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Summary of Dissertation

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

MUDr. Tomáš Hrnčíř
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Field:	Immunology
Chairman of the Supervisory Committee:	
	Doc. RNDr. Vladimír Holáň, DrSc.
Supervising Body:	Institute of Microbiology of the ASCR, v.v.i. Department of Immunology and Gnotobiology 549 22 Novy Hradek
Author:	MUDr. Tomáš Hrnčíř
Supervisor:	Prof. MUDr. Helena Tlaskalová-Hogenová, DrSc.
Opponents:	Doc. MUDr. Ludmila Prokešová, CSc. Prof. RNDr. Aleš Macela, DrSc.

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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 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

ABSTRAKT V ČEŠTINĚ

Savci se rodí bezmikrobní, avšak rychle po porodu jsou jejich epitelové povrchy osídleny obrovským množstvím baktérií. Nejrozsáhlejší společenství mikrobů se nachází v distálním střevě. Střevní mikroorganismy svým počtem desetkrát převyšují naše somatické a zárodečné buňky. Vztah mezi hostitelem a mikroorganismy se vyvinul tak, že obě strany z něj profitují. Studie na bezmikrobních myších prokázaly, že střevní mikroorganismy jsou nezbytné pro řádný vývoj imunitního systému. Klíčový význam přirozené imunity při komplexních a dynamických interakcích hostitele a mikroorganismů je stále patrnější.

Hlavními cíli této studie bylo: zaprvé, zjistit, zda sterilní dieta s vysokým obsahem lipopolysacharidu může podpořit vývoj imunitního systému u bezmikrobních myší, zadruhé, objasnit, zda střevní mikroorganismy a dieta s vysokým obsahem lipopolysacharidu ovlivňují citlivost k endotoxinovému šoku, a konečně, zhodnotit roli adaptivní imunity při jeho patogenezi.

Provedené experimenty jasně dokazují, že jak živé střevní mikroorganismy, tak také sterilní dieta s vysokým obsahem lipopolysacharidu zvyšují citlivost k endotoxinovému šoku. Též bylo prokázáno, že imunodeficitní SCID myši, které postrádají zralé B a T lymfocyty, jsou citlivější k endotoxinovému šoku než imunokompetentní Balb/c myši. Tato studie potvrzuje naši hypotézu, že nejen živé střevní mikroorganismy, ale také sterilní dieta s vysokým obsahem lipopolysacharidu stimuluje vývoj, maturaci a funkci imunitního systému. Získané výsledky jsou v souladu, a dále rozšiřují "hygienickou hypotézu" tím, že dokazují, že nejen živé organismy, ale také sterilní mikrobiální antigeny stimulují vývoj imunitního systému. Na závěr bychom chtěli zdůraznit, že kvalita diety by měla být pravidelně testována, protože obsah lipopolysacharidu v dietě může významným způsobem ovlivnit výsledky experimentů.

ABSTRACT IN ENGLISH

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.

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.

CHAPTER ONE

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 pa-

thogenic 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 microbi-cidal 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.

Gut Microbiota
Effects on the Adaptive Immune System

Studies in germ-free (GF) mice have shown that gut microbiota play a crucial role in the development and maturation of the immune system [14, 33-40]. 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, 41-47]. Peyer's patches are hypoplastic with few germinal centers [48]. 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 [49-51].

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 cen-

ters [6, 37, 38, 52, 53]. Spleens of GF mice are also smaller and the content of CD4+ T cells is reduced [35]. 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.

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 [54, 55]. A role of gut microbiota in establishing this equilibrium has been postulated [35, 56, 57]. 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 [58, 59].

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 [60-64]. The latest research suggests that Tregs are best defined by the expression of the transcription factor Foxp3 [62-64]. 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.

CHAPTER TWO

SIGNIFICANCE AND AIMS 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” [65]. 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]

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: the group of GF mice fed a LPS low diet, the group of GF mice fed a LPS-rich diet and the group of CV mice fed a LPS-rich diet. To determine the role of the 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.

The Specific Aims

To determine the concentration of LPS in mouse pelleted diets

To analyze the effect of live gut microbiota and LPS content of the sterile diet on the weight and cellularity of lymphoid organs

To investigate the effect of live gut microbiota and LPS content of the sterile diet on the susceptibility to intraperitoneal LPS challenge in immunocompetent and immunodeficient mice

To investigate the effect of live gut microbiota and LPS content of the sterile diet on the *in vitro* susceptibility of Balb/c and SCID spleen cells to stimulation with LPS

To analyze the frequency of main lymphocyte subpopulations in Peyer's patches, mesenteric lymph nodes, spleen, peritoneal cavity and thymus

To analyze the effect of live gut microbiota and LPS content of the sterile diet on the proportion of Foxp3-expressing regulatory T cells

To investigate whether the live gut microbiota and LPS content of the sterile diet shift the T_H1/T_H2 balance and influence a production of anti-inflammatory cytokines

CHAPTER THREE

MATERIALS AND METHODS

Mice

Both conventional and germ-free Balb/c and SCID mice were bred at the animal facility of the Institute of Microbiology of the ASCR in Novy Hradek and were used at the age of 8-10 weeks.

Determination of LPS Content of Mouse Feed Pellets

We have tested all the pelleted diets which are commonly used in our animal facility. 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.

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. The ST1 diet is a grain-based diet, which is based on wheat, oat, corn, wheat flour, snail clover fodder, soya pollard, bone meal and scrap cake. The AIN-93G diet has almost 100 times lower content of 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. Red blood cells in spleen cell suspensions were lysed with ACK lysing buffer. All the cells were washed twice in a culture medium. To harvest resident peritoneal cells, 10 ml culture medium per mouse was injected into a 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 96-well flat-bottom culture plate in RPMI-1640 medium containing 10% FBS, 2mM 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).

Proliferation Assay

Freshly isolated lymphocytes were resuspended in CFDA-SE solution (5 µM final concentration) and mixed rapidly. After 5 min at room temperature, the cells were washed three times with 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.

Flow Cytometry and Intracellular Cytokine Staining

Phenotypic analysis of cells isolated from spleen, thymus, MLNs, PPs and peritoneal cavity was performed by flow cytometry. Cells were pre-incubated with anti-mouse CD16/CD32 mAb (eBioscience, USA) and then stained with FITC, PE or PE-Cy5 conjugated mAbs. 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 FACSCalibur 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.

Data Analysis

The Student's 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.

CHAPTER FOUR

RESULTS

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

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.

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).

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 were not affected by the LPS content of the sterile diet nor gut microbiota. 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.

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.

LPS-rich Sterile Diet Increases *In Vivo* Susceptibility of GF 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).

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.

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.

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. 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. The proportion of CD19+ B cells in Peyer's patches, spleen and peritoneal cells remains constant irrespective of the degree of microbial stimulation.

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

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. We have not observed a significant increase in the proportion of CD4+ T cells of CV spleen compared to GF spleen. The proportion of CD4+ T cells in Peyer's patches, thymus and peritoneal cells remains constant regardless of the level of microbial stimulation.

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 [66]. In addition, we show that the proportion of CD8+ T cells not influenced by gut colonization or LPS content of the sterile diet also in other lymphoid organs including MLNs, Peyer's patches, thymus and peritoneal cells.

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

We have found that both gut microbiota and the LPS-rich diet drive the expansion of CD4+Foxp3+ Tregs in MLNs. We have not detected any significant difference in the proportion of Tregs in other lymphoid organs.

Gut Microbiota Stimulate 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 effect of LPS-rich diet on the expansion of CD8+Foxp3+ Tregs in all lymphoid organs of GF mice was not significant.

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.

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

CFDA-SE-stained spleen cells were stimulated with ConA and LPS. After 72 h the CFDA-SE staining profile was analyzed by flow cytometry. No significant difference was found between the groups of CV and GF mice fed either diet. Thus we conclude that neither gut microbiota nor LPS content of the sterile diet influence non-specific proliferative response of spleen cells *in vitro*.

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.

Gut Microbiota Stimulate 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) was 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 significant. LPS stimulated spleen cells did not produce any detectable levels of IL-10.

CHAPTER FIVE

DISCUSSION

The Role of Adaptive Immunity and Regulatory T Cells in Sepsis

The role of conventional T cells and the involvement of sub-populations 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 [67, 68].

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 [62-64]. 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.

Regarding the functional role of Treg cells in injury-induced, dysregulated immune response, Heuer et al. [69] 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. [70] recently reported that burn injury primes innate immune cells for a progressive increase in TLR4 and TLR2 agonist-induced pro-inflammatory 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 GF Mice

It is generally accepted that live gut microbiota are essential for the development and maturation of the mammalian immune system [14, 33-40]. 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, 35, 45, 71-73]. PPs and MLNs are smaller, less cellular (lower numbers of B and T cells) and do not have germinal centers [6, 10, 37, 38, 48, 74]. Spleens of GF mice are also smaller, less cellular and the proportion of CD4+ T cells is reduced [35]. 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 [35].

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 [75]. It has been recently reported that gut microbiota is crucial for the generation and expansion of Tregs [76]. 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 [77]. Paradoxically, it was reported that CD25+ Tregs from GF mice are as suppressive and protective as those from CV mice [78, 79] 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 [80].

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 [81, 82]. The proliferative response does not require APCs and is augmented by TCR triggering and IL-2 stimulation [81]. 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. 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” [65]. 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 micro-

organisms and parasites during early postnatal development. Early childhood use of antibiotics is associated with an increased risk of developing asthma and allergic disorders [83]. The extensive use of cleaning products, including chlorination products, is also linked to the rise of childhood asthma [84]. The widespread use of antimicrobial 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 [85]. 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 [86].

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 NLR 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 NLR 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

Finally, 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 SIX

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.

CHAPTER SEVEN

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PUBLICATIONS

Publications *in Extenso* Related to the Present Study

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.

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None.