0.01
	CD4+Foxp3+	0.03	±	0.01	0.03	±	0.00	0.03	±	0.00
	CD8+Foxp3+	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Thymus	Total	207.03	±	9.23	205.76	±	8.46	205.76	±	7.98
	CD4-CD8-	8.70	±	0.30	10.72	±	0.50	11.87	±	0.20
	CD4+CD8+	155.27	±	8.13	151.59	±	7.26	146.52	±	7.18
	CD4+CD8-	30.23	±	1.82	28.81	±	1.72	31.39	±	2.39
	CD8+CD4-	13.04	±	0.67	11.93	±	0.57	9.88	±	0.57
	CD4+Foxp3+	1.35	±	0.05	1.25	±	0.03	1.15	±	0.02
	CD8+Foxp3+	0.66	±	0.02	0.66	±	0.02	0.65	±	0.01

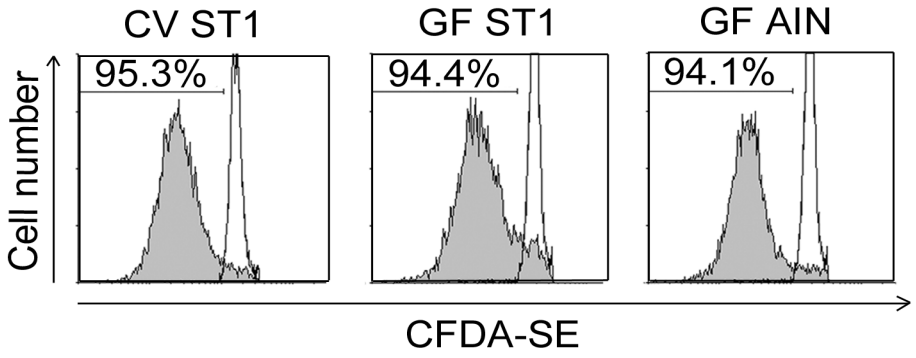
In Vitro Proliferative Response of Spleen Cells Is Not Influenced by Gut Microbiota or LPS Content of the Sterile Diet

Spleen cells isolated from CV mice and GF mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1) were stained with CFDA-SE fluorescein and stimulated with concanavalin A (ConA). After 72 h the CFDA-SE staining profile of spleen cells negative for CD19 antigen (B cell marker) was analyzed by flow cytometry. No significant difference was found between the groups of CV and GF mice fed either diet (Fig. 19). Thus we conclude that neither gut microbiota nor LPS content of the sterile diet influence non-specific proliferative response of spleen cells *in vitro*.

Figure 19

In vitro proliferative response of spleen cells is not influenced by gut microbiota or LPS content of the sterile diet

Spleen cells isolated from CV mice and GF mice fed either diet were stained with CFDA-SE fluorescein and stimulated with ConA. After 72 h the cells were analyzed by FACS. Histograms in this figure show the CFDA-SE staining profile of lymphocytes negative for CD19 antigen (B cell marker). Proliferation was measured as the percentage of cells showing decreased staining intensity of CFDA-SE compared to the intensity of the CFDA-SE^{bright} population. Open histograms represent unstimulated control cells. The presented data are from a representative experiment. Each experiment was repeated at least three times with similar results.



The Effect of Gut Microbiota and the LPS-rich Diet on T_H1/T_H2 Balance and T_H17 cells

To characterize the effect of LPS content of the sterile diet on T_H1/T_H2 balance, we have stimulated splenocytes with ConA and LPS and determined cytokine profiles. We have found that the "default" germ-free T_H2-skewed cytokine profile is partially corrected in GF mice fed the LPS-rich diet (ST1) compared to GF mice fed the low LPS diet (AIN-93G). Further we show that the production of IL-12, which is a key factor that drives T_H1-cell differentiation, is decreased in the absence of gut microbiota or the LPS-rich diet (Fig. 20 – 23). It has been recently shown that IL-12 and IFNγ inhibit T_H17-cell development, thus suppressing T_H17- mediated autoimmune diseases [181, 182].

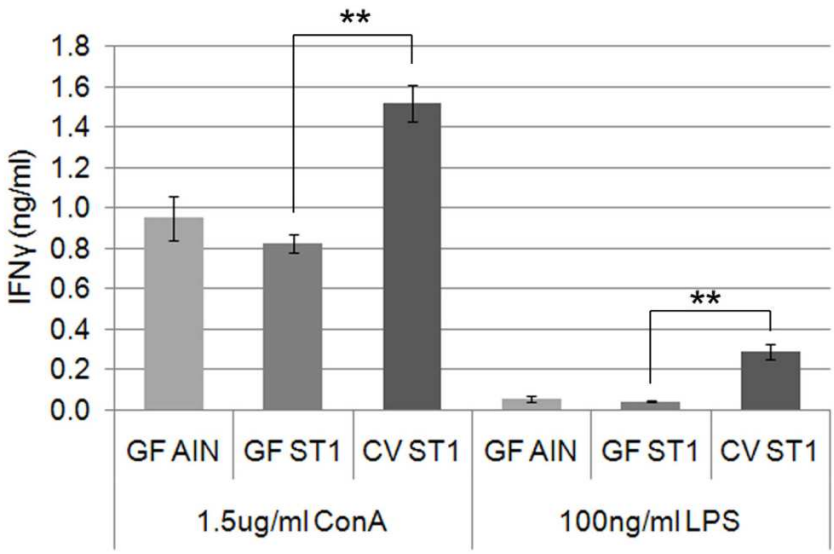
Gut Microbiota Stimulates the Production of Anti-inflammatory Cytokine Interleukin-10

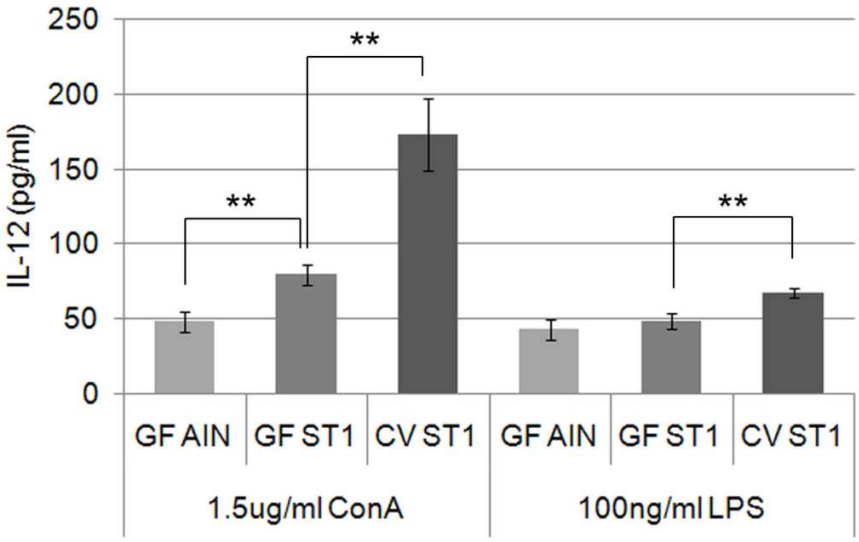
Spleen cells from CV and GF mice fed either a low LPS diet (AIN-93G) or a LPS-rich diet (ST1) were stimulated for 48 h with ConA and LPS. We have found that ConA stimulated spleen cells from CV mice produce significantly higher concentrations of IL-10. The effect of LPS content of the sterile diet on IL-10 production was not sig-

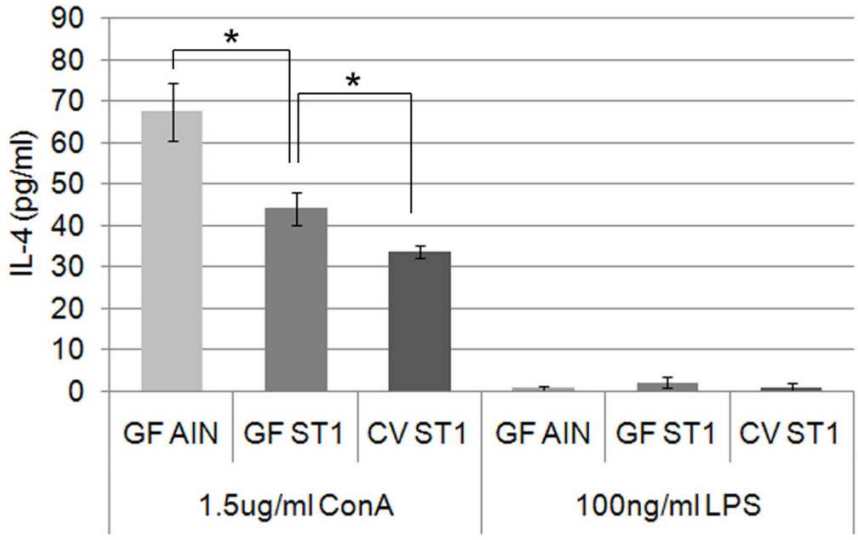
nificant. LPS stimulated spleen cells did not produce any detectable levels of IL-10 (Fig. 23).

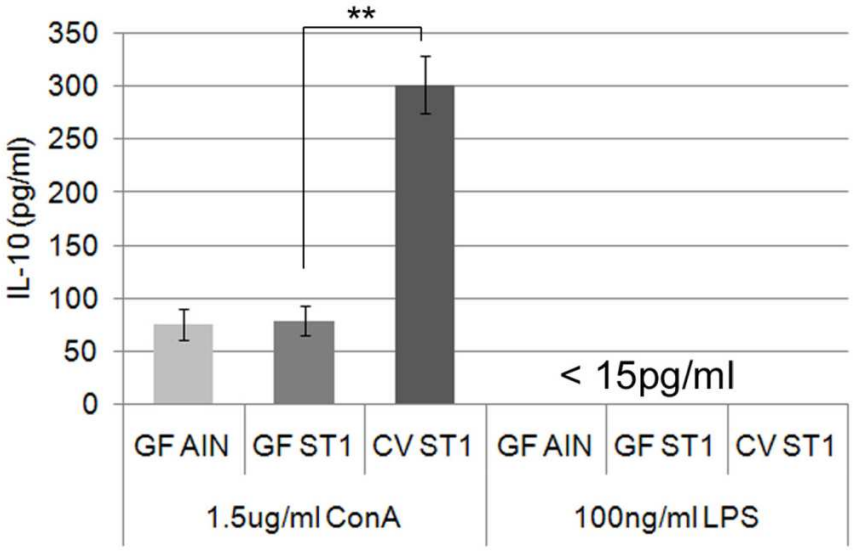
Figure 20 - 23
Microbial stimulation corrects T_H1/T_H2 imbalance present in germ-free mice and induces production of interleukin-10

*Spleen cells from CV and GF mice fed the low LPS diet (AIN-93G) or LPS-rich diet (ST1) have been stimulated with ConA and LPS for 48 h. The production of IFNγ, IL-4, IL-10 and IL-12 cytokines was determined by Luminex analyzer. Results represent the mean (±SE) of at least 6 mice/group. Statistical analyses were performed with the Student's t-test (unpaired t-test) and one-way analysis of variance (ANOVA). * indicates $p<0.05$ and ** indicates $p<0.01$.*




250
200
150
100
50
0
IL-12 (pg/ml)
**
**
**
GF AIN
GF ST1
CV ST1
GF AIN
GF ST1
CV ST1
1.5ug/ml ConA
100ng/ml LPS


90
80
70
60
50
40
30
20
10
0
IL-4 (pg/ml)
*
*
GF AIN
GF ST1
CV ST1
GF AIN
GF ST1
CV ST1
1.5ug/ml ConA
100ng/ml LPS


**
350
300
250
200
150
100
50
0
IL-10 (pg/ml)
< 15pg/ml
GF AIN
GF ST1
CV ST1
GF AIN
GF ST1
CV ST1
1.5ug/ml ConA
100ng/ml LPS

CHAPTER SEVEN

DISCUSSION

The Role of Adaptive Immunity and Regulatory T Cells in Sepsis

Sepsis syndrome is a common and frequently fatal clinical condition. It represents a major health care problem worldwide. Around 750,000 people are affected by sepsis each year in the United States [183]. In France, the incidence of severe sepsis has been estimated at nearly 60,000 episodes in 2001 [184]. These are highly lethal diseases, and mortality ranges from 20% in sepsis to over 60% in septic shock [185]. Severe sepsis itself represents the number one cause of mortality in noncoronary European intensive care units [186].

Inflammation was thougt initially to play a key role in the host response to septic challenge [187]. Subsequently, several anti-inflammatory agents were tested for the treatment of sepsis resulting in the failure of numerous clinical trials [188]. This suggests that we still do not adequately understand the underlying pathological process of sepsis, which leads to multiple organ dysfunctions.

It is now generally accepted that along with the intense inflammatory response aimed to eliminate the pathogen, regulatory mechanisms are initiated to control this potentially damaging response. It has been suggested that this regulatory process can, if itself not controlled properly, result in serious dysfunctions of immunological responses during sepsis. In fact, patients with sepsis present features of immunodeficiency; these abnormalities include T cell anergy, a shift to T_H2 response, lower expression of MHC

class II molecules, increased production of anti-inflammatory cytokines such as IL-10 and increased apoptosis of immune cells [187].

The role of conventional T cells and the involvement of subpopulations of regulatory T cells in the pathogenesis of sepsis have been underestimated during the last decades. As a result of their ability to interact not only with cells of the innate immune system but also with other cells of the adaptive immune system, T cells play a central role in the anti-infectious immune response as effectors and regulators of this response. The regulatory role of the adaptive immunity in sepsis has been demonstrated by the description of an increased mortality, a decreased bacterial clearance, and a dysregulated, pro-inflammatory immune response after polymicrobial septic challenge in the model of immunodeficient Rag1-/- mice lacking mature B and T cells [189, 190].

Regulatory T cells are a component of the immune system that suppresses activation of other immune cells and thus maintains immune system homeostasis. The latest research suggests that Tregs are best defined by the expression of the transcription factor Foxp3 [170-172]. The large majority of Foxp3-expressing Tregs is found within CD4+ helper T cell population and expresses high levels of the interleukin-2 receptor alpha chain (CD25). Regulatory T cells comprise about 5-10% of the mature CD4+ helper T cell subpopulation in mice. Mutations in the gene encoding Foxp3 result in the development of overwhelming systemic autoimmunity in the first year of life in both humans and mice. However, regulatory T cells have been shown to play a role not only in autoimmunity, but also in cancer, allergy, and transplantation in animal models and in humans. They may also play a role in infectious diseases. In particular, it has been recently dem-

onstrated that LPS can directly activate Treg cells via TLRs [191, 192].Thus, these cells could directly respond to “danger-like signals”.

Regarding the functional role of Treg cells in injury-induced, dysregulated immune response, Heuer et al. [193] reported that in a mouse model of septic shock induced by cecal ligation and puncture (CLP), the adoptive transfer of Treg cells before and after CLP had a protective and dose-dependent effect on survival. Murphy et al. [194] recently reported that burn injury primes innate immune cells for a progressive increase in TLR4 and TLR2 agonist-induced proinflammatory cytokine production and that this inflammatory phenotype is exaggerated in adaptive immune system-deficient (Rag1(-/-)) mice. They have shown that CD4+CD25+ T cells when adoptively transferred to Rag1-/- recipient mice are capable of reducing TLR-stimulated cytokine production levels to wild-type levels, whereas CD4+CD25- T cells have no regulatory effect. These findings suggest a previously unsuspected role for CD4+CD25+ T regulatory cells in controlling host inflammatory responses after injury.

Our observations of significantly increased LPS susceptibility of immunodeficient SCID mice compared to immunocompetent Balb/c mice are consistent with the published studies and demonstrate the essential role of adaptive immunity and regulatory T cells in the control of sepsis.

Based on the known properties of Treg cells , on the results of the mentioned studies and our results, one could postulate a role of Treg cells in the supression of pro-inflammatory cytokine production after septic challenge or severe injury.However, the

precise mechanisms (IL-10/TGFβ production, CTLA4 interaction, apoptosis) involved in this process remain to be elucidated.

LPS-Driven Lymphocyte Expansion in PPs and MLNs of Germ-free Mice

It is generally accepted that live gut microbiota is essential for the development and maturation of the mammalian immune system [14, 139-146]. Multiple studies revealed that animals kept under germ-free conditions have reduced cellular components of mucosal and systemic immunity. GF mice have lower numbers of CD4+ T cells, IgA producing B cells and intraepithelial T cells in gut lamina propria [6, 141, 151, 195-197]. PPs and MLNs are smaller, less cellular (lower numbers of B and T cells) and do not have germinal centers [6, 10, 143, 144, 155, 198]. Spleens of GF mice are also smaller, less cellular and the proportion of CD4+ T cells is reduced [141]. Our studies confirm the important role played by gut microbiota. In addition, we show that microbiota-derived antigens present in the sterile diet stimulate the development of the immune system even in the absence of live gut microbiota. In the present study, we show that LPS-rich sterile diet partially corrects the profound immunological deficiences found in GF mice. We demonstrate that LPS-rich diet stimulates the expansion of all major lymphocyte subpopulations in GF MLNs and PPs, including CD19+ B cells, CD8+ T cells and CD4+ T cells. The proportions of CD19+ B cells, CD8+ T cells and CD4+ T cells in PPs of GF mice remain constant during the LPS-driven expansion. In contrast, we observed a significant increase in the proportion of CD4+ T cells at the expense of CD19+ B cells in MLNs of GF mice. Our observation of the significant increase in the proportion of CD4+ T cells in spleen of GF mice fed LPS-rich diet is in line with recent studies showing that

monocolonization of germ-free animals with *Bacteroides fragilis* results in CD4+ T cell expansion [141].

The Effect of Gut Microbiota and LPS-rich Diet on the Development of Tregs

It is still controversial whether gut microbiota and microbiota-derived antigens play a role in the development and maturation of Tregs. In the transfer model of colitis developing in CD4+CD45RB^{high} T cell reconstituted immune-deficient SCID mice we have shown that the presence of normal gut microbiota enhances a functional potency of the Treg population. The inhibitory activity of CD4+CD45RB^{low} T cells from GF mice was significantly impaired compared to the population isolated from specific-pathogen free mice [173]. It has been recently reported that gut microbiota is crucial for the generation and expansion of Tregs [174]. Ostman et al. reported that CD25+ Tregs from GF mice are less effective in suppressing proliferation of responder CD4+CD25- T cells. However, they did not find any difference in the proportion of CD4+Foxp3+ T cells between CV and GF mice. The only deficit of CD4+Foxp3+ T cells in GF mice was detected in the liver-draining celiac lymph nodes [175]. Paradoxically, it was reported that CD25+ Tregs from GF mice are as suppressive and protective as those from CV mice [176, 177] and Booki et al. recently reported that peptide antigens derived from intestinal microorganisms are not essential for the generation, in vivo proliferation or suppressive activity of Tregs [178].

Recently, it has been reported that CD4+CD25+ Treg cells express several TLRs including TLR4 and that the exposure of CD4+CD25+ Treg cells to the TLR4 ligand

LPS induced up-regulation of several activation markers and enhanced their survival or proliferation [199, 200]. The proliferative response does not require APCs and is augmented by TCR triggering and IL-2 stimulation [199]. These findings provide the first evidence that CD4+CD25+ Treg cells respond directly to pro-inflammatory bacterial products. Thus, the observed expansion of Tregs, driven by gut microbiota and their antigens, might be mediated not only indirectly via DCs but also directly via Tregs‘ TLRs.

Our results are consistent with the results of Strauch et al. and Ostman et al., which demonstrate the important role of gut microbiota in the development and function of Tregs. We show that both live gut microbiota and LPS-rich sterile diet expand the absolute cell numbers of Foxp3-expressing CD4+ T cells in MLNs and PPs (Table 1). In addition, we observed that the stimulating effect of both gut microbiota and LPS-rich sterile diet significantly increased the proportion of CD4+Foxp3+ Tregs in MLNs. However, we would like to emphasize that the ratio of CD4+Foxp3- T cells to CD4+Foxp3+ Tregs remains constant in all lymphoid organs irrespective of the level of microbial stimulation.

Microbiota-derived Antigens in Sterile Diet and the Hygiene Hypothesis

A lack of early childhood exposure to infectious agents increases susceptibility to allergic diseases. This theory was first published by David P. Strachan in 1989 and called “hygiene hypothesis” [179]. Now it is used to explain the higher incidence of allergic diseases, inflammatory bowel disease, multiple sclerosis, and type I diabetes in developed

countries. The hygiene hypothesis has expanded to include also symbiotic bacteria including gut microbiota and parasites as important modulators of immune system development. The increase of hygienic standards and effective medical care over the last decades have diminished or eliminated exposure to these microorganisms and parasites during early postnatal development. Early childhood use of antibiotics is associated with an increased risk of developing asthma and allergic disorders [201]. The extensive use of cleaning products, including chlorination products, is also linked to the rise of childhood asthma [202]. The widespread use of anti-microbial food additives and other methods of food preservation reduced diversity and modified the composition of gut microbiota influencing thus the host-gut microbiota subtle balance. In addition, the extensive use of food preservatives reduced not only the load of ingested live microbiota but also the content of microbiota-derived antigens leading thus to decreased stimulation of the immune system. The major proposed mechanism explaining the "hygiene hypothesis" is that the developing immune system must receive sufficient stimuli in order to adequately develop Tregs, or it will be more susceptible to autoimmune diseases and allergic diseases, because of insufficiently repressed T_H1 and T_H2 responses, respectively [203]. Our data confirm the proposed mechanism as we have found that both gut microbiota as well as LPS-rich diet drive the expansion of CD4+Foxp3+ Tregs in MLNs. In addition, we observed a stimulating effect of gut microbiota on IL-10 production from spleen and a shift of T_H1/T_H2 balance from the "default" T_H2 towards T_H1-mediated response. Our results are in line with the results of Bamias et al. who observed a significant decrease in the frequency of Tregs and IL-10 production from MLNs of GF mice and conclude that the

regulatory component of the mucosal immune system, in the absence of live gut microbiota, is compromised [204].

How Microbiota-derived Antigens Influence the Maturation of Immune System

Several mechanisms by which gut microbiota and their antigens may influence the development of the immune system have been proposed. According to the current knowledge the nature of TLR and NOD ligands selectively determines the cytokine production by DCs and thus modulates T-cell differentiation.

Gut intraluminal antigens are sampled by DCs in the Peyer's patches and the intestinal epithelium and carried to the MLNs via the afferent lymphatics. In the MLNs the DCs induce T-cell activation and differentiation. The DCs are activated through the recognition of microbial antigens, such as LPS, via TLR and NOD receptors. The activation of DCs leads to the production of cytokines and expression of co-stimulatory molecules. The presentation of processed antigens bound to MHC class II results in the activation and differentiation of T cells. The cytokines secreted by activated DCs play a critical role in T-cell differentiation. The pivotal cytokines that control T-cell differentiation are IFNγ and IL-12 (T_H1), IL-4 (T_H2) and TGF-β and IL-6 (T_H17) and TGF-β (Tregs). The activated CD4+ T cells then migrate to effector tissues where they help to orchestrate the immune responses.

MLNs are the key site for the induction of mucosal tolerance to intestinal antigens. Our data show that the stimulating effect of gut microbiota and microbiota-derived

antigens is essential for the maturation of CD4+ T cell subpopulations including Tregs in MLNs.

LPS Content of the Sterile Diet and Gnotobiotic Animal Models

Last but not least, we would like to emphasize that the content of microbiota-derived antigens in sterile diets has a significant effect on the development and function of the immune system under germ-free conditions and thus the quality of diet should be tested in all gnotobiotic models.

CHAPTER EIGHT

CONCLUSIONS

Our results clearly show that both live gut microbiota and LPS-rich sterile diet increase susceptibility to LPS. Further, we demonstrate that immunodeficient SCID mice, which lack mature B and T cells, are more sensitive to LPS challenge compared to immunocompetent Balb/c mice. Thus we conclude that the adaptive immune responses play a key role in the pathogenesis of endotoxin shock.

In addition, we demonstrate that the microbiota-derived antigens, such as LPS, present in the sterile diet induce the development and maturation of the mammalian immune system in the absence of live gut microbiota. Our data show that LPS-rich sterile diet in germ-free mice promotes the expansion of CD19+ B cells, CD4+ and CD8+ T cells including Foxp3-expressing T cells in MLNs and PPs, increases the proportion of CD4+ T cells in MLNs and spleen, increases the proportion of CD4+Foxp3+ T cells in MLNs and shifts the T_H2-mediated immune response towards T_H1-mediated response.

The stimulating effects of LPS-rich sterile diet mimic the effects of live gut microbiota. Thus our results are in line with, and further expand the "hygiene hypothesis" by demonstrating that not only live organisms including gut microbiota and parasites but also the sterile microbial antigens contaminating diet during food processing stimulate the maturation of the immune system. We speculate that the higher incidence of both T_H1 and T_H2-mediated diseases seen in developed nations might be due to an immune dysregulation caused by insufficient microbial stimulation during the early development of the immune system. The very low numbers of CD4+Foxp3+ Tregs found in MLNs of GF

mice fed the low LPS diet could thus lead to an impaired functioning of the immune system.

APPENDICES

Appendix A

Abbreviations

AIN-93G: a purified diet (the low LPS diet)

CFDA-SE: carboxyfluorescein diacetate, succinimidyl ester

CV: conventional

DAMPs: danger-associated molecular patterns

DCs: dendritic cells

ECs: epithelial cells

GF: germ-free

LBP: lipopolysaccharide binding protein

LPS: lipopolysaccharide

MAMPs: microbial-associated molecular patterns

MASP: mannose-binding lectin (MBL)-associated serin protease

MLNs: mesenteric lymph nodes

NLRs: nucleotide-binding domain, leucin rich repeat (LRR) containing proteins

PC: peritoneal cells

PPs: Peyer's patches

PRRs: pattern recognition receptors

ST1: a grain-based diet (LPS-rich diet)

TLRs: Toll-like receptors

Tregs: regulatory T cells

Appendix B

Curriculum Vitae

Born: January 27, 1973, Prague, Czech Republic

Education and Employment:

1991-1998	2nd Faculty of Medicine, Charles University in Prague
1998-1999	Department of Neurology, Hospital Kralovske Vinohrady, Prague PhD. Project – The diagnostics and therapy of brain tumors
1999-2000	Military service – General practitioner at Military point in Stribro
2000-2008	Institute of Microbiology of the AS CR, v.v.i. Department of Immunology and Gnotobiology, Novy Hradek PhD. Project (2000 - 2002) – The study of etiology and pathogenesis of experimental models of autoimmunity (IBD and ANKENT) PhD. Project (2004 - 2008) – The role of gut microbiota in the development and maturation of the immune system – the defense of PhD. thesis planned in September 2008

Stays:

1995	Oxford School of English, Oxford, United Kingdom
1996	Department of Neurosurgery, University of Würzburg, Germany
2002-2003	Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, the Netherlands, Marie Curie Fellowship

Research projects:

Generation of Hsp-specific IL-10 producing CD4+ regulatory T cells *in vitro* and *in vivo*

Intestinal flora as a regulator of peripheral tolerance for self-antigens

2003 Department of Immunology and Histology, University of Groningen, the Netherlands.

Current projects:

Project number: 202020 (7^{th} framework programme of the EU)

Project title: IPODD—IBD: proteases offer new targets for drug discovery (2008 – 2010)

Project number: S500200572 (GAAV)

Project title: The development of new tools for the diagnostics and therapy of Crohn's diseases and ulcerative colitis

Field expertise:

High experience in the field of mucosal immunology. Extensive training in cell and molecular biology and immunology.

Language skills:

English – fluent (TOEFL), German and Russian – working level

Appendix C

Publications

Hrncir, T., Kverka, M., Stepankova, R., Kozakova H., Hudcovic T., Tlaskalova-Hogenova H. **2008**. The role of lipopolysaccharide content of the diet in the susceptibility to endotoxin shock: studies in germ-free immunocompetent and immunocompromised mice. Manuscript under preparation.

Hrncir, T., Stepankova, R., Kozakova H., Hudcovic T., Tlaskalova-Hogenova H. **2008**. Gut microbiota and lipopolysaccharide content of the diet influence T cell development: studies in germ-free mice. Manuscript submitted to *BMC Immunology*.

Kverka, M., Sokol, D.,Zakostelska, Z., Frolova, L., Klimesova, K., Hudcovic, T., **Hrncir, T.**, Jelen, P., Rossmann, P., Mrazek, J., Kopecny, J., Tlaskalova-Hogenova, H. **2008**. *Bacteroides distasonis* components ameliorate experimental intestinal inflammation in BALB/c mice. Manuscript submitted to *Infection and Immunity*.

Repa, A., Kozakova, H., Hudcovic, T., Stepankova, R., **Hrncir, T**., Tlaskalova-Hogenova, H., Pollak, A., Wiedermann, U. **2008**. Susceptibility to nasal and oral tolerance induction to Bet v 1 is not dependent on the presence of the microflora. *Immunology Letters* 117:50-56. **Impact Factor: 2.4**

Hudcovic, T., Kozakova, H., Kolinska, J., Stepankova, R., **Hrncir, T.**, and Tlaskalova-Hogenova, H. **2008**. Monocolonization with Bacteroides ovatus protects immunodeficient SCID mice from mortality in chronic intestinal inflammation caused by long-lasting dextran sodium sulfate treatment. *Physiol Res*. **Impact Factor: 2.1**

Stepankova, R., Powrie. F., Kofronova, O., Kozakova, H., Hudcovic, T., **Hrncir, T.**, Uhlig, H., Read, S., Rehakova, Z., Benada, O., Heczko, P., Strus, M., Bland, P., and Tlaska-

lova-Hogenova, H.. **2007**. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RB(high) CD4+ T cells. *Inflammatory bowel diseases* 13:1202-1211. **Impact Factor: 3.9**

Hudcovic, T., Stepankova, R., Kozakova, H., **Hrncir, T**., Tlaskalova-Hogenova, H. **2007**. Effects of *Escherichia coli* strains (*E. coli* O6K13 and *E. coli* Nissle 1917) mono-colonization on the development of experimentally induced acute and chronic intestinal inflammation in germ–free immunocompetent and immunodeficient mice. *Folia Microbiol.* 6. **Impact Factor: 0.9**

Tlaskalova-Hogenova, H., Tuckova, L., Mestecky, J., Kolinska, J.,. Rossmann, P.,. Stepankova, R., Kozakova, H., Hudcovic, T., **Hrncir, T.**, Frolova, L., and Kverka, M. **2005**. Interaction of mucosal microbiota with the innate immune system. *Scandinavian journal of immunology* 62 Suppl 1:106-113. **Impact Factor: 2.0**

Tlaskalova-Hogenova, H., L. Tuckova, R. Stepankova, T. Hudcovic, L. Palova-Jelinkova, H. Kozakova, P. Rossmann, D. Sanchez, J. Cinova, **T. Hrncir**, M. Kverka, L. Frolova, H. Uhlig, F. Powrie, and P. Bland. **2005**. Involvement of innate immunity in the development of inflammatory and autoimmune diseases. *Annals of the New York Academy of Sciences* 1051:787-798. **Impact Factor: 1.9**

REFERENCES

1. DC Savage: **Microbial ecology of the gastrointestinal tract**. *Annu Rev Microbiol* 1977, **31**:107-33.

2. JC Meslin, E Sacquet: **Effects of microflora on the dimensions of enterocyte microvilli in the rat**. *Reprod Nutr Dev* 1984, **24**:307-14.

3. F Backhed, H Ding, T Wang, LV Hooper, GY Koh, A Nagy, CF Semenkovich, JI Gordon: **The gut microbiota as an environmental factor that regulates fat storage**. *Proc Natl Acad Sci U S A* 2004, **101**:15718-23.

4. JC Meslin, E Sacquet, JL Guenet: **[Action of bacterial flora on the morphology and surface mucus of the small intestine of the rat]**. *Ann Biol Anim Biochim Biophys* 1973, **13**:203-14.

5. HA Gordon, E Bruckner-Kardoss: **Effect of normal microbial flora on intestinal surface area**. *Am J Physiol* 1961, **201**:175-8.

6. JR Glaister: **Factors affecting the lymphoid cells in the small intestinal epithelium of the mouse**. *Int Arch Allergy Appl Immunol* 1973, **45**:719-30.

7. GD Abrams, H Bauer, H Sprinz: **Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice**. *Lab Invest* 1963, **12**:355-64.

8. S Lesher, HE Walburg, Jr., GA Sacher, Jr.: **Generation Cycle in the Duodenal Crypt Cells of Germ-Free and Conventional Mice**. *Nature* 1964, **202**:884-6.

9. B Wostmann, E Bruckner-Kardoss: **Development of cecal distention in germfree baby rats**. *Am J Physiol* 1959, **197**:1345-6.

10. JA Hudson, TD Luckey: **Bacteria Induced Morphologic Changes**. *Proc Soc Exp Biol Med* 1964, **116**:628-31.

11. BS Wostmann, PL Knight: **Antagonism between vitamins A and K in the germfree rat**. *J Nutr* 1965, **87**:155-60.

12. BS Wostmann: **The germfree animal in nutritional studies**. *Annu Rev Nutr* 1981, **1**:257-79.

13. BE Gustafsson: **Vitamin K deficiency in germfree rats**. *Ann N Y Acad Sci* 1959, **78**:166-74.

14. H Tlaskalova-Hogenova, R Stepankova, T Hudcovic, L Tuckova, B Cukrowska, R Lodinova-Zadnikova, H Kozakova, P Rossmann, J Bartova, D Sokol, et al: **Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases**. *Immunol Lett* 2004, **93**:97-108.

15. SE Williams, TI Brown, A Roghanian, JM Sallenave: **SLPI and elafin: one glove, many fingers**. *Clin Sci (Lond)* 2006, **110**:21-35.

16. T Ganz: **Defensins: antimicrobial peptides of innate immunity**. *Nat Rev Immunol* 2003, **3**:710-20.

17. MW Hornef, S Normark, B Henriques-Normark, M Rhen: **Bacterial evasion of innate defense at epithelial linings**. *Chem Immunol Allergy* 2005, **86**:72-98.

18. B Agerberth, GH Gudmundsson: **Host antimicrobial defence peptides in human disease**. *Curr Top Microbiol Immunol* 2006, **306**:67-90.

19. E Voss, J Wehkamp, K Wehkamp, EF Stange, JM Schroder, J Harder: **NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2**. *J Biol Chem* 2006, **281**:2005-11.

20. A Uehara, Y Fujimoto, A Kawasaki, S Kusumoto, K Fukase, H Takada: **Meso-diaminopimelic acid and meso-lanthionine, amino acids specific to bacterial peptidoglycans, activate human epithelial cells through NOD1**. *J Immunol* 2006, **177**:1796-804.

21. A Uehara, Y Fujimoto, K Fukase, H Takada: **Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce antimicrobial peptides, but not proinflammatory cytokines**. *Mol Immunol* 2007, **44**:3100-11.

22. A Iwasaki, R Medzhitov: **Toll-like receptor control of the adaptive immune responses**. *Nat Immunol* 2004, **5**:987-95.

23. JH Fritz, RL Ferrero, DJ Philpott, SE Girardin: **Nod-like proteins in immunity, inflammation and disease**. *Nat Immunol* 2006, **7**:1250-7.

24. MT Abreu, M Fukata, M Arditi: **TLR signaling in the gut in health and disease**. *J Immunol* 2005, **174**:4453-60.

25. E Cario, DK Podolsky: **Intestinal epithelial TOLLerance versus inTOLLerance of commensals**. *Mol Immunol* 2005, **42**:887-93.

26. M Lotz, D Gutle, S Walther, S Menard, C Bogdan, MW Hornef: **Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells**. *J Exp Med* 2006, **203**:973-84.

27. M Lotz, T Konig, S Menard, D Gutle, C Bogdan, MW Hornef: **Cytokine-mediated control of lipopolysaccharide-induced activation of small intestinal epithelial cells**. *Immunology* 2007, **122**:306-15.

28. J Lee, JH Mo, K Katakura, I Alkalay, AN Rucker, YT Liu, HK Lee, C Shen, G Cojocaru, S Shenouda, et al: **Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells**. *Nat Cell Biol* 2006, **8**:1327-36.

29. SL Brown, TE Riehl, MR Walker, MJ Geske, JM Doherty, WF Stenson, TS Stappenbeck: **Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury**. *J Clin Invest* 2007, **117**:258-69.

30. S Rakoff-Nahoum, J Paglino, F Eslami-Varzaneh, S Edberg, R Medzhitov: **Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis**. *Cell* 2004, **118**:229-41.

31. E Cario, G Gerken, DK Podolsky: **Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function**. *Gastroenterology* 2007, **132**:1359-74.

32. AJ Macpherson, NL Harris: **Interactions between commensal intestinal bacteria and the immune system**. *Nat Rev Immunol* 2004, **4**:478-85.

33. Inohara, Chamaillard, C McDonald, G Nunez: **NOD-LRR proteins: role in host-microbial interactions and inflammatory disease**. *Annu Rev Biochem* 2005, **74**:355-83.

34. GB Johnson, GJ Brunn, Y Kodaira, JL Platt: **Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4**. *J Immunol* 2002, **168**:5233-9.

35. C Termeer, F Benedix, J Sleeman, C Fieber, U Voith, T Ahrens, K Miyake, M Freudenberg, C Galanos, JC Simon: **Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4**. *J Exp Med* 2002, **195**:99-111.

36. S Akira, H Hemmi: **Recognition of pathogen-associated molecular patterns by TLR family**. *Immunol Lett* 2003, **85**:85-95.

37. E Latz, A Schoenemeyer, A Visintin, KA Fitzgerald, BG Monks, CF Knetter, E Lien, NJ Nilsen, T Espevik, DT Golenbock: **TLR9 signals after translocating from the ER to CpG DNA in the lysosome**. *Nat Immunol* 2004, **5**:190-8.

38. A Marshak-Rothstein: **Toll-like receptors in systemic autoimmune disease**. *Nat Rev Immunol* 2006, **6**:823-35.

39. S Akira, K Takeda: **Toll-like receptor signalling**. *Nat Rev Immunol* 2004, **4**:499-511.

40. CA Janeway, Jr., R Medzhitov: **Innate immune recognition**. *Annu Rev Immunol* 2002, **20**:197-216.

41. PJ Sansonetti: **The innate signaling of dangers and the dangers of innate signaling**. *Nat Immunol* 2006, **7**:1237-42.

42. S Akira, S Uematsu, O Takeuchi: **Pathogen recognition and innate immunity**. *Cell* 2006, **124**:783-801.

43. V Apostolopoulos, IF McKenzie: **Role of the mannose receptor in the immune response**. *Curr Mol Med* 2001, **1**:469-74.

44. JC Kagan, R Medzhitov: **Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling**. *Cell* 2006, **125**:943-55.

45. ZJ Chen: **Ubiquitin signalling in the NF-kappaB pathway**. *Nat Cell Biol* 2005, **7**:758-65.

46. M Yamamoto, T Okamoto, K Takeda, S Sato, H Sanjo, S Uematsu, T Saitoh, N Yamamoto, H Sakurai, KJ Ishii, et al: **Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling**. *Nat Immunol* 2006, **7**:962-70.

47. S Sato, H Sanjo, K Takeda, J Ninomiya-Tsuji, M Yamamoto, T Kawai, K Matsumoto, O Takeuchi, S Akira: **Essential function for the kinase TAK1 in innate and adaptive immune responses**. *Nat Immunol* 2005, **6**:1087-95.

48. JH Shim, C Xiao, AE Paschal, ST Bailey, P Rao, MS Hayden, KY Lee, C Bussey, M Steckel, N Tanaka, et al: **TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo**. *Genes Dev* 2005, **19**:2668-81.

49. A Takaoka, H Yanai, S Kondo, G Duncan, H Negishi, T Mizutani, S Kano, K Honda, Y Ohba, TW Mak, et al: **Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors**. *Nature* 2005, **434**:243-9.

50. A Schoenemeyer, BJ Barnes, ME Mancl, E Latz, N Goutagny, PM Pitha, KA Fitzgerald, DT Golenbock: **The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling**. *J Biol Chem* 2005, **280**:17005-12.

51. DB Stetson, R Medzhitov: **Recognition of cytosolic DNA activates an IRF3-dependent innate immune response**. *Immunity* 2006, **24**:93-103.

52. RM O'Connell, SA Vaidya, AK Perry, SK Saha, PW Dempsey, G Cheng: **Immune activation of type I IFNs by Listeria monocytogenes occurs independently of TLR4, TLR2, and receptor interacting protein 2 but involves TNFR-associated NF kappa B kinase-binding kinase 1**. *J Immunol* 2005, **174**:1602-7.

53. S Stockinger, B Reutterer, B Schaljo, C Schellack, S Brunner, T Materna, M Yamamoto, S Akira, T Taniguchi, PJ Murray, et al: **IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism**. *J Immunol* 2004, **173**:7416-25.

54. KS Kobayashi, M Chamaillard, Y Ogura, O Henegariu, N Inohara, G Nunez, RA Flavell: **Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract**. *Science* 2005, **307**:731-4.

55. D Mackey, BF Holt, 3rd, A Wiig, JL Dangl: **RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis**. *Cell* 2002, **108**:743-54.

56. HS Kim, D Desveaux, AU Singer, P Patel, J Sondek, JL Dangl: **The Pseudomonas syringae effector AvrRpt2 cleaves its C-terminally acylated target, RIN4, from Arabidopsis membranes to block RPM1 activation**. *Proc Natl Acad Sci U S A* 2005, **102**:6496-501.

57. J Bertin, WJ Nir, CM Fischer, OV Tayber, PR Errada, JR Grant, JJ Keilty, ML Gosselin, KE Robison, GH Wong, et al: **Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB**. *J Biol Chem* 1999, **274**:12955-8.

58. N Inohara, T Koseki, L del Peso, Y Hu, C Yee, S Chen, R Carrio, J Merino, D Liu, J Ni, et al: **Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB**. *J Biol Chem* 1999, **274**:14560-7.

59. SE Girardin, IG Boneca, J Viala, M Chamaillard, A Labigne, G Thomas, DJ Philpott, PJ Sansonetti: **Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection**. *J Biol Chem* 2003, **278**:8869-72.

60. N Inohara, Y Ogura, A Fontalba, O Gutierrez, F Pons, J Crespo, K Fukase, S Inamura, S Kusumoto, M Hashimoto, et al: **Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease**. *J Biol Chem* 2003, **278**:5509-12.

61. M Hasegawa, K Yang, M Hashimoto, JH Park, YG Kim, Y Fujimoto, G Nunez, K Fukase, N Inohara: **Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments**. *J Biol Chem* 2006, **281**:29054-63.

62. N Inohara, G Nunez: **NODs: intracellular proteins involved in inflammation and apoptosis**. *Nat Rev Immunol* 2003, **3**:371-82.

63. SE Girardin, IG Boneca, LA Carneiro, A Antignac, M Jehanno, J Viala, K Tedin, MK Taha, A Labigne, U Zahringer, et al: **Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan**. *Science* 2003, **300**:1584-7.

64. B Opitz, A Puschel, W Beermann, AC Hocke, S Forster, B Schmeck, V van Laak, T Chakraborty, N Suttorp, S Hippenstiel: **Listeria monocytogenes activated p38**

MAPK and induced IL-8 secretion in a nucleotide-binding oligomerization domain 1-dependent manner in endothelial cells. *J Immunol* 2006, **176**:484-90.

65. SE Girardin, R Tournebize, M Mavris, AL Page, X Li, GR Stark, J Bertin, PS DiStefano, M Yaniv, PJ Sansonetti, et al: **CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri**. *EMBO Rep* 2001, **2**:736-42.

66. L Franchi, A Amer, M Body-Malapel, TD Kanneganti, N Ozoren, R Jagirdar, N Inohara, P Vandenabeele, J Bertin, A Coyle, et al: **Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages**. *Nat Immunol* 2006, **7**:576-82.

67. EA Miao, CM Alpuche-Aranda, M Dors, AE Clark, MW Bader, SI Miller, A Aderem: **Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf**. *Nat Immunol* 2006, **7**:569-75.

68. F Martinon, V Petrilli, A Mayor, A Tardivel, J Tschopp: **Gout-associated uric acid crystals activate the NALP3 inflammasome**. *Nature* 2006, **440**:237-41.

69. S Mariathasan, DS Weiss, K Newton, J McBride, K O'Rourke, M Roose-Girma, WP Lee, Y Weinrauch, DM Monack, VM Dixit: **Cryopyrin activates the inflammasome in response to toxins and ATP**. *Nature* 2006, **440**:228-32.

70. TD Kanneganti, N Ozoren, M Body-Malapel, A Amer, JH Park, L Franchi, J Whitfield, W Barchet, M Colonna, P Vandenabeele, et al: **Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3**. *Nature* 2006, **440**:233-6.

71. TD Kanneganti, M Lamkanfi, YG Kim, G Chen, JH Park, L Franchi, P Vandenabeele, G Nunez: **Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling**. *Immunity* 2007, **26**:433-43.

72. FS Sutterwala, Y Ogura, M Szczepanik, M Lara-Tejero, GS Lichtenberger, EP Grant, J Bertin, AJ Coyle, JE Galan, PW Askenase, et al: **Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1**. *Immunity* 2006, **24**:317-27.

73. L Franchi, TD Kanneganti, GR Dubyak, G Nunez: **Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria**. *J Biol Chem* 2007, **282**:18810-8.

74. ED Boyden, WF Dietrich: **Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin**. *Nat Genet* 2006, **38**:240-4.

75. B Faustin, L Lartigue, JM Bruey, F Luciano, E Sergienko, B Bailly-Maitre, N Volkmann, D Hanein, I Rouiller, JC Reed: **Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation**. *Mol Cell* 2007, **25**:713-24.

76. JV McCarthy, J Ni, VM Dixit: **RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase**. *J Biol Chem* 1998, **273**:16968-75.

77. N Inohara, L del Peso, T Koseki, S Chen, G Nunez: **RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis**. *J Biol Chem* 1998, **273**:12296-300.

78. M Thome, K Hofmann, K Burns, F Martinon, JL Bodmer, C Mattmann, J Tschopp: **Identification of CARDIAK, a RIP-like kinase that associates with caspase-1**. *Curr Biol* 1998, **8**:885-8.

79. Y Ogura, N Inohara, A Benito, FF Chen, S Yamaoka, G Nunez: **Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB**. *J Biol Chem* 2001, **276**:4812-8.

80. F Martinon, K Burns, J Tschopp: **The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta**. *Mol Cell* 2002, **10**:417-26.

81. DP Cerretti, CJ Kozlosky, B Mosley, N Nelson, K Van Ness, TA Greenstreet, CJ March, SR Kronheim, T Druck, LA Cannizzaro, et al: **Molecular cloning of the interleukin-1 beta converting enzyme**. *Science* 1992, **256**:97-100.

82. T Vorup-Jensen, JC Jensenius, S Thiel: **MASP-2, the C3 convertase generating protease of the MBLectin complement activating pathway**. *Immunobiology* 1998, **199**:348-57.

83. M Takahashi, D Iwaki, K Kanno, Y Ishida, J Xiong, M Matsushita, Y Endo, S Miura, N Ishii, K Sugamura, et al: **Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway**. *J Immunol* 2008, **180**:6132-8.

84. M Matsushita, T Fujita: **The role of ficolins in innate immunity**. *Immunobiology* 2002, **205**:490-7.

85. M Matsushita, Y Endo, T Fujita: **Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease**. *J Immunol* 2000, **164**:2281-4.

86. C Garlanda, B Bottazzi, G Salvatori, R De Santis, A Cotena, L Deban, V Maina, F Moalli, A Doni, T Veliz-Rodriguez, et al: **Pentraxins in innate immunity and inflammation**. *Novartis Found Symp* 2006, **279**:80-6; discussion 86-91, 216-9.

87. A Mantovani, C Garlanda, A Doni, B Bottazzi: **Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3**. *J Clin Immunol* 2008, **28**:1-13.

88. GD Gomez, JL Balcazar: **A review on the interactions between gut microbiota and innate immunity of fish**. *FEMS Immunol Med Microbiol* 2008, **52**:145-54.

89. O Salazar, JA Asenjo: **Enzymatic lysis of microbial cells**. *Biotechnol Lett* 2007, **29**:985-94.

90. J Harder, R Glaser, JM Schroder: **The role and potential therapeutical applications of antimicrobial proteins in infectious and inflammatory diseases**. *Endocr Metab Immune Disord Drug Targets* 2007, **7**:75-82.

91. K Takeda, T Kaisho, S Akira: **Toll-like receptors**. *Annu Rev Immunol* 2003, **21**:335-76.

92. S Thoma-Uszynski, S Stenger, O Takeuchi, MT Ochoa, M Engele, PA Sieling, PF Barnes, M Rollinghoff, PL Bolcskei, M Wagner, et al: **Induction of direct antimicrobial activity through mammalian toll-like receptors**. *Science* 2001, **291**:1544-7.

93. T Ayabe, DP Satchell, CL Wilson, WC Parks, ME Selsted, AJ Ouellette: **Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria**. *Nat Immunol* 2000, **1**:113-8.

94. L Alexopoulou, AC Holt, R Medzhitov, RA Flavell: **Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3**. *Nature* 2001, **413**:732-8.

95. F Heil, H Hemmi, H Hochrein, F Ampenberger, C Kirschning, S Akira, G Lipford, H Wagner, S Bauer: **Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8**. *Science* 2004, **303**:1526-9.

96. SS Diebold, T Kaisho, H Hemmi, S Akira, C Reis e Sousa: **Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA**. *Science* 2004, **303**:1529-31.

97. DJ Lenschow, TL Walunas, JA Bluestone: **CD28/B7 system of T cell costimulation**. *Annu Rev Immunol* 1996, **14**:233-58.

98. Y Liu, CA Janeway, Jr.: **Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells**. *Proc Natl Acad Sci U S A* 1992, **89**:3845-9.

99. J Banchereau, RM Steinman: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**:245-52.

100. RM Steinman, D Hawiger, MC Nussenzweig: **Tolerogenic dendritic cells**. *Annu Rev Immunol* 2003, **21**:685-711.

101. C Pasare, R Medzhitov: **Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells**. *Science* 2003, **299**:1033-6.

102. CM Snapper, WE Paul: **Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production**. *Science* 1987, **236**:944-7.

103. J Stulik, J Bures, P Jandik, F Langr, H Kovarova, A Macela: **The different expression of proteins recognized by monoclonal anti-heat shock protein 70 (hsp70) antibody in human colonic diseases**. *Electrophoresis* 1997, **18**:625-8.

104. K Edfeldt, J Swedenborg, GK Hansson, ZQ Yan: **Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation**. *Circulation* 2002, **105**:1158-61.

105. RC Bone: **Sepsis, the sepsis syndrome, multi-organ failure: a plea for comparable definitions**. *Ann Intern Med* 1991, **114**:332-3.

106. RL Paterson, NR Webster: **Sepsis and the systemic inflammatory response syndrome**. *J R Coll Surg Edinb* 2000, **45**:178-82.

107. GS Martin, DM Mannino, S Eaton, M Moss: **The epidemiology of sepsis in the United States from 1979 through 2000**. *N Engl J Med* 2003, **348**:1546-54.

108. BM Sultzer: **Endotoxin-induced resistance to a staphylococcal infection: cellular and humoral responses compared in two mouse strains**. *J Infect Dis* 1968, **118**:340-8.

109. O Westphal: **Bacterial endotoxins. The second Carl Prausnitz Memorial Lecture**. *Int Arch Allergy Appl Immunol* 1975, **49**:1-43.

110. NK Surana, JW St Geme: **Lymphangitis after self-administration of lipopolysaccharide**. *N Engl J Med* 2005, **352**:944-5.

111. SM Opal: **The clinical relevance of endotoxin in human sepsis: a critical analysis**. *J Endotoxin Res* 2002, **8**:473-6.

112. DL Dunn: **Prevention and treatment of multiple organ dysfunction syndrome: lessons learned and future prospects**. *Surg Infect (Larchmt)* 2000, **1**:227-36; discussion 236-7.

113. NC Riedemann, RF Guo, PA Ward: **Novel strategies for the treatment of sepsis**. *Nat Med* 2003, **9**:517-24.

114. RV McCloskey, RC Straube, C Sanders, SM Smith, CR Smith: **Treatment of septic shock with human monoclonal antibody HA-1A. A randomized, double-blind, placebo-controlled trial. CHESS Trial Study Group**. *Ann Intern Med* 1994, **121**:1-5.

115. SN Vogel, CT Hansen, DL Rosenstreich: **Characterization of a congenitally LPS-resistant, athymic mouse strain**. *J Immunol* 1979, **122**:619-22.

116. AS Cross, JC Sadoff, N Kelly, E Bernton, P Gemski: **Pretreatment with recombinant murine tumor necrosis factor alpha/cachectin and murine interleukin 1 alpha protects mice from lethal bacterial infection**. *J Exp Med* 1989, **169**:2021-7.

117. I Smirnova, N Mann, A Dols, HH Derkx, ML Hibberd, M Levin, B Beutler: **Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility**. *Proc Natl Acad Sci U S A* 2003, **100**:6075-80.

118. I Smirnova, MT Hamblin, C McBride, B Beutler, A Di Rienzo: **Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans**. *Genetics* 2001, **158**:1657-64.

119. E Lorenz, JP Mira, KL Frees, DA Schwartz: **Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock**. *Arch Intern Med* 2002, **162**:1028-32.

120. NC Arbour, E Lorenz, BC Schutte, J Zabner, JN Kline, M Jones, K Frees, JL Watt, DA Schwartz: **TLR4 mutations are associated with endotoxin hyporesponsiveness in humans**. *Nat Genet* 2000, **25**:187-91.

121. M Guha, N Mackman: **LPS induction of gene expression in human monocytes**. *Cell Signal* 2001, **13**:85-94.

122. ST Smiley, JA King, WW Hancock: **Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4**. *J Immunol* 2001, **167**:2887-94.

123. M Watari, H Watari, I Nachamkin, JF Strauss: **Lipopolysaccharide induces expression of genes encoding pro-inflammatory cytokines and the elastin-degrading enzyme, cathepsin S, in human cervical smooth-muscle cells**. *J Soc Gynecol Investig* 2000, **7**:190-8.

124. S Sasu, D LaVerda, N Qureshi, DT Golenbock, D Beasley: **Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation**. *Circ Res* 2001, **89**:244-50.

125. D Grenier, L Grignon: **Response of human macrophage-like cells to stimulation by Fusobacterium nucleatum ssp. nucleatum lipopolysaccharide**. *Oral Microbiol Immunol* 2006, **21**:190-6.

126. A Velayudham, I Hritz, A Dolganiuc, P Mandrekar, E Kurt-Jones, G Szabo: **Critical role of toll-like receptors and the common TLR adaptor, MyD88, in induction of granulomas and liver injury**. *J Hepatol* 2006, **45**:813-24.

127. AM Diehl: **Cytokine regulation of liver injury and repair**. *Immunol Rev* 2000, **174**:160-71.

128. SM Riordan, NA Skinner, J Kurtovic, S Locarnini, CJ McIver, R Williams, K Visvanathan: **Toll-like receptor expression in chronic hepatitis C: correlation**

with pro-inflammatory cytokine levels and liver injury. *Inflamm Res* 2006, **55**:279-85.

129. GL Su: **Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation**. *Am J Physiol Gastrointest Liver Physiol* 2002, **283**:G256-65.

130. A Tsung, RA Hoffman, K Izuishi, ND Critchlow, A Nakao, MH Chan, MT Lotze, DA Geller, TR Billiar: **Hepatic ischemia/reperfusion injury involves functional TLR4 signaling in nonparenchymal cells**. *J Immunol* 2005, **175**:7661-8.

131. A Shimamoto, AJ Chong, M Yada, S Shomura, H Takayama, AJ Fleisig, ML Agnew, CR Hampton, CL Rothnie, DJ Spring, et al: **Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury**. *Circulation* 2006, **114**:I270-4.

132. JS Park, F Gamboni-Robertson, Q He, D Svetkauskaite, JY Kim, D Strassheim, JW Sohn, S Yamada, I Maruyama, A Banerjee, et al: **High mobility group box 1 protein interacts with multiple Toll-like receptors**. *Am J Physiol Cell Physiol* 2006, **290**:C917-24.

133. PL De Jager, D Franchimont, A Waliszewska, A Bitton, A Cohen, D Langelier, J Belaiche, S Vermeire, L Farwell, A Goris, et al: **The role of the Toll receptor pathway in susceptibility to inflammatory bowel diseases**. *Genes Immun* 2007, **8**:387-97.

134. A Araki, T Kanai, T Ishikura, S Makita, K Uraushihara, R Iiyama, T Totsuka, K Takeda, S Akira, M Watanabe: **MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis**. *J Gastroenterol* 2005, **40**:16-23.

135. MM Fort, A Mozaffarian, AG Stover, S Correia Jda, DA Johnson, RT Crane, RJ Ulevitch, DH Persing, H Bielefeldt-Ohmann, P Probst, et al: **A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease**. *J Immunol* 2005, **174**:6416-23.

136. RW Summers, DE Elliott, K Qadir, JF Urban, Jr., R Thompson, JV Weinstock: **Trichuris suis seems to be safe and possibly effective in the treatment of inflammatory bowel disease**. *Am J Gastroenterol* 2003, **98**:2034-41.

137. E Furrie, S Macfarlane, A Kennedy, JH Cummings, SV Walsh, A O'Neil D, GT Macfarlane: **Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates**

resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005, **54**:242-9.

138. W Kruis, P Fric, J Pokrotnieks, M Lukas, B Fixa, M Kascak, MA Kamm, J Weismueller, C Beglinger, M Stolte, et al: **Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine**. *Gut* 2004, **53**:1617-23.

139. JJ Cebra: **Influences of microbiota on intestinal immune system development**. *Am J Clin Nutr* 1999, **69**:1046S-1051S.

140. LV Hooper: **Bacterial contributions to mammalian gut development**. *Trends Microbiol* 2004, **12**:129-34.

141. SK Mazmanian, CH Liu, AO Tzianabos, DL Kasper: **An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system**. *Cell* 2005, **122**:107-18.

142. H Tlaskalova-Hogenova, J Cerna, L Mandel: **Peroral immunization of germfree piglets: appearance of antibody-forming cells and antibodies of different isotypes**. *Scand J Immunol* 1981, **13**:467-72.

143. NA Bos, H Kimura, CG Meeuwsen, H De Visser, MP Hazenberg, BS Wostmann, JR Pleasants, R Benner, DM Marcus: **Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet**. *Eur J Immunol* 1989, **19**:2335-9.

144. NA Bos, VA Ploplis: **Humoral immune response to 2,4-dinitrophenyl--keyhole limpet hemocyanin in antigen-free, germ-free and conventional BALB/c mice**. *Eur J Immunol* 1994, **24**:59-65.

145. H Tlaskalova-Hogenova, L Tuckova, J Mestecky, J Kolinska, P Rossmann, R Stepankova, H Kozakova, T Hudcovic, T Hrncir, L Frolova, et al: **Interaction of mucosal microbiota with the innate immune system**. *Scand J Immunol* 2005, **62 Suppl 1**:106-13.

146. H Tlaskalova-Hogenova, J Sterzl, R Stepankova, V Dlabac, V Veticka, P Rossmann, L Mandel, J Rejnek: **Development of immunological capacity under germfree and conventional conditions**. *Ann N Y Acad Sci* 1983, **409**:96-113.

147. MC Moreau, R Ducluzeau, D Guy-Grand, MC Muller: **Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin**. *Infect Immun* 1978, **21**:532-9.

148. JL Fahey, S Sell: **The Immunoglobulins of Mice. V. the Metabolic (Catabolic) Properties of Five Immunoglobulin Classes**. *J Exp Med* 1965, **122**:41-58.

149. PA Crabbe, DR Nash, H Bazin, H Eyssen, JF Heremans: **Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice**. *Lab Invest* 1970, **22**:448-57.

150. L Helgeland, E Dissen, KZ Dai, T Midtvedt, P Brandtzaeg, JT Vaage: **Microbial colonization induces oligoclonal expansions of intraepithelial CD8 T cells in the gut**. *Eur J Immunol* 2004, **34**:3389-400.

151. Y Umesaki, H Setoyama, S Matsumoto, Y Okada: **Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus**. *Immunology* 1993, **79**:32-7.

152. A Imaoka, S Matsumoto, H Setoyama, Y Okada, Y Umesaki: **Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ-free mice**. *Eur J Immunol* 1996, **26**:945-8.

153. M Kawaguchi, M Nanno, Y Umesaki, S Matsumoto, Y Okada, Z Cai, T Shimamura, Y Matsuoka, M Ohwaki, H Ishikawa: **Cytolytic activity of intestinal intraepithelial lymphocytes in germ-free mice is strain dependent and determined by T cells expressing gamma delta T-cell antigen receptors**. *Proc Natl Acad Sci U S A* 1993, **90**:8591-4.

154. L Prokesova, P Mlckova, I Stankova, A Chloubova, V Novotna, P Ladmanova, P Chalupna, M Mara: **Effect of Bacillus firmus on antibody formation after mucosal and parenteral immunization in mice**. *Immunol Lett* 1998, **64**:161-6.

155. HA Gordon: **Morphological and physiological characterization of germfree life**. *Ann N Y Acad Sci* 1959, **78**:208-20.

156. LV Hooper, JI Gordon: **Commensal host-bacterial relationships in the gut**. *Science* 2001, **292**:1115-8.

157. LV Hooper, TS Stappenbeck, CV Hong, JI Gordon: **Angiogenins: a new class of microbicidal proteins involved in innate immunity**. *Nat Immunol* 2003, **4**:269-73.

158. TS Stappenbeck, LV Hooper, JI Gordon: **Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells**. *Proc Natl Acad Sci U S A* 2002, **99**:15451-5.

159. BS Wostmann, JR Pleasants: **The germ-free animal fed chemically defined diet: a unique tool**. *Proc Soc Exp Biol Med* 1991, **198**:539-46.

160. BS Wostmann, JR Pleasants, P Bealmear: **Dietary stimulation of immune mechanisms**. *Fed Proc* 1971, **30**:1779-84.

161. MF Neurath, S Finotto, LH Glimcher: **The role of Th1/Th2 polarization in mucosal immunity**. *Nat Med* 2002, **8**:567-73.

162. A Sheikh, DP Strachan: **The hygiene theory: fact or fiction?** *Curr Opin Otolaryngol Head Neck Surg* 2004, **12**:232-6.

163. LM Bowman, PG Holt: **Selective enhancement of systemic Th1 immunity in immunologically immature rats with an orally administered bacterial extract**. *Infect Immun* 2001, **69**:3719-27.

164. GA Rook, LR Brunet: **Give us this day our daily germs**. *Biologist (London)* 2002, **49**:145-9.

165. L Prokesova, P Mlckova, I Stankova, P Ladmanova, J Jezkova, P Chalupna, O Novotna, D Cechova, J Julak: **Immunostimulatory effect of Bacillus firmus on mouse lymphocytes**. *Folia Microbiol (Praha)* 2002, **47**:193-7.

166. LE Harrington, RD Hatton, PR Mangan, H Turner, TL Murphy, KM Murphy, CT Weaver: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages**. *Nat Immunol* 2005, **6**:1123-32.

167. B Stockinger, M Veldhoen: **Differentiation and function of Th17 T cells**. *Curr Opin Immunol* 2007, **19**:281-6.

168. CL Bennett, J Christie, F Ramsdell, ME Brunkow, PJ Ferguson, L Whitesell, TE Kelly, FT Saulsbury, PF Chance, HD Ochs: **The immune dysregulation, po-**

lyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001, **27**:20-1.

169. ME Brunkow, EW Jeffery, KA Hjerrild, B Paeper, LB Clark, SA Yasayko, JE Wilkinson, D Galas, SF Ziegler, F Ramsdell: **Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse**. *Nat Genet* 2001, **27**:68-73.

170. S Hori, T Nomura, S Sakaguchi: **Control of regulatory T cell development by the transcription factor Foxp3**. *Science* 2003, **299**:1057-61.

171. JD Fontenot, MA Gavin, AY Rudensky: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells**. *Nat Immunol* 2003, **4**:330-6.

172. JD Fontenot, JP Rasmussen, LM Williams, JL Dooley, AG Farr, AY Rudensky: **Regulatory T cell lineage specification by the forkhead transcription factor foxp3**. *Immunity* 2005, **22**:329-41.

173. B Singh, S Read, C Asseman, V Malmstrom, C Mottet, LA Stephens, R Stepankova, H Tlaskalova, F Powrie: **Control of intestinal inflammation by regulatory T cells**. *Immunol Rev* 2001, **182**:190-200.

174. UG Strauch, F Obermeier, N Grunwald, S Gurster, N Dunger, M Schultz, DP Griese, M Mahler, J Scholmerich, HC Rath: **Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis**. *Gut* 2005, **54**:1546-52.

175. S Ostman, C Rask, AE Wold, S Hultkrantz, E Telemo: **Impaired regulatory T cell function in germ-free mice**. *Eur J Immunol* 2006, **36**:2336-46.

176. O Annacker, O Burlen-Defranoux, R Pimenta-Araujo, A Cumano, A Bandeira: **Regulatory CD4 T cells control the size of the peripheral activated/memory CD4 T cell compartment**. *J Immunol* 2000, **164**:3573-80.

177. M Gad, AE Pedersen, NN Kristensen, MH Claesson: **Demonstration of strong enterobacterial reactivity of CD4+CD25- T cells from conventional and germ-free mice which is counter-regulated by CD4+CD25+ T cells**. *Eur J Immunol* 2004, **34**:695-704.

178. B Min, A Thornton, SM Caucheteux, SA Younes, K Oh, J Hu-Li, WE Paul: **Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis**. *Eur J Immunol* 2007, **37**:1916-23.

179. DP Strachan: **Hay fever, hygiene, and household size**. *Bmj* 1989, **299**:1259-60.

180. P Pereira, L Forni, EL Larsson, M Cooper, C Heusser, A Coutinho: **Autonomous activation of B and T cells in antigen-free mice**. *Eur J Immunol* 1986, **16**:685-8.

181. CT Weaver, RD Hatton, PR Mangan, LE Harrington: **IL-17 family cytokines and the expanding diversity of effector T cell lineages**. *Annu Rev Immunol* 2007, **25**:821-52.

182. E Bettelli, M Oukka, VK Kuchroo: **T(H)-17 cells in the circle of immunity and autoimmunity**. *Nat Immunol* 2007, **8**:345-50.

183. DC Angus, WT Linde-Zwirble, J Lidicker, G Clermont, J Carcillo, MR Pinsky: **Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care**. *Crit Care Med* 2001, **29**:1303-10.

184. C Brun-Buisson, P Meshaka, P Pinton, B Vallet: **EPISEPSIS: a reappraisal of the epidemiology and outcome of severe sepsis in French intensive care units**. *Intensive Care Med* 2004, **30**:580-8.

185. C Brun-Buisson, F Roudot-Thoraval, E Girou, C Grenier-Sennelier, I Durand-Zaleski: **The costs of septic syndromes in the intensive care unit and influence of hospital-acquired sepsis**. *Intensive Care Med* 2003, **29**:1464-71.

186. JL Vincent, Y Sakr, CL Sprung, VM Ranieri, K Reinhart, H Gerlach, R Moreno, J Carlet, JR Le Gall, D Payen: **Sepsis in European intensive care units: results of the SOAP study**. *Crit Care Med* 2006, **34**:344-53.

187. RS Hotchkiss, IE Karl: **The pathophysiology and treatment of sepsis**. *N Engl J Med* 2003, **348**:138-50.

188. JL Vincent, Q Sun, MJ Dubois: **Clinical trials of immunomodulatory therapies in severe sepsis and septic shock**. *Clin Infect Dis* 2002, **34**:1084-93.

189. O Shelley, T Murphy, H Paterson, JA Mannick, JA Lederer: **Interaction between the innate and adaptive immune systems is required to survive sepsis and control inflammation after injury**. *Shock* 2003, **20**:123-9.

190. RS Hotchkiss, KC Chang, PE Swanson, KW Tinsley, JJ Hui, P Klender, S Xanthoudakis, S Roy, C Black, E Grimm, et al: **Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte**. *Nat Immunol* 2000, **1**:496-501.

191. S Sakaguchi: **Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors**. *J Exp Med* 2003, **197**:397-401.

192. I Caramalho, T Lopes-Carvalho, D Ostler, S Zelenay, M Haury, J Demengeot: **Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide**. *J Exp Med* 2003, **197**:403-11.

193. JG Heuer, T Zhang, J Zhao, C Ding, M Cramer, KL Justen, SL Vonderfecht, S Na: **Adoptive transfer of in vitro-stimulated CD4+CD25+ regulatory T cells increases bacterial clearance and improves survival in polymicrobial sepsis**. *J Immunol* 2005, **174**:7141-6.

194. TJ Murphy, N Ni Choileain, Y Zang, JA Mannick, JA Lederer: **CD4+CD25+ regulatory T cells control innate immune reactivity after injury**. *J Immunol* 2005, **174**:2957-63.

195. R Dobber, A Hertogh-Huijbregts, J Rozing, K Bottomly, L Nagelkerken: **The involvement of the intestinal microflora in the expansion of CD4+ T cells with a naive phenotype in the periphery**. *Dev Immunol* 1992, **2**:141-50.

196. CS Probert, AM Williams, R Stepankova, H Tlaskalova-Hogenova, A Phillips, PW Bland: **The effect of weaning on the clonality of alpha beta T-cell receptor T cells in the intestine of GF and SPF mice**. *Dev Comp Immunol* 2007, **31**:606-17.

197. AM Williams, CS Probert, R Stepankova, H Tlaskalova-Hogenova, A Phillips, PW Bland: **Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse**. *Immunology* 2006, **119**:470-8.

198. H Hooijkaas, R Benner, JR Pleasants, BS Wostmann: **Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet**. *Eur J Immunol* 1984, **14**:1127-30.

199. G Liu, Y Zhao: **Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells**. *Immunology* 2007, **122**:149-56.

200. NK Crellin, RV Garcia, O Hadisfar, SE Allan, TS Steiner, MK Levings: **Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells**. *J Immunol* 2005, **175**:8051-9.

201. JH Droste, MH Wieringa, JJ Weyler, VJ Nelen, PA Vermeire, HP Van Bever: **Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease?** *Clin Exp Allergy* 2000, **30**:1547-53.

202. M Nickmilder, A Bernard: **Ecological association between childhood asthma and availability of indoor chlorinated swimming pools in Europe**. *Occup Environ Med* 2007, **64**:37-46.

203. F Guarner, R Bourdet-Sicard, P Brandtzaeg, HS Gill, P McGuirk, W van Eden, J Versalovic, JV Weinstock, GA Rook: **Mechanisms of disease: the hygiene hypothesis revisited**. *Nat Clin Pract Gastroenterol Hepatol* 2006, **3**:275-84.

204. G Bamias, A Okazawa, J Rivera-Nieves, KO Arseneau, SA De La Rue, TT Pizarro, F Cominelli: **Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis**. *J Immunol* 2007, **178**:1809-18.