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THE INFLUENCE OF LACTOBACILLI AND *STAPHYLOCOCCUS AUREUS* ON IMMUNE RESPONSIVENESS *IN VITRO*

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SUMMARY

Alteration of gut microbiota has been associated with development of immune mediated diseases, such as allergy. In part, this could be due to the influence of microbes in shaping the immune response. In paper I, we investigated the association of early-life gut colonization with bacteria, and numbers of IL-4, IL-10 and IFN-γ producing cells at two years of age in response to PBMC stimulation with phytohemagglutinin (PHA) *in vitro*. Early *Staphylococcus (S) aureus* colonization was directly proportional to increased numbers of IL-4 and IL-10 secreting cells, while early co-colonization with lactobacilli and *S. aureus* associated with a decrease in IL-4, IL-10 and IFN-γ secreting cells compared to *S. aureus* alone. This was also confirmed in *in vitro* stimulation of PBMC with *Lactobacillus* and/or *S. aureus* strains, where *S. aureus*-induced IFN-γ production by Th cells was down regulated by co-stimulation with *Lactobacillus*. In paper II, we investigated the effects of UV-killed and/or culture supernatant (sn) of *Lactobacillus* strains and *S. aureus* strains on IEC and immune cell responses. IEC exposed to *S. aureus-sn* produced CXCL-1/GRO-α and CXCL-8/IL-8, while UV-killed bacteria had no effect. MyD88 gene silencing of IEC dampened *S. aureus*-induced CXCL-8/IL-8 production, indicating the involvement of TLR signaling. Further, PBMC from healthy donors exposed to *Lactobacillus-sn* and *S. aureus-sn* were able to produce a plethora of cytokines, but only *S. aureus* induced the T-cell associated cytokines: IL-2, IL-17, IFN-γ and TNF-α; which were down regulated by the simultaneous presence of any of the different *Lactobacillus* strains. Intracellular staining of T cells further confirmed *S. aureus* induced IFN-γ and IL-17 production by Th cells, and up regulation of CTLA-4 expression and IL-10 production by Treg cells.

In conclusion, we show that colonization with gut microbiota at early age modulates the cytokine response in infancy. In addition, bacterial species influence cytokine response in a species-specific manner and we demonstrate that lactobacilli modulate *S. aureus*-induced immune response away from an inflammatory phenotype.

LIST OF PAPERS

This thesis is based on the two original papers listed below, which will be referred to by their roman numerals in the text.

I. Maria A. Johansson, Shanie Saghafian-Hedengren, Yeneneh Haileselassie, Stefan Roos, Marita Troye-Blomberg, Caroline Nilsson, Eva Sverremark-Ekström.

Early-Life Gut Bacteria Associate with IL-4-, IL-10- and IFN-γ Production at Two Years of Age. Plos One. 2012; 7(11). e49315.

II. Yeneneh Haileselassie, Maria A Johansson, Christine L Zimmer, Sophia Björkander, Dagbjort H Petursdottir, Johan Dicksved, Mikael Petersson, Jan-Olov Persson, Carmen Fernandez, Stefan Roos, Ulrika Holmlund, Eva Sverremark-Ekström.

Lactobacilli regulate *Staphylococcus aureus* 161:2-induced pro-inflammatory T-cell responses *in vitro.* Plos One: *In press*

TABLE OF CONTENT

ABBREVIATIONS

AHR	airway hyper-responsiveness
AMP	anti-microbial peptides
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
BAFF	B-cell-activating factor
BCR	B cell receptors
CBMC	cord blood mononuclear cells
CP	crypt patches
DC	dendritic cell
FAE	follicular associated epithelium
GF	germ free
IBS	irritable bowel syndrome
IEL	intraepithelial lymphocytes
IFN	interferon
IL	interleukin
IDO	indoleamine 2,3-dioxygenase
IEC	intestinal epithelial cells
ILC	innate lymphoid cells
ILF	isolated lymphoid follicles
LI	large intestine
LP	lamina propria
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MAMP	microbial-associated molecular pattern
NEC	necrotizing enterocolitis
NFκB	nuclear factor-κB
NK cell	natural killer cell
NLR	nucleotide oligomerization domain-like receptor
NOD	nucleotide oligomerization domain
PBMC	peripheral blood mononuclear cells
PGN	peptidoglycan
PHA	phytohemaglutinin
PP	Peyer's patches
PRR	pattern recognition receptor
RORγt	retinoic-acid-receptors γt
SN	supernatant
SI	small intestine
Tc cell	T cytotoxic cell
Th cell	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg cell	T regulatory cell

INTRODUCTION

Mammals have elaborate defense mechanisms against pathogens, which involve both the innate and the adaptive immune system (1). As a first line of defense, the innate immune response involves physiological barriers such as pH and temperature, and physical barriers such as the mucous layer and the underlying single layer of epithelium (2). These barriers prevent the entry of the pathogens to the body. The gut mucosa is in direct contact with the external environment. Thus, it is continuously exposed to dietary products, environmental antigens, pathogens and commensal microbes. In addition, the gut mucosa harbors the largest immune organ known as the gut associated lymphoid tissues (GALT) (1). GALT is rich in both innate and adaptive immune cells, which are of hematopoietic origin. These cells respond rapidly to clear the pathogens by phagocytosis and by secreting biological soluble factors (3). In addition to the hematopoietic cells, both epithelial cells and paneth cells are involved in innate immune responses in the gut by secreting biological factors such as antimicrobial products and cytokines (4). Compared to innate immune cells, adaptive immune cells are slower to act and recognize specific antigens, rather than common patterns. They respond by secreting antibodies, by cytolytic killing and/or by secreting cytokines that could facilitate in killing of the pathogens (5).

There is a strong communication between the innate and adaptive immune cells. For instance, the innate immune cells initiate the adaptive immune response by processing and presenting antigens to adaptive immune cells, while cytokines and antibodies secreted by these cells improve the phagocytic and killing activity of the innate immune cells. However, while protecting against pathogens is the main goal of the immune system, the immune system in the gut needs to develop hypo-responsiveness to innocuous antigens and commensal microbes (6).

Innate immune system

The innate immune system is the first line of defense that facilitates a response to ultimately clear the pathogen. It orchestrates the activation and attraction of different immune cells, including adaptive immune cells. Cells of the innate immune system include monocytes, macrophages, dendritic cells (DC), natural killer (NK) cells, mast cells, basophils, neutrophils and eosinophils.

Cells of the innate branch respond rapidly when challenged and recognize patterns common to most pathogens and microbes known as microbial-associated molecular patterns (MAMP). Pattern recognition receptors (PRR) present in or at the surface of immune cells recognize and bind to MAMP. Activation of cells via interaction of PRR with MAMP enhance phagocytosis of the microbes, and the release of soluble factors that facilitate chemotaxis and activation of immune cells to the site. Hence, activation of cells via PRR, mediates a cascade of function by different immune cells. The major PRR are the Toll like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors. Up to now, 13 murine TLRs and 10 human TLRs have been identified on the surface or in intracellular compartment of cells (7). Each TLR recognize distinct molecular components of microbes. For example TLR4 binds to lipopolysaccharides (LPS), TLR2 to peptidoglycan (PGN), TLR5 to flagellin and TLR9 to CpG DNA (8). Recognition of these MAMP by TLR activates downstream molecules such as MyD88 and nuclear factor-κB (NFκB) that lead to synthesis and secretion of cytokines (9). NOD receptors are intracellular receptors that recognize bacterial components and activation of cells via NOD also lead to production of inflammatory molecules (7).

Adaptive immune system

Compared to the innate immune system, the adaptive immune system is slower to respond. Hallmarks of the adaptive immune system are its ability to deliver antigen specific response and having immunological memory. Immunological memory enables adaptive immune cells to respond rapidly and efficiently when they encounter the pathogen again in latter times. T cells and B cell are the major adaptive immune cells.

Conventional T cells

T cells are lymphocytes that originate from hematopoietic progenitor cells in the bone marrow and mature in the thymus. Conventional T cells have αβ-T cell receptor on their surface that enables them to recognize antigen presented in the context of major histocompatibility complex (MHCI or MHCII) on the surface of either infected cells or on

antigen presenting cells (APC, i.e DC and macrophages), respectively. There are two major subgroups of conventional T cells, namely T (Th) helper cells and T (Tc) cytotoxic cells. They can be distinguished by expression of CD4 and CD8, respectively. Tc cells are involved in cytolytic killing of infected and transformed cells, while Th cells facilitate the initiation of both humoral and cell mediated immunity by controlling the activation of cells, such as B cells, Tc cells and macrophages via cell-cell interaction and/or through release of cytokines.

CD4+T cells

The major subsets of CD4+T cells are Th1, Th2, Th17 and regulatory T (reg) cells. They are distinguished by their distinct regulatory transcription factors, cytokine profile and distinct function. For instance Th1 cells are controlled by transcription factor, T-bet. Th1 produce IFN-γ and are important for clearing intracellular bacteria. Th2 cells are controlled by GATA-binding protein 3 (GATA3) and produce interleukin (IL) 4, IL-5 and IL-13, and are necessary to clear extracellular pathogens, such as helminths. Th17 cells are crucial for host defense against bacteria, viruses and fungi and Th17 cells produce IL-17 A, IL-17 F and IL-22. Further, retinoic-acid-receptor γt (RORγt) is the transcription factor that control Th17 cell differentiation (10). There are additional subsets of Th cells; namely Th22, Th9 and T follicular helper (Tfh) cells (11). Th22 cells secrete IL-22 and are critical for host defense against Gram-negative bacterial organisms and intestinal epithelial layer repair. Th9 cells secrete IL-9 and are linked to immune-mediated diseases, such as autoimmunity and asthma, while T follicular helper cells play a vital role in the formation of germinal centers in secondary lymphoid organs. Treg cells are a subset of Th cells that play major role in active suppression of immune responses. Treg cells are classified into natural Treg cells and inducible Treg cells. Natural Treg cells develop in the thymus, and express the Forkhead box P3 (Foxp3) transcription factor that is crucial for the development and function of these cell types. Inducible Treg develop in the periphery from mature CD4+ T cells in response to signals from regulatory cytokines, or APC conditioned by a regulatory micro milieu (microbial products or local regulatory cytokines). Treg cells set their inhibitory effect by expressing CTLA-4 receptor that could down regulate the expression of co-stimulatory molecules; CD80 and CD86 by APC. In addition, Treg cells secrete regulatory cytokines, such as IL-10 and TGF-β (12).

B cells

B cells are crucial for the humoral immune response and are produced and mature in the bone marrow (13). They migrate to secondary lymphoid tissues where they are activated and differentiate into antibody secreting plasma cells and memory cells upon antigen encounter.

Activation of B cells requires antigen recognition by B cell receptors (BCR) and co-stimulatory signal provided by CD40-CD40L interaction with Th cells (also known as T cell dependent activation) or the antigen itself (T cell independent activation). The different isotypes of antibodies (Ig) that exist are IgM, IgD, IgG, IgE and IgA. These antibodies have various functions, such as facilitating opsonization of microbes, activating complement proteins or antibody-dependent cell mediated cytotoxicity (ADCC). In addition, some of the antibodies (such as IgA and IgM) can be transported to the extracellular side of the body including the lumen of the gut and respiratory tract and can also be found in extracellular fluids; tears and breast milk. They play major roles in clearing microbes out of the body (14). However, antibodies can also be involved in the pathology of immune-mediated disease. For instance in allergic individuals, when IgE antibodies that are bound to high affinity IgE-receptors on e.g mast cells recognize innocuous antigens, the antibodies will cross-link. The cross-linking results in receptor activation and the secretion of mediators that causes allergic symptoms (15). On the other hand, IgE antibodies are necessary to defend against extracellular parasites such as helminths.

Unconventional T cells and innate lymphoid cells

Unconventional T cells and innate lymphoid cells are hematopoietic cells that lie in the intersection between the innate and the adaptive arm, as they share features from both the innate and adaptive immune cells. Unconventional T cells are further subdivided into mucosa-associated invariant T (MAIT) cells, γδ T cells and NKT cells (16). Little is known about MAIT cells, but along γδ T cells, they are the first immune cells found in the fetus and provide protection to newborns prior to activation of the adaptive immune system (17). γδ T cells are much more prevalent at mucosal and epithelial sites and account for 50% of the total intraepithelial lymphocyte (IEL) population in the gut. γδ T cells do not need to see antigen in complex with MHC in the same manner as conventional T cells. Instead, γδ-TCR serve as pattern recognition receptors, recognizing conserved phosphoantigens of bacterial metabolites and cell damage products. On the other hand, NKT cells share properties with both NK cells and T cells. They recognize glycolipids when presented by CD1d on APC.

Innate lymphoid cells (ILC) are a heterogeneous population that include lymphoid tissue inducers, NK cells and other ILC members that are subdivided depending on their controlling transcription factors and their function, just like the subsets of conventional T cells (18). They develop from hematopoietic precursors, do not express TCR and IL-7 is important

for their development. Like the γδ T cells, they are abundant at the mucosal surface, and are mainly involved in organogenesis and mediate immune response against pathogens (19).

Immune cell communication

In order to orchestrate immune responses, cells of the immune system communicate either through cell-cell surface contact or through production and secretion of cytokines and chemokines. Cytokines are soluble proteins that are produced to effect target cells that express specific receptors for the cytokines. The binding of cytokine to its relevant receptor on the target cell results in a cascade of intracellular downstream signals, that could influence the gene expression of the target cell, and will eventually alter the target cells' function. Cytokine action can be paracrine or autocrine, depending on the target cells, and therefore cytokines can have a multitude of effects. In addition, different cytokines can exert similar effect on target cell, showing redundancy. Chemokines, on the other hand, are cytokines with particular function; they exert a chemotactic effect on a target cell (3).

The intestinal epithelium

The intestinal epithelium is mainly composed of a single layer of intestinal epithelial cells (IEC), which are connected by tight junctions (20). IEC are not just passive barriers; they control the transport of luminal content to the body and the release of secretory-antibodies to the lumen. The IEC are equipped with major PRRs that enable them to sense conserved MAMP and transfer signals to the underlying immune system. They therefore play a major role in immune responses by secreting cytokines and antimicrobial peptides in response to interaction with microbial components (4). IEC are also believed to express major histocompatibility complex (MHC) class I and II, and CD1d on their surface, which are important for antigen presentation (21). Thus IEC can interact actively with underlying immune cells either via secreting factors or cell surface interaction. These communications are important for the maintenance of intestinal homeostasis. In addition to IEC, the epithelium contains mucous secreting goblet cells, hormone secreting enteroendocrine cells, and paneth cells that are found in the crypt of the small intestine (SI) and produce high amounts of antimicrobial peptides (22). In addition, intraepithelial lymphocytes (IEL) reside between IEC, and subsets of APC extend their dendrites between the IEC to directly sample luminal antigens (23) (24). Moreover, the intestinal epithelium is associated with several types of lymphoid organs, collectively known as the gut associated lymphoid tissues (GALT) (Figure 1).

Gut associated lymphoid tissues (GALT)

GALT is the largest immune structures in the host. It is very rich in both innate and adaptive immune cells (1) and has inductive and effector sites. The inductive sites include; Peyer's patches (PP), crypt patches (CP) and isolated lymphoid follicles (ILF); are all located within the mucosa. The effector sites encompass lymphocytes scattered throughout the epithelium, and the lamina propria (LP), a layer of loose connective tissue that underlie the epithelium.

PP are macroscopic collections of lymphoid tissue located in the submucosa along the length of the SI. They are secondary lymphoid organs that contain large B cell follicles and T cell areas. ILF, on the other hand, are microscopic and found in the mucosal surface of both SI and large intestine (LI). A single layer of columnar epithelial cells called follicular associated epithelium (FAE) and a more diffuse area immediately bellow the epithelium, known as subepithelium dome, separates the PPs from the lumen of the SI. FAE has specialized epithelial cells, called M cells that are devoid of microvilli and a thick mucus layer. M cells facilitate transport of exogenous antigens from the lumen to APC such as DC in the PP. However, there are macrophages and subsets of DC in the LP that can directly sample luminal antigens (24). Immune cells from the PP and LP drain via afferent lymphatics to the mesenteric lymph nodes (MLN). There is evidence that DC migrate to the MLN to imprint gut homing phenotype to naïve T cells, both under steady state, and during intestinal inflammation (25).

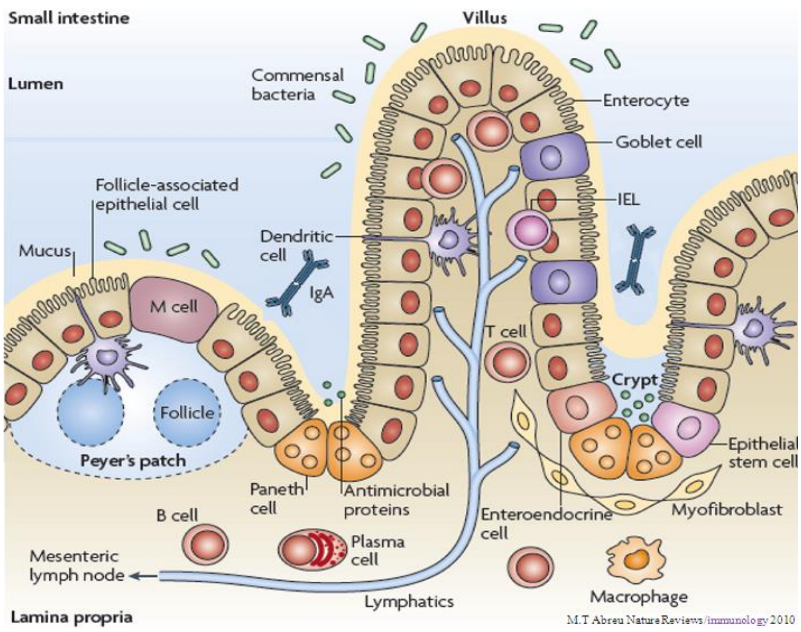

Figure1 Schematic representation of the intestinal immune system:
A single layer of IEC separates luminal content from the underlying immune cells. There are goblet cells, enteroendocrine cells and paneth cells along the epithelium. The intestinal lumen contains nutrients, commensal bacteria and secretory IgA. A goblet cell-produced mucus layer covers the apical side of the epithelium. Beneath the IEC, effector immune cells are scattered sparsely throughout the lamina propria and epithelium. IEL and APC localize between the IEC. A specialized epithelium termed follicle-associated epithelium and M cells overlie the Peyer's patches (20).

Colonization of the gut

In the mother womb, the fetus is in a sterile environment. The transition from this sterile environment to one that is rich in microorganisms starts during delivery (26) (27). During and after birth, the baby is colonized by different microbes, mostly bacteria, on the skin, in the gut and at mucosal surfaces. The early infant microbiota composition exhibits high dynamics, instability and low diversity. In general, the members of the gut bacteria can be categorized as allochthonous bacteria or autochthonous bacteria. Allochthonous bacteria reside transiently in the gut, while autochthonous bacteria are indigenous residents of the gut (28). The early composition is dominated by facultative anaerobes. Following consumption of the oxygen in the gut by facultative anaerobes, the environment becomes more favorable for strict anaerobes to dominate (29). Eventually, there will be more than 100 trillion individual commensal bacteria inhabiting an adult human gut, correlating to more than ten times the total number of cells in the host body. The composition of the bacteria along the gut varies, with the highest number of bacterial colonies residing in the colon. The firmicutes and bacteroidetes phyla dominate the bacterial groups present in the human intestine, while proteobacteria, actinobacteria, fusobacteria and verrucomicrobia are also common in human distal gut as minor constituents.

Although the composition and temporal patterns of the microbial communities vary widely among individual babies, particular genera of microbes like proteobacteria (eg. *Escherichia coli*) and actinobacteria (eg. *Bifidobacterium* (B.) species) tend to predominate (26).

The composition of the microbiota at early age can be influenced by different factors. Four factors with significant influence on gut colonization are diet, mode of delivery, hygienic condition and antibiotics use.

1. <u>Mode of delivery</u>

   The mode of delivery determines the composition of the early postnatal intestinal microbiota. The early gut microbiota composition of infants born with vaginal delivery resembles that of the maternal vaginal or gut microbiota, while those born with Caesarean section acquire skin or environmental bacteria at early age. Infants delivered vaginally harbor mainly *Lactobacillus*, *Prevotella*, or *Sneathia* spp., while infants delivered by Caesarean section are dominated by *Staphylococcus*, *Corynebacterium*, and

Propionibacterium spp (27). A recent study has also shown that Cesarean section delivered infants lack *Bacteroidetes* in their gut and the colonization with these bacteria were delayed for at least one year in some of the infants. In addition, Caesarean section delivered infants had lower total microbial diversity, compared to vaginally delivered infants (30).

2. Diet

The main benefit that the microbe gains from the host is nutrients. Members of the microbiota have their specificity in nutritional requirement that could determine their survival in the niche. Thus diet has a role in determining the composition of the microbiota in the host. At early age of the infant, the effect of diet in influencing the composition is much more prominent. Breast milk is one source of bacteria for the infant gut, including staphylococci, streptococci, and lactic acid bacteria (31) (32). Moreover, the content of the breast milk provides the appropriate nutrients for commensal bacteria to sustain in the gut (33). For instance oligosaccharides from breast milk favor the growth of *Bifidobacterium* species. Later with weaning, a more diverse microbiota is obtained, which is relatively established throughout life and more related to choice of diet (34). For example, the composition of the intestinal microbiota of children in rural Africa was dominated by bacteroidetes over firmicutes compared to age matched Italian children. The African children consumed plant-based diet, which is high in cellulose and xylans. Members of the bacteroidetes are known to digest these fibers and generate certain metabolites (such as SCFA), which are essential for gut homeostasis (35). On the other hand, in fecal samples from mice fed with high fat and high carbohydrate (westernized diet) firmicutes was more abundant than bacteroidetes. Firmicutes is better in energy consumption than bacteroidetes (36). In addition, fatty acids from dietary fats can induce inflammatory response, which can indirectly shape the microbial community (37).

3. Hygiene standard

The composition of the early gut microbiota can be affected by hygienic standards. Infants born in less affluent countries encounter enormous load of bacteria starting from birth that could shape the pattern of colonization in the gut. A previous study has shown that Pakistani infants harbor a more diverse microbiota at early age compared to Swedish infants (38). In addition, a comparison between genetically close populations in two different countries, such as Estonia and Sweden revealed that Estonian infants were highly

colonized with lactobacilli, while Swedish infants were mainly colonized with *C. difficile* (39). Moreover, exposure to pets, number of siblings and being raised in a rural area could influence the hygienic condition, which could further affect the microbial composition in the early gut (40) (41).

4. Antibiotics

Administration of antibiotics to treat infection could also clear out the indigenous commensal bacteria. In addition, the persistent use of antibiotics will led to the rise of antibiotic resistant bacteria, paving way for the dominance of pathobionts (potential pathogens) in the gut (42). Previous studies using cultivation-based analysis of the fecal samples have shown that antibiotic treatment resulted in a decrease in the number of clostridia and bifidobacteria and increases in the enterococci that persisted for four weeks post treatment. With a much more elaborate technique using microbial DNA analysis, Jakobsson et al has confirmed similar pattern in the relative abundance of these bacteria after treatment (43). In addition, alteration of the intestinal microbiota of mice by antibiotic treatment altered the anatomy of the gut, including large caecal (caecal enlargement), intestinal hyperplasia and altered villus length and width (44).

Methods for studying microbial composition in the gut

Most of our standing knowledge of the composition and function of the microbiota associated with humans are derived from cultivating microbial populations in the laboratory. The drawback of this technique is that, due to selective growth requirement, the majority of the gut microbes resist cultivation in the laboratory. However, recent use of culture independent approaches has helped enormously to characterize microbial diversity. The most commonly used culture independent method is the use of 16S rRNA gene, which is highly conserved among bacterial species. 16S rRNA genes contain conserved and variable regions that enable the identification of bacteria up to species level. The technique involves extraction of DNA from samples, amplification of 16S rRNA gene using primers, followed by sequencing to reveal bacterial identity (45). Sequencing of the 16S rRNA gene for identification of bacterial species present in the gut is making way for new high-throughput sequencing methods that allows sequencing of all genes present in the population. These techniques have enabled scientists in the field to visualize the diversity of the microbial gene and the organisms in the gut (44). Understanding the composition of the microbial community alone does not necessarily give a full picture of its function. Using high throughput metagenomics, it is

possible to sequence the total microbial community DNA, and even match the sequences to known functional genes. But unless it is supported by a proteomic or metabolic study, evidence on functional capabilities from metagenomic studies will remain to be prediction. Therefore future metagenomics studies should encompass protein and metabolite profiling (46).

Microbiota-host interactions

In the interlinked mutualistic relationship of the microbiota and the host, the microbiota contribute to the digestion and fermentation of indigestible carbohydrates, production of vitamins, organogenesis and protection against pathogens. The host will provide niches and nutrients, which are essential for the survival of the microbes. In addition, commensal microbes are believed to contribute to the maturation and regulation of the host immune system (5).
The relationship between the microbiota and the host is undoubtedly complex; involving the interaction among individual members of the microbiota, the epithelium and the mucosal and systemic immune system.

Microbiota-epithelial cell interaction

Commensal microbes have various mechanisms to influence the function and development of IEC. It is already mentioned above that the commensal microbes are involved in digestion of complex polysaccharides that cannot be digested by the host. The resulting metabolites from these complex molecules aid in the maintenance of intestinal homeostasis. For instance, butyrate, a short chain fatty acid metabolite derived from commensal microbes, enhances the integrity of intestinal epithelium barrier, by facilitating rapid repair and tight junction assembly (47). In order to keep the immune responses subdue, it is important to reduce unwanted activation of the immune cells. That is why keeping the intestinal barrier intact is important to prevent foreign antigen from interacting with the underlying immune cells. This can be achieved by facilitating a repair mechanism and also through induction of antimicrobial factors or mucous production that could protect epithelial layer from damage (21).

To avoid inappropriate response to gut microbes, the intestinal epithelium down regulates the expression of TLR2 and TLR4 by the epithelial cells (20). In concordance with dampening of TLR signaling, during the postnatal period, epithelial cells increase the expression of the NFκB inhibitor IκBα, to prevent the expression of pro-inflammatory cytokines regulated by NFκB (transcription factor)(21). Commensal bacteria have acquired mechanisms to interact with the epithelial cells without setting an alarm. *Bacteroides thetaiotaomicron* attenuate CXCL-8/IL-8 and TNF production by epithelial cells by inducing the expression of peroxisome-proliferation-activated receptor γ (PPARγ) that transport NFκB to

the cytoplasm (48). Introducing LPS from gram-negative bacteria to pups leads to an upregulated expression of microRNA-146a (miR-146a) by intestinal epithelium. The increased expression of microRNA-146a contributes for innate immune tolerance by inhibiting TLR signaling through its ability to suppress the translation of TLR signaling molecule IL1-associated kinase 1 (IRAK1) (49). Further, an *in vitro* study has shown that G+ and G– commensals induced TGF-β1 and thymic stromal lymphoietin (TSLP) production by IEC, which subsequently generated a tolerogenic DC phenotype (50).

Epithelial cell-regulation of immune cell function

IEC are the first cells to encounter the microbiota. Upon interaction with the microbiota, IEC can tone the function of the underlying immune cells. There are cells, such as DC and IELs that are distributed within the epithelial layer. The recruitment, maturation and function of these cells are regulated by the IEC. IEC produced retinoic acid and TGF-β modulate subsets of DC to induce Treg cells (51). Since DC are in close proximity with IEC, the IEC lead the major role in modulating the DC function. *In vitro* investigation showed that TSLP and TGF-β from either primary IEC or from polarized IEC-line (Caco2) modulated the immature human monocyte-derived DC to a tolerogenic phenotype (52). IEC-conditioned DC released less IL-12, thus losing its ability to polarize Th1 responses against bacteria. In concordance, patients with Crohns' disease have IEC that appear to be TSLP negative, which could be attributed to the pro-inflammatory profile of the DC. This shows that the surrounding micromileu can influence the function of DC to preserve gut homeostasis. In addition, IEC constitutively produce CCL-25, a chemokine important for recruiting IEL that has gut homing receptors (CCR9+) (25) (53).

Presence of poly Ig receptors on the surface of IEC enables them to shuttle antibodies, such as IgA to the lumen. Secretory IgA minimize excessive inflammation in the gut induced by gut microbes by facilitating the clearance of microbes via the fecal stream. In addition, activated IEC secreted B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) can influence B cells to undergo IgA class-switching in the gut (54)(55).

As already mentioned above, IEC express MHC molecules to present processed antigen. However, IEC lack the co-stimulatory molecules for MHC-TCR complex (56). Antigen presentation by IEC in the absence of co-stimulation may promote anergy, which might aid in local and systemic T cell tolerance. Alternatively, memory T cells are less in need for co-stimulatory molecules, thus the ability of IEC to present antigen may be more important for memory T cell function.

Microbiota-immune system interaction

The microbiota plays a multifactorial role in influencing structural and functional development of the immune system in the gut. Experimental setups using animal models have enabled us to investigate the interaction of microbes and the host immune system, in detail. Study with germ-free (GF) mice revealed that the gut microbiota is required for the normal generation and/or maturation of GALT (57) (58). Although the PP in GF mice is smaller in size than those of specific-pathogen-free (SPF) mice, functionality and maturation of PP are not affected by the presence or absence of gut microbiota. PP develop before birth, but their size and the development of germinal center require postnatal microbial colonization of the gut (59). Unlike PP, the maturation of ILF and CP requires stimulation by the gut microbiota. Specifically, the development of ILF is impaired in mice lacking pattern recognition receptors (PRRs) such as, TLR and nod like receptors (NLR) (60).

Effects of microbiota on mucosal immune function

The benefits of microbiota have been indicated in mucosal immune development since the absence of microbial signal in GF mice affected both the innate and adaptive arm of the mucosal immune system. GF mice have a reduced number of local resident DC and macrophages. In addition, GF mice have decreased numbers of Treg in the LP, indicating the involvement of gut microbiota in the generation of Treg in the gut. Treg are needed to diminish excessive pro-inflammatory response in the gut (61) (62). Short chain fatty acids derived from commensal microbes, such as butyrate mitigate pro-inflammatory responses by inducing regulatory cytokines production, such as IL-10 (63). The numbers of IgA secreting plasma cells are also affected in GF mice, owing to the reduced amount of IgA (64).

Colonization of GF mice with a single species of bacterium is enough to restore the mucosal immune system, including increased numbers of intraepithelial lymphocytes and to modulate the activity of local APC. For instance, introducing *B. infantis* to mice was enough to induce CD4+ T cells differentiation to FOXP3+ Treg cells (65). In addition, oral inoculation of mice with a defined mixture of Clostridium strains initiated the differentiation of Treg cells in the colon (61) (66). Further, administration of polysaccharide A, a surface structure of *Bacteroides fragilis,* was enough to induce functional Treg cells proliferation (67). Segmented filamentous bacteria (SFB) on the other hand, reside in the distal ileum of mice and promote Th17 cells differentiation (68). SFB interaction with the host epithelium induces serum amyloid A protein (SAA), which indirectly facilitate Th17 cell development, by enhancing IL-6 and IL-23 production by LP dendritic cells (69). In addition, ATP secreted from commensal bacteria endorses Th17 cell generation in the gut (70).

As already mentioned above, DC are present throughout the GALT, including the LP and PP. Most mucosal resident DC are immature and less immunogenic. They express low MHC molecules and co-stimulatory molecules. Moreover DC subsets in the gut can sample antigen directly from the lumen (71). This unique function is achieved by the expression of the fractalkine receptor (CX3CR1). The interaction with bacteria or bacterial products triggers the functional maturation of DC that enables the DC to modulate the surrounding immune system by secreting cytokines and through antigen presentation. Interaction of MAMP with PRR on DC leads to high levels of MHC, co-stimulatory molecules and cytokines needed for antigen presentation and T cell activation. Mucosal DC promote T cell priming and induce B cells to secrete immunoglobulin A (IgA) through T cell dependent and independent mechanisms.

Effects of microbiota on systemic immune function

The influence of the microbes is not only limited to mucosal immunity. It extends its effect on the systemic immune system. It has been shown that NOD1 deficient mice have reduced systemic neutrophil killing capacity. This was attributed to lack of microbial signal, as the effect was the same for GF and antibiotic treated mice (72). In addition, higher intensity of *Bacteroides (B) fragilis* at early age were found to inversely related to TLR4 mRNA expression in PBMC from 12 month infants. As a consequence, LPS induced CXCL-8/IL-8 and IL-17 levels were also negatively correlated with *B. fragilis* colonization for a week after birth (73). This could indicate an influence of bacterial colonization in the induction of tolerance. Further, LPS has been shown to induce mild inflammation that could lead to insulin resistant Type 1 diabetes, whose etiology are linked to alteration of microbial composition at early age (74) (5). Interestingly, gut microbe depletion with antibiotics treatment of mice lead to the expansion of basophils in the peripheral blood and an increase in serum IgE levels (75). Thus, as a consequence, the allergic airway inflammation that was triggered by exposure to house dust mite allergen were worsened. In addition, upon direct interaction with B cells, the microbiota can activate MyD88 signalling to restrict IgE class-switching in B cells, that could be crucial in preventing allergic inflammation (75).

Gut microbiota in health and disease

In the past 30- 40 years, the incidence and prevalence of chronic inflammatory diseases have increased markedly in affluent countries, in particular inflammatory disorders, which are associated with the mucosa of the airway and the gut, such as asthma and inflammatory bowel diseases (IBD) (76) (5). In part, this can be explained by the hygiene hypothesis-a standard of living related changes, such as: modern hygiene, dietary and medical practices contribute to the

alteration of the composition of the gut microbiota at early age. Absence of key commensal bacterial populations during this window, along with genetic and epigenetic factors, may deprive signals important for proper immune system development and function, which leads to disease susceptibility. The association of the change of the gut flora composition to the rise of both Th1 dependent autoimmunity and Th2 driven allergy indicate that the effect is not solely on the imbalance between Th1 and Th2 response, but rather on lack of induction of a regulatory response.

Role of gut microbiota in allergy development

The immune system of newborn infant is inexperienced and immature. The neonatal immune system is Th2 skewed, which is also evident in GF mice. The immune pathology of atopic diseases is characterized by Th2-driven inflammatory responses against environmental or dietary allergens (77). A pioneer study in Sweden and Estonia has shown that allergic children had a distinct population of commensal bacteria at early age in comparison to those non-allergic children from either region (78). The primary notion of this study is that the microbiota composition is the underlying factor in the development of allergic diseases, in spite of other environmental variations that exists in these two countries. Similarly other epidemiological studies have also shown that certain strains of bacteria; namely *Clostridium* (C.) *difficile* and *Staphylococcus aureus* are mainly dominant in children that later develop allergy (79) (80). On the other hand, children that develop allergy tend to have lower levels of lactic acid producing bacteria (LAB) and enterococci in their stools, than infants not developing allergy (81) (82). In addition, our group has shown that early colonization with lactobacilli reduces the risk for allergy development later in life, irrespective of allergic heredity (83).

Further, investigation on children from anthroposophic schools in New Zealand aged 5–10, showed that recurrent uses of antibiotics during the first year of life, contributed to the development of atopic disease later in childhood. Interestingly, the microbiota of those children that were not treated with antibiotics contained higher levels of lactic acid bacteria than those that were treated (84). In animal models, pups treated with antibiotics tend to have a Th2 biased immune system and elevated levels of food allergen-specific IgE and IgG1 (44). The mechanisms behind the associations are not fully elucidated but these studies show the importance of specific microbial signals for proper immune development and response.

Atopic allergy and its underlying complexity

Understanding the immunological pathways that lead to an allergic response is important for developing effective treatment and prevention. Although they play major role,

Th2 cells are not the sole factors responsible for the development of IgE-dependent allergic inflammation. For instance, since the entrance of the allergen into the body is via the mucosal surface or the skin, breaching of these barriers contributes to the development of allergic reactions. Noteworthy, there is increased gut epithelial barrier permeability in both pediatric and adult allergic patients (77). Further, epithelial cells secreted TSLP modulate APC, such as DC to favor a Th2 response (85). At steady state, TSLP is expressed in the lung and intestine, and contributes to the control of a Th1 response at these sites. Overexpression of TSLP at local sites has been linked to the development of allergic diseases, such as airway hyper-responsiveness (AHR) and atopic dermatitis (86). Once the allergen enters the body, the professional APC take up the allergen to process and present it as peptides on MHC class II molecules to naïve Th cells. This leads to the differentiation of naïve Th cells to Th2 cells and production of cytokines; such as IL-3, IL-4, IL-5, IL-13 and GM-CSF upon activation. All these cytokines, together or alone, contribute to the development of allergy, either by promoting IgE class-switching in B cells (IL-4 and IL-13), recruiting mast cells (IL-4, IL-9 and IL-13) or being involved in the maturation of eosinophils (IL-3, IL-5 and GMCSF) and basophils (IL-3 and IL-4) (87).

For long, it was believed that shifting the balance to a Th1 phenotype could serve as a protection. But the process is proving far more complex. For instance, the rise of both Th2-driven allergic disease and Th1-driven autoimmune diseases, to epidemic levels in developed countries, can serve as indication that allergic development is not solely due to the imbalance between Th2 and Th1 responses (88). Although known for its suppression of Th2 response, IFN-γ released by Th1 cells is involved in the pathology of atopic dermatitis by causing damage to the keratinocytes (89). In addition, in mice, the augmentation of IFN-γ production in the presence of a Th2 cell response worsened allergic inflammation. This was caused by IFN-γ-induced damage of the epithelial barrier, leading to easy penetration of the allergen (90). Another subset of Th cells that are recently linked to allergy is Th17 cells. As it has already been mentioned, Th17 cells produce IL-17A. In the asthmatic airway, IL-17A is elevated. Normally, IL-17A is responsible for the induction of CXCL-8/IL-8 production by epithelial cells. At steady state CXCL-8/IL-8 promote epithelial cell proliferation and repair, but overexpression of CXCL-8/IL-8 leads to recruitment of neutrophils that result in neutrophilic-airway-inflammation (91). On the other hand, Treg plays pivotal role in suppressing allergic responses. As described above, Treg cells suppress inflammatory responses either by secreting TGF-β and IL-10 or by cell-cell contact inhibition via expression of CTLA-4 (11).

Lactobacilli and *S. aureus* in the gut

Lactobacilli, member of the phylum firmicutes, are detected in variable amounts ranging from 10^5 to 10^8 CFU/g in infants faeces. Among the species, *L. salivarius, L. rhamnosus* and *L. paracasei* are the dominant ones (92). Lactobacilli in general play an important role inhibiting the growth of a wide spectrum of pathogenic bacteria by competing for adhesion and nutrients, and through the production of antimicrobial compounds, such as bacteriocins, organic acids, or hydrogen peroxide. Lactobacilli contribute to keeping intestinal barrier integrity (93) (94) (95) and co-culturing different *Lactobacillus* strains with pathogenic bacteria has been shown to prevent pathogen-induced reduction in trans-epithelial resistance across epithelial monolayers (92).

S. aureus is commonly found on the skin, but recently there has been an increased rate of isolation of *S. aureus* from western infants' stools (96). It was detected in the stool samples of more than 80% of the infants at any time during their first year of life. *S. aureus* is not a passive resident of the gut. Early intestinal colonization with *S. aureus* is associated with an increase in the level of circulating soluble CD14 (sCD14) in infants. sCD14 is a co-receptor for both TLR2 and TLR4. This could pertain to the influence of *S. aureus* colonization in the development of the immune system and subsequent allergy development (97). Staphylococci can also induce inflammation. *In vitro* stimulation of monocytes with toxins from staphylococci induced IL-17 production by T cells (98). Its toxins are capable of functioning as super antigens activating large number of non-specific T lymphocytes in the gut (99). A previous *in vitro* study has shown that a group of live LAB inhibited staphylococcus enterotoxin A (SEA) induced secretion of Th2-cytokines (IL-4 and IL-5) (100).

The knowledge of the mechanisms of how lactobacilli modulate the immune response is still at an early stage. A joint work of *in vitro* and *in vivo* experiments needs to be performed to understand microbe-microbe or microbe-host interactions and how to manipulate it for therapeutic benefit.

In vitro studies reveal the ability of *Lactobacillus* strains to induce a regulatory cytokine profile, evident by a high ratio of IL-10/IL-12 production by immune cells. In addition, these strains were able to attenuate chronic inflammation in an animal model (101).

Oral administrations of lactobacilli influence the production of cytokines such as TGF-β and TSLP, which shape the phenotype of DC in the LP. In addition, a *Lactobacillus strain* can ameliorate systemic anaphylaxis in a food allergy model in animal by suppressing serum IgE and IgG1 responses (102).

Oral treatment of rat pups with *L. reuteri* DSM 17938 increased the frequency of Foxp3+ Treg Cells in the intestine and the mesenteric lymph node, resulting in ablation of the induced inflammatory status (103). Moreover, oral administration of a mixture of bifidobacteria, lactobacilli, and *Streptococcus salivarius* to mice enhanced the percentage of Treg cells (104). Since these probiotic strains are transient bacteria (allochothonous), their mode of influence needs to be fully elucidated. They could be important at early age in educating the immune system directly or in shaping the microbial ecology within the gut that could indirectly influence immune homeostasis.

The impact of probiotics and prebiotics on the development of allergy

Probiotics are defined as live microorganisms that have beneficial effects on health. Supplementation of probiotics looks promising in prevention or treatment of diarrhea, IBS, necrotizing enterocolitis (NEC) and certain bacterial infection (105). The precise mechanisms on how probiotics provide protection against the development of allergy have not yet been clearly understood. But collectively, probiotics could contribute to increased intestinal barrier integrity, enhance gut-specific IgA responses, and enhance TGF-β and IL-10 production by Treg cells (106). In addition, probiotic bacteria might reduce the risk of allergic disease development by the degradation of luminal antigen that might have immunomodulatory effect. For instance, an *in vitro* study has shown that enzyme derived from *Lactobacillus GG* hydrolyze cow's milk casein, which lead to reduction in lymphocyte proliferation and Th2 cytokine production (107). Furthermore, the immunomuodulatory effects of probiotics have been shown in a study where supplementation with *L. rhamnosus* species as probiotics were given to the mother from 35 weeks of gestation and then to the baby for two years, reduced the risk of eczema (108). Oral supplementation with *L. rhamnosus* GG and *B. lactis* Bb-12 to infants at the time of weaning increased mucosal cow's milk –specific IgA production (109). This might contribute to the clearance of antigen, thus favoring the formation of tolerance. Further, the probiotic supplements extend their effect by modifying the innate immunity as observed by augmented concentration of sCD14 in the serum. Interaction of microbial products from these probiotics with TLR2 and CD14 might be the causative agent for increment in TGF-β levels that further influenced the IgA production.

In contention there are other reports showing no effect of probiotic in reducing allergic development (110) (111). The efficacy of probiotics in neonates and infants is dependent on different factors: the probiotic strain used, the doses, the age at which probiotic was supplemented and the duration (112).

Prebiotics are indigestible nutrients that specifically favor the growth of resident bacterial species in the gut. For instance, oral administration of prebiotics such as inulin and fructo-oligosaccharides has been shown to facilitate the growth of bifidobacteria and lactobacilli (113). In another study, administration of a mixture of four *Lactobacillus* strains with prebiotic oligosaccharides mitigated the risk for eczema by age 2 without extended effect by age 5 (114). Despite this, there are still contentions in the use of prebiotic as a treatment, since oral administration of these substrates could favor the growth of unwanted microbes that could induce pro-inflammatory response.

PRESENT STUDY

General aim:

- To investigate the influence of commensal bacteria on immune responsiveness during infancy

Specific aims:

- To study the association of early-life gut colonization to cytokine responses at two years of age. (paper I).
- To investigate how different species of bacteria influence the immune response of gut epithelial cells and peripheral immune cells *in vitro* (paper II).

Material and Methods summary

Detailed description of the material and methods are found in the specified sections of paper I and II, respectively. In paper I, fecal samples from infants (n=30) during the first two months of life were analyzed. DNA from these fecal samples was extracted and amplified using primers for *Bifidobacterium (B.) adolescentis*, *B. breve*, *B. bifidum*, a group of lactobacilli (*L. casei*, *L. paracasei* and *L. rhamnosus*) and *Staphylococcus (S.) aureus* and quantified using real time PCR. In conjunction, peripheral blood mononuclear cells (PBMC) isolated from these children at two years of age were stimulated with phytohaemagglutinin (PHA) to measure the number of IL-4−, IL-10− and IFN-γ secreting cells using ELISpot.

In paper II, we used seven *Lactobacillus (L.)* strains (*L. reuteri* DSM 17938, *L. reuteri* ATCC PTA 4659, *L. rhamnosus* kx151A1, *L. rhamnosus* GG, *L. casei* LMG 6904, *L. casei* Shirota, *L. paracasei* F19) and three *Staphylococcus (S.) aureus* strains (*S. aureus* 139:3, *S. aureus* 151:2 *and S. aureus* 161:2), which were grown in their respective growth media (MRS broth and BHI broth). Intestinal epithelial cell lines (IEC) (HT29 and SW480) were exposed to *Lactobacillus (L.)* strains-sn and *S. aureus* strains-sn. In addition, IEC were stimulated with suspension of UV-killed bacteria (*L. reuteri* DSM 17938 and/or *S. aureus* 161:2) with or without the respective bacteria-sn. We investigated the TLR signal involvement in the IEC response to *S. aureus* by using siRNA to silence the MyD88 gene in the IEC. Further, PBMC and cord blood mononuclear cells (CBMC) from healthy donors were stimulated directly with bacteria-sn or with bacteria conditioned IEC-sn. The level of cytokines and chemokines in the IEC and PBMC/CBMC-sn were analyzed using human proteomic array (36 cytokines) and ELISA.

PBMC were also stimulated with bacterial supernatants *in vitro* and intracellular staining of T cells for IL-4 and IFN-γ (paper I) and IL-10, IFN-γ and IL-17 (paper II) was performed and analyzed by flow cytometry. In addition, $CD25^{high}CD127^{low}FoxP3^{+}$ cells were analyzed for intracellular IL-10 and CTLA-4 and considered as Treg cells (paper II). 7AAD-binding (BD Via-Probe 7) (paper I) or the LIVE/DEAD Fixable Dead Cell Stain Kit-Aqua (Invitrogen) was used to investigate the viability of the cells (paper I) (paper II).

The role of histamine in lactobacilli-mediated immune modulation was also investigated in paper II. Histamine levels were measured in bacteria-sn. Further, PBMC were pre-incubated with ranitidine (the histamine receptor blocking agent) before stimulating the cells with the bacteria-sn. To evaluate if the *Lactobacillus*-sn could degrade cytokines, rIL-17

and rIFN-γ were pre-incubated with *L. reuteri* DSM 17938. The respective proteins levels were then measured with ELISA.

Results and discussions summary

Paper I:

The mutual relationship between commensal bacteria and intestinal epithelial cells plays an important role in the development and maintenance of gut homeostasis (21). The commensal bacteria contribute to the metabolic activity (115), are involved in the development of an intact intestinal architecture (116) and also serve as a first line of defense by competing with pathogenic bacteria (117). In addition, a lot of data support a significant role for commensal microbes in shaping immune system development and responses. Besides a defect in immune development and responses in GF mice, clinical and epidemiological studies have shown that alteration of early colonization associates with the risk for the development of immune-mediated diseases, such as allergy (77). Recent work done by our group has shown that early colonization with lactobacilli is associated with a reduced allergy risk at five years of age. We further observed that early lactobacilli colonization seemed to protect against allergy development: In a group of children with double allergic heredity (both parents allergic) we saw that those children who were early colonized with lactobacilli did not develop allergy, while those who were not early colonized with lactobacilli did develop allergy (83). The immunological mechanisms behind the above associations are still an enigma. However, we had data pointing towards that the early-life microbiota associated with both systemic and mucosal immunity during childhood in a species-specific manner (73). Allergic disorders are strongly associated with an altered immune profile, with Th2 dominance and aberrant Treg capacity. Therefore, we set out to investigate this further and in paper I, our main aim was to know whether early-life colonization with lactobacilli, bifidobacteria and *S. aureus* could influence the shaping of T cell-associated immune responses during childhood. Thus we investigated the association of early-life gut colonization to cytokine responses at two years of age by examining the number of IL-4, IL-10 and IFN-γ secreting cells following a general PHA stimulation. Early colonization with lactobacilli was inversely associated with the numbers of IL-4, IL-10 and IFN-γ producing cells at two years of age, while the opposite was seen when the children were grouped based on *S. aureus* colonization. Similar trends could be seen for relative amounts of both lactobacilli (for IL-4) and *S. aureus* (for IL-10). For bifidobacteria, colonization or relative amounts did not associate with cytokine-producing cell numbers.

Due to the observed variation of early colonization with lactobacilli and *S. aureus* in their association to allergy development as well as to the number of cytokine-secreting cells at two years of age, we wanted to see how co-colonization with lactobacilli and *S. aureus* at an early age associated with the numbers of IL-4, IL-10 and IFN-γ secreting cells in comparison to one ore none of the bacterial species. Interestingly, the presence of *S. aureus* in the absence of lactobacilli was associated with significantly more IL-4 and IFN-γ producing cells. Noteworthy, in children with the absence of both species at early age, the numbers of cytokine-secreting cells were lower in a similar manner to children colonized with lactobacilli. This might indicate the sole perpetration of *S. aureus* in triggering an increase in number of cytokine-producing cells, but that the presence of lactobacilli can divert its effect. Thus, given the observed opposite effect of *S. aureus* and lactobacilli colonization, we analyzed both secretion and intracellular production of IL-4 and IFN-γ after stimulating PBMC with *L. rhamnosus* GG and *S. aureus* 161:2 to investigate the immunostimulatory effect of these bacteria *in vitro.* In addition, IL-10 levels were measured in the PBMC-sn post stimulation. *S. aureus* 161.2-sn alone induced a higher percentage of IFN-γ and tended to increase the amount of IL-4 producing CD4+ T helper cells compared to *L. rhamnosus* GG-sn alone. Interestingly, co-stimulation of PBMC with *S. aureus* 161:2 and *L. rhamnosus* GG resulted in a down regulation of these responses. Similarly, the levels of IFN-γ were higher in *S. aureus* conditioned PBMC-sn. On the contrary, *L. rhamnosus* GG-sn induced higher IL-10 production by PBMC. IL-4 levels were undetectable or very low in the PBMC-sn. The *in vitro* PBMC stimulation experiment confirms the ability of lactobacilli in modulating a *S. aureus* induced response. The varying outcome on IL-10 responses between the experimental setup could be attributed to the fact that *S. aureus-sn* might induce other cells among the PBMC such as monocytes to produce IL-10, while PHA is potent in promoting a T cell cytokine response.

In paper I, we managed to show that colonization with gut microbiota at early age associates with cytokine response in infancy. In addition, different species can alter cytokine response differently and counteract each other to keep the immune response at bay.

Paper II:

To have a deeper understanding on the mechanism of how early co-colonization with lactobacilli and *S. aureus* affect the host immune system, *in vitro* cell stimulation experiments were important. In addition, since IEC interact with bacterial component and have immunomodulatory capacity, we were also interested in the response of IEC toward these bacteria and how this further influenced the response of PBMC/CBMC. We first screened the

response of IEC upon stimulation with *S. aureus* 161:2-sn and *L. reuteri* DSM 17938-sn using human cytokine proteome array (including 36 different cytokines and chemokines). Both IEC-lines (HT29 and SW480) produced a restricted pattern of factors upon stimulation, but only *S. aureus* induced the production of the pro-inflammatory chemokines CXCL-8/IL-8 and CXCL1/GROα above background levels. We also investigated the levels of TSLP, APRIL and TGF-β1 in IEC-sn with ELISA as these factors are suggested to be produced by IEC and have immune modulatory functions, but none of these cytokines were produced by IEC upon stimulation with any of the bacteria-sn tested. To confirm the finding that *S. aureus*-sn but not *Lactobacillus*-sn induces a pro-inflammatory response in IEC, we stimulated HT-29 with seven different strains of *Lactobacillus and* 3 strains of *S. aureus*, and measured the production of CXCL-8/IL-8 by ELISA. Only, *S. aureus* 161:2 induced CXCL-8/IL-8 production by IEC.

To analyze the involvement of surface structures of the bacteria in influencing the pro-inflammatory response of the IEC, we compared the response of IEC toward UV-killed bacteria, bacteria-sn or a combination of both. The result showed that the *S. aureus*-induced IEC response was mediated by secreted bacteria products and not by the UV-killed bacteria. In addition, a combination of both UV-killed bacteria and the bacteria-sn showed no additive effect compared to the effect of the supernatant alone. Microbial recognition by TLRs on IEC contributes to the maintenance of intestinal barrier and induction of cytokine production (118) (20). Our experiments showed that MyD88-silencing partially dampened the *S. aureus* induced IEC response. This indicates that *S. aureus-sn* induce CXCL-8/IL-8 production by IEC via TLR-signaling.

To elucidate how different bacteria-sn influence cytokine responses by immune cells, and whether IEC-secreted factors could influence these responses, PBMC from healthy donors were stimulated with *L. reuteri* DSM 17938-sn and *S. aureus* 161:2-sn directly or with supernatants from HT-29 cultures exposed to the same bacteria. In both conditions, *Lactobacillus* and *S. aureus strains* were able to induce a wide range of cytokines, but only *S. aureus* induced the T-cell associated cytokines IL-2, IL-17 and IFN-γ. The inclusion of IEC-derived factors did not alter the response of PBMC toward bacteria-sn. Lack of IEC-line influence on the PBMC response and its (IEC-line) hypo-responsiveness to bacterial stimulation in our study could be attributed to the limitations of using IEC-lines and not primary cells.

Both *S. aureus* 161:2 and the lactobacilli were able to induce IL-6 production by PBMC, as expected. However, only *S. aureus* induced IL-17 (*S. aureus* 161:2 and 139:3), IFN-γ, IL-2 and TNF-α production (*S. aureus* 161:2), while the lactobacilli induced none or low

levels of these cytokines. The ability of *S. aureus* to induce cytokine production by immune cells could be attributed to its toxins acting as superantigens and causing a non-specific T-cell activation by cross-linking of the TCR. But only *S. aureus* 161:2, out of the two toxin containing strains was effective in inducing strong T cell associated responses. This might indicate that other mechanisms of activation are involved. By linking the ability of *S. aureus* to produce extracellular ATP (119) with the potential of ATP to induce Th17 in the gut (70), we investigated the contribution of ATP in *S. aureus* induction of IL-17. However, our observation refutes the hypothesis of the involvement of ATP in inducing IL-17 in our experimental setup (unpublished observation). Further, lipoprotein from *S. aureus* has been shown to induce T cell activation indirectly by activating DC via TLR2 signaling (120). This could be interesting to investigate in the future.

In paper I, we observed that early co-colonization with lactobacilli and *S. aureus* strains are linked to immune regulation. Thus, here in paper II we stimulated the cells with a combination of *S. aureus* 161:2-sn and the different *Lactobacillus*-sn. The *S. aureus* induced IL-17, IFN-γ, IL-2 and TNF-α production by PBMC were significantly down regulated by a simultaneous exposure to *Lactobacillus*-secreted factors. Similarly *S. aureus* induced IFN-γ response by CBMC was reduced by the tested lactobacilli. However, none of the *Lactobacillus*-sn altered *S. aureus* 161:2 induced CXCL-8/IL-8 production by IEC-line.

Flow cytometry analysis of intracellular staining of T cells revealed that IFN-γ and IL-17 production following *S. aureus* stimulation was attributed to CD4+Tcells and simultaneous stimulation with *L. reuteri* DSM 17938-sn and *S. aureus* 161:2-sn decreased the percentage of IFN-γ secreting cells. In addition, *S. aureus* affected the Treg cell population by up regulating CTLA-4 and inducing the production of IL-10.

Understanding the mechanism of how lactobacilli modulate the *S. aureus* induced immune response was at the heart of our study. Regulated production of IL-17 and IFN-γ in the gut is important for lymphocyte homeostasis and epithelial cell repair (121). On the contrary uncontrolled production of IL-17 and IFN-γ could be detrimental, resulting in excessive inflammation (122). Lactobacilli contribute to reduction of intestinal inflammation either by competing against the expansion of pathobionts such as *S. aureus* (123) or modulating the immune response induced by toxins from *S. aureus* (100). Previous work has shown that histamine derived from a *Lactobacillus* strain down-regulated inflammatory immune responses in human monocytes by binding to H2 receptors (124). We examined histamine production from our different strains of bacteria. However, only one out of our original set of seven lactobacilli (*L. reuteri* ATCC PTA 4659) produced histamine. In addition, blocking H2

receptors and subsequently stimulating PBMC with a combination of *S. aureus* 161:2-sn and *L. reuteri* DSM 17938-sn did not alter lactobacilli regulation of the PBMC response against *S. aureus*. We have also tested the proteolytic ability of lactobacilli-sn, but pre-incubation of recombinant IL-17 and IFN-γ with *Lactobacillus*-sn did not alter their detection by specific ELISA. In addition, T cell viability was not affected by *Lactobacillus*-sn. Although the mechanisms need to be further elucidated, these studies present a possible role for lactobacilli in induction of immune cell regulation.

In paper II, we demonstrated that *S. aureus* can induce a strong pro-inflammatory response by IEC and an excessive T cell associated response. Lactobacilli managed to curb the *S. aureus*-induced Th1/Th17 response, further indicating that lactobacilli can mitigate inflammatory conditions.

In these studies, we primarily used a non-polarized IEC-line obtained from an adult. However, recently we have tested the response of the fetal IEC-line (FHS-74 int) and the polarized adult IEC-line (Caco2) to the bacterial-sn. Interestingly, *S. aureus*-sn was able to stimulate IEC-line regardless of degree of maturity or formation of tight junctions. In addition it is worth mentioning that there is an ongoing work in our laboratory investigating the response of primary murine gut epithelial cells toward bacteria-sn. The use of PBMC is another limitation, in the context of the normal physiology of the gut; the local immune cells residing in the gut are influenced by the surrounding micro-milieu. Thus one can argue that the local resident immune cells might not respond in a similar manner.

General conclusion

In general, these papers shed light on the mechanisms behind gut microbial influence on host immune homeostasis. In the first paper, we demonstrated that colonization with lactobacilli early in life is associated with a lower cytokine response, while *S. aureus* colonization in infancy acted in the opposite way, and early co-colonization with lactobacilli and *S. aureus* associated with reduced cytokine responses. Similarly in paper II, it was the *S. aureus* that induced a strong pro-inflammatory response by IEC and a T cell-associated response by immune cells. Interestingly, as we have seen in paper I, co-colonization with lactobacilli and *S. aureus* associated with a decrease in numbers of cytokine-secreting cells. Likewise, in paper II co-stimulation with *Lactobacillus* strains and *S. aureus* downregulated the Th1/Th17 response by immune cells. This reveals the importance of lactobacilli in modulating the immune response away from excessive inflammation. Today, *S. aureus* is frequently found in infants' gut. However, delayed or altered colonization with lactobacilli could

deprive the immune system with an important signal protecting the host from developing an inflammatory phenotype. In addition, although further investigation are needed to confirm these findings, our results might recommend the use of lactobacilli as a potential therapeutic aid to treat premature infants that experience aggressive inflammation, such as NEC in their gut. Treatment with lactobacilli could ameliorate the aggressive inflammation in these infants.

The gut microbiota is complex and these two species are not the only species that could influence the immune response in infants' gut. However, it supports the notion that a balanced gut microbiota is crucial for proper maturation and function of the immune system.

FUTURE PERSPECTIVES

The current studies laid foundation for future studies to understand the interaction of the microbiota, the epithelium and immune cells in detail.

Identifying the modulatory factors in the supernatants of *Lactobacillus* and *S. aureus*

In the recent work, we were not able to identify the biological factor from lactobacilli and *S. aureus* that modulated the immune response. We are going to implement molecular biology techniques to identify these factors and evaluate their effect *in vitro*.

The effect of *Lactobacillus* and *S. aureus* on the epithelial barrier integrity

Intact gut epithelial barrier is important to prevent unwanted immune response. We will evaluate in detail the effect of the bacteria-sn on the morphology of intestinal epithelial cells and the expression of surface molecules that are involved in the tight junction formation between epithelial cells.

The effect of breast milk in modulating the immune response

Breast milk not only serves as a source of nutrition for infants, it also provides passive immunity at early age. Breast milk contains antimicrobial compounds and antibodies from the mother that could provide protection against infection at early age. In addition, breast milk contains both anti-inflammatory and pro-inflammatory cytokines that can influence the immune response. We have previously shown the potential of breast milk in modulating the immune response to microbial challenge. We will investigate in detail the effect of breast milk on the maturation of immature IEC and its modulatory effect on the response of IEC and immune cells towards bacteria.

***In vivo* assessment on the effect of *S. aureus* and lactobacilli**

In the context of the normal physiology of the gut; the local immune cells residing in the gut are influenced by the surrounding micro-milieu. We are planning to tackle this question by colonizing GF mice with these bacteria (lactobacilli and/or *S. aureus*) and follow the effect on the immune response.

In addition, our group has shown that early colonization with lactobacilli reduces the risk for allergy development later in life, irrespective of allergic heredity (83). We will transfer microbes from our cohorts' (allergic and non-allergic children) fecal sample to GF mice and investigate the immune development and responses in these animals and their offspring.

The interaction of gut microbes with DC

DC in the gut play a major role in orchestrating immune response that could determine the homeostasis in the gut. Our current knowledge about the interaction of microbes and DC are mainly obtained from animal studies. Due to technical difficulties in obtaining gut DC from human, for long people used *in vitro* monocyte derived DC to assess the interaction. But these DC already have a pro-inflammatory phenotype that differ them from the DC in the gut at steady state. However, not so long ago, Rescigno et al were able to obtain tolerogenic DC from monocytes that was able to induce Treg differentiation (52). We will investigate whether the interaction with bacterial factors could influence the phenotype of these DC and subsequently influence the T cell response.

Unconventional T cell function and gut microbiota composition

Since, MAIT cells and γδ T cells are among the first immune cells found in the fetus and are located in the gut mucosa; we are interested in following their development and functionality from birth in relation to gut colonization. We will phenotype them and follow their function at three different time points: at birth, two-years of age and adulthood. We will characterize their function in children with different gut-flora composition at two years of age.

Probiotic treatment of premature-born infants

We have initiated studies on the development of a gut microbiota, gut homeostasis and immune development in very premature infants. Very premature infants are included in a randomized double-blind placebo-controlled study on probiotics. We will analyze the establishment of their gut microbiota by real-time PcR on fecal samples, gut homeostasis and local immune activity in gut biopsies and systemic immune maturation in peripheral blood cells.

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