The immunomodulatory function of staphylococcal superantigen on oral tolerance

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Abstract

Exposure to soluble proteins via the gut gives rise to systemic tolerance, a phenomenon called oral tolerance. Failure of oral tolerance results in allergy, a disease that has increased during the last decades in Western industrialized countries. The cause of this rapid increase is unknown. However, according to the hygiene hypothesis and many epidemiological studies, there is a clear correlation between a hygienic lifestyle and the prevalence of allergy. The hygiene hypothesis is also supported by the results of animal studies. Specifically, germ-free mice have been found to be deficient in the development of oral tolerance and have less functional regulatory T cells. We have observed that Swedish infants have a less diverse gut microbial flora and a slower strain turnover compared to infants in developing countries, suggesting that certain microbes may have particularly strong immunoregulatory potential. Thus, neonatal colonization by *Staphylococcus aureus* (*S. aureus*) in the gut protects against food allergy development. Most *S. aureus* strains can produce one or more toxins with superantigenic function, including staphylococcal enterotoxins (SE) A, B, C, D, and E, as well as toxic shock syndrome toxin-1 (TSST-1). Superantigens are the strongest known T cell stimulants, as they stimulate 5-30% of all T cells in an antigen-independent manner by cross-linking MHC class II molecules on antigen-presenting cells with the Vβ region of the T cell receptor.

The purpose of this thesis was to study the immunomodulatory role of staphylococcal superantigen on oral tolerance in animal models of allergy. Newborn pups were exposed to SEA during the first two weeks of life. Oral tolerance was induced at 6 weeks of age by feeding the mice the model antigen ovalbumin (OVA). Oral tolerization was followed by sensitization and challenge according to an airway allergy model or a food allergy model. Neonatal SEA treatment resulted in enhanced development of oral tolerance, as evidenced by decreased sensitization in both allergy models. Further, when colonizing germ-free mice with superantigen-producing *S. aureus*, improved oral tolerance induction in the food allergy model was observed compared to mice colonized by a non toxin-producing strain. To investigate the long-term effect of SEA on the immune system, immune cells were studied at the time for oral tolerization. We found that mice neonatally treated with SEA had higher proportions of lymphocytes expressing the gut migratory markers chemokine receptor CCR9 and integrin α4β7. This was associated with higher numbers of $FoxP3^+$ regulatory T cells in the small intestinal lamina propria. In addition, neonatal SEA treatment rendered dendritic cells (DCs) more tolerogenic demonstrated by lower expression of co-stimulatory markers, higher expression of MHC class II, and reduced T cell stimulatory properties.

A subpopulation of gut DCs expressing CD103 have been suggested to be important for oral tolerance. This DC subset specifically imprints gut migratory potential on stimulated T cells and can convert naïve T cells into regulatory T cells. The unique properties of the $CD103^+$ DCs depend on their expression of retinal dehydrogenases (RALDHs), enzymes that convert vitamin A to retinoic acid (RA). By interfering with the vitamin A metabolism *in vivo* by giving mice the RALDH inhibitor Citral in their drinking water, the improvement in oral tolerance noted after neonatal SEA treatment was lost. In addition, Citral intake affected gut DCs by lowering the expression of MHC class II, suggesting that high expression of antigens via MHC class II is important for oral tolerance.

In conclusion, neonatal exposure to superantigen or colonization of germ-free mice by superantigen-producing *S. aureus* confers an increased ability for oral tolerance several weeks after treatment. This improvement is likely dependent upon an interaction between gut-residing DCs and gut-migrating lymphocytes, particularly regulatory T cells. SEA treatment affects gut DCs inducing prolonged capacity in this subset to evoke gut-homing potential to T cells. In addition, the improved oral tolerance observed following neonatal SEA treatment might also be dependent on functional vitamin A metabolism.

Original papers

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

I. Anna Lönnqvist*, Sofia Östman*, Nina Almqvist, Susanne Hultkrantz, Esbjörn Telemo, Agnes E. Wold and Carola Rask
Neonatal exposure to staphylococcal superantigen improves induction of oral tolerance in a mouse model of airway allergy
Eur J Immunol, 2009 Feb; 39(2):447-56#

II. Anna Stern, Agnes E. Wold and Sofia Östman
Accumulation of FoxP3+ Tregs in the gut of mice neonatally treated with *S. aureus* superantigen
Submitted

III. Anna Stern, Agnes E. Wold and Sofia Östman
Oral tolerance improved by staphylococcal superantigen depends on functional vitamin A metabolism
In manuscript

IV. Anna Stern*, Erika Lindberg*, Fredrik Bäckhed, Agnes E. Wold and Sofia Östman
Superantigen-producing *Staphylococcus aureus* promotes oral tolerance in mice
In manuscript

* These authors contributed equally to the study.

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Abbreviations

APC	Antigen presenting cell
BALf	Bronchoalveolar lavage fluid
CFU	Colony-forming units
CTLA-4	Cytotoxic T lymphocyte antigen 4
CCR	Chemokine receptor
DC	Dendritic cell
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iTreg	Induced regulatory T cell
MadCAM-1	Mucosal addressin cell adhesion molecule 1
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
nTreg	Natural regulatory T cell
OVA	Ovalbumin
PCA	Passive cutaneous anaphylaxis
PP	Peyer's patches
RA	Retinoic acid
RAR	Retinoic acid receptor
RALDH	Retinaldehyde dehydrogenase
SAg	Superantigen
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSST-1	Toxic shock syndrome toxin 1

Introduction

During the last few decades, immunoregulatory disorders such as allergy, inflammatory bowel disease, and organ-specific autoimmune diseases, have become more prevalent in the Western world [1-3]. For example, IgE-dependent allergies have tripled the last 20 years [4, 5].

Although the cause of this rapid increase is unknown, it was noted in the 19th century that poor people and farmers seldom were allergic. In 1989 David Strachan formulated the hygiene hypothesis to explain the rise in allergy in industrialized countries throughout the 20th century [6]. He observed that having many older siblings was associated with lower prevalence of hay fever and proposed that early infections were required to properly mature the immune system. Since then, several epidemiological studies have supported the hygiene hypothesis [5-12]. For example, allergies are less common in children attending day care before two years of age [11], being raised on a farm with livestock [12], and growing up with pets [10].

The living conditions during the first years of life determine the propensity to develop allergy, even if the allergic disease presents at a later age [5]. However, the nature of the protective microbial factor remains elusive. Stimulation of the gut-associated immune system seems important. Individuals with antibodies towards infectious matters in water and food, such as *Helicobacter pylori*, *Toxoplasma gondii* and hepatitis A virus, have a lower prevalence of allergy compared to individuals lacking these antibodies. On the contrary, exposure to airborne viruses such as measles, rubella and mumps do not confer protection and are sometimes associated with increased risk of developing allergy [7]. Another question is whether infections are required for proper immune activation and maturation, or whether the normal bacterial flora may provide the crucial stimulation for proper maturation of the neonatal immune system. Based on the observation that Swedish infants are colonized later and by a less varied bacterial fecal flora than infants in developing countries [13, 14], our group proposed that changes in the gut microbiota might underlie the rise in allergies in Western industrialized countries [15].

The intestinal immune system

The fundamental role of the immune system is to protect us from infectious microorganisms. The elimination of microbes creates inflammation, which is damaging to our own tissues. In order to avoid unnecessary inflammatory responses, potentially harmful agents (microbes) should be distinguished from harmless antigens, such as self-antigens and environmental antigens. The latter should evoke no, or a weak, non-inflammatogenic immune response (i.e. tolerance). The balance between immunity and tolerance is believed to be orchestrated by an interplay between dendritic cells (DCs), regulatory T cells and effector T cells.

The intestinal immune system encounters more types of antigens than other parts of the immune system, including commensal and pathogenic microbes and harmless antigens, such as food proteins. Approximately 50 kg of food proteins reach the human intestine in a year, and 130-190 g of these proteins are absorbed daily in the gut [16]. The commensal microbiota in the large intestine is composed of approximately 10^{11} bacteria while the small intestine contains 10^{4}-10^{7} bacteria/g of intestinal contents.

Oral tolerance

The gastrointestinal immune system tolerates repeated exposure to food antigens while maintaining the capacity to initiate strong immune responses against microbes. Consequently, exposure to soluble proteins via the gut normally gives rise to systemic tolerance toward this antigen, a phenomenon called oral tolerance. Development of oral tolerance was described in 1911 by Wells, who noticed that systemic anaphylaxis in egg-sensitized guinea pigs was prevented by previous feeding of egg protein [17]. Although extensive research has been performed since then, the mechanisms behind the development of oral tolerance are still unclear.

Organization of the intestinal immune system

A single layer of columnar epithelium forms the lining of the intestinal mucosa. The cells are connected by tight junctions to create a barrier to the luminal external environment. The surface area is enlarged by folds, crypts, villi and microvilli and covers approximately 200 m^2 in an adult human [18], making it more than 100 times larger than

the area of the skin. Under the epithelium is a thin layer of richly vascularised, loose connective tissue, the lamina propria, that contains both blood and lymph capillaries. Lymphoid cells in the gut are located in different compartments (Fig. 1), collectively referred to as the gut-associated lymphoid tissue (GALT). The GALT consists of both lymphocytes scattered throughout the lamina propria and the epithelium and organized lymphoid tissue where immune responses are induced. The latter includes the Peyer's patches (PP) and the gut draining mesenteric lymph nodes (MLN), as well as smaller isolated lymphoid follicles situated beneath the epithelium. Immune responses are triggered by an interaction between antigen-presenting cells, T cells and B cells in organized lymphoid tissue, which provides an optimal environment for this type of interaction.

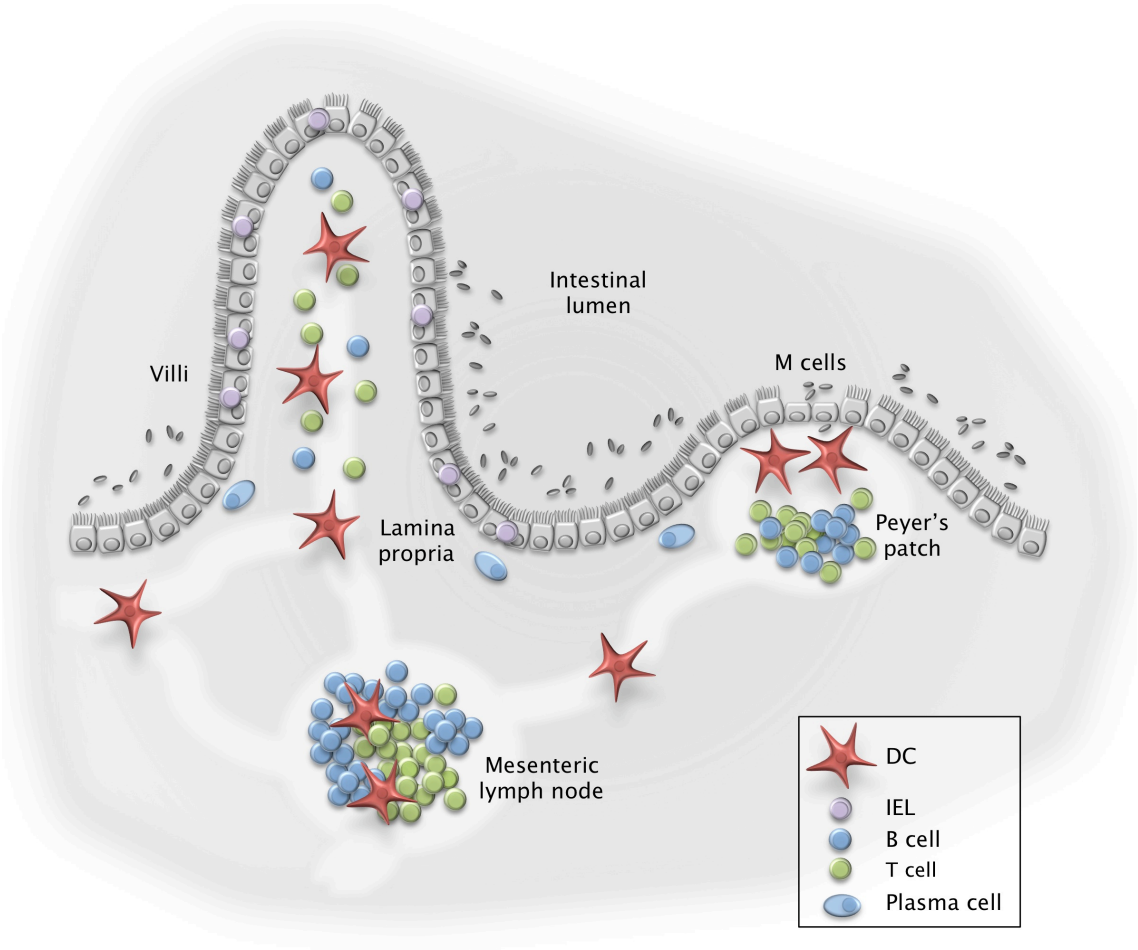

Figure 1. Schematic depiction of the lymphoid tissue of the small intestine with lymphocytes (B cells and T cells), DCs, and plasma cells scattered in the lamina propria, intraepithelial lymphocytes (IEL) located within the epithelium, and organized lymphoid organs, such as Peyer's patches and draining mesenteric lymph nodes.

Dendritic cells

Antigen-presenting cells (APCs) are present at all mucosal surfaces and sample microbes and soluble antigens and carry them to the nearest lymph node, where the antigens are presented to circulating T cells. DCs are highly specialized APC with a unique capacity to prime naïve $CD4^+$ T cells. DC maturation is characterized by upregulated expression of MHC class II molecules and co-stimulatory molecules, such as CD80, CD86, and the chemokine receptor CCR7 [19-22]. This maturation occurs in parallel with the migration of the DCs, and the upregulation of CCR7 guides the migratory DCs to the lymph nodes [23]. CCL19 and CCL21 are the ligands for CCR7 expressed by lymphatic endothelium and/or within lymph nodes by stromal cells, endothelial cells, and DCs themselves [24].

In addition to mature DCs migrating in response to infection or inflammation, "semi-mature" DCs migrate continuously under normal conditions [25, 26]. Presentation of an antigen by activated or mature APCs result in T cell priming and induction of an immune response to the corresponding antigen, whereas presentation of the same antigen during steady state by semi-mature APCs induces antigen-specific T cell tolerance [27, 28].

DCs in the GALT are found in the PP, MLN, and isolated lymphoid follicles, as well as in the lamina propria [29, 30]. DCs in the PP are exposed to microbial and other antigens that are transported via specialized epithelial cells known as microfold or M cells [31] from the lumen into the intestine. There are other routes through which DCs can obtain access to luminal antigens. For example, they may acquire antigens indirectly via internalization of apoptotic epithelial cells [32] or uptake of exosomes shed from epithelial cells [33, 34]. In addition, lamina propria DCs can directly open tight junctions and extend dendrites into the gut lumen [35-37].

A subpopulation of DCs in the GALT express CD103 (αEβ7 integrin) [38, 39] and represents approximately 40% of the total DC population in the MLN. The majority of the $CD103^+$ DCs are believed to originate in the lamina propria and represent a tissue-derived migratory population, whereas most $CD103^{neg}$ DCs represent a resident population [40]. Once in the lymph node, the DCs can present antigens to naïve T cells and induce differentiation of these cells by secreting appropriate cytokines [41].

T cells

T cells originate from precursor cells in the bone marrow, and migrate at an early stage to the thymus to mature. During the maturation process, they undergo both positive and negative selection to ensure that T cells leaving the thymus will have a functional T cell receptor capable of responding to presented antigens, but still ignoring self-antigens. T cells leaving the thymus express one of the co-receptors CD4 or CD8. $CD4^+$ T cells are helper cells that are primarily involved in activating other cell types, such as APCs or B cells, whereas $CD8^+$ T cells have the ability to kill cells infected with virus or intracellular pathogens.

Migration of T cells into distinct tissues is important for the initiation and maintenance of an effective immune response. Immune responses are mainly initiated in secondary lymphoid structures, such as MLN, PP and spleen. Here circulating naïve T cells are interacting with APCs and become activated when they meet their cognate antigen. Upon activation, T cells migrate into peripheral tissues and perform their effector function. The homing capacity of T cells depends on the local lymphoid environment. Therefore, T cells activated in gut-associated lymph nodes, such as PP and MLN, demonstrate an enhanced ability to enter intestinal tissues [42, 43], whereas T cells activated in the skin-draining lymph nodes display an enhanced capacity to migrate to non-intestinal tissues, such as the inflamed skin [42].

T cell migration

The migration and entry of T cells into peripheral tissues, in which immune responses are initiated and controlled, are mediated by the interaction of cellular adhesion receptors on circulating effector T cells and their ligands on vascular endothelial cells. In order to leave the circulation, lymphocytes must undergo four distinct adhesion steps: rolling, activation, firm adhesion and transmigration. Selectins and integrins are responsible for the initial tethering and rolling of cells along the vessel wall by binding to their respective ligands on the T cells. This weak interaction facilitates the interaction between chemokine receptors present on lymphocytes and chemokines bound to the endothelial surface. This interaction leads to conformational changes of the T cell surface integrins, resulting in higher affinity for their ligands. This mediates a firm

adhesion, and the T cells stop rolling, spread out, and prepare to migrate through the endothelium in response to a gradient of chemokines produced in the tissue.

Gut tropism

Several cellular adhesion receptor pairs have been implicated in regulating T cell entry to the intestinal lamina propria and epithelium (Fig. 2). T cells expressing the integrin α4β7 are directed to the gut because α4β7 binds to MadCAM-1 (mucosal adressin cell-adhesion molecule 1) [44, 45], which is highly expressed on high endothelial venules, PP, and MLN [46]. In parallel, the T cells upregulate the chemokine receptor CCR9 [47], allowing them to respond to CCL25 (TECK, thymus expressed chemokine) expressed by epithelial cells in the small intestine [48, 49]. DCs from PP and MLN can imprint a gut-homing potential on T cells *in vitro* [50-52]. The CD103$^+$ GALT DCs are particularly potent in this respect [38-40].

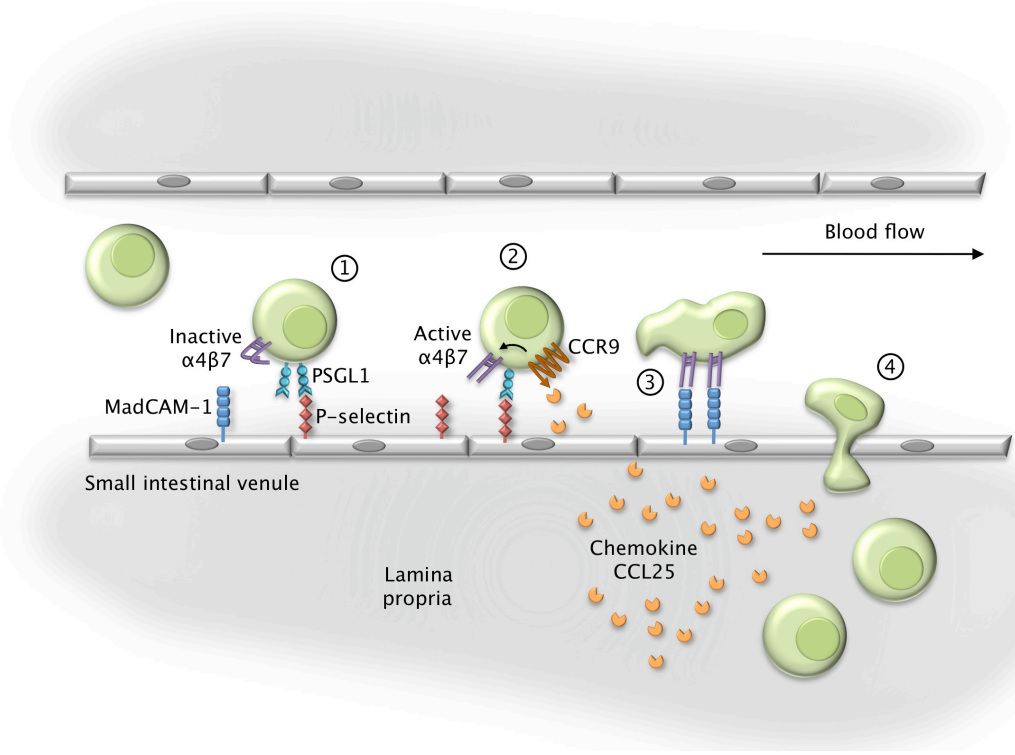

Figure 2. Migration of T cells into the gut-associated lymphoid tissue involves four steps. P-selectin interacts with PSGL1 (P-selectin glycoprotein ligand 1) on the T cells, which mediates weak tethering and rolling (1). This action facilitates the interaction between chemokine receptor 9 (CCR9) and its ligand CCL25. The binding of CCL25 by CCR9 leads to the activation and conformational changes of the integrin α4β7 (2). The interaction between active α4β7 and MadCAM-1 mediates a firm adhesion (3), and the T cell stops rolling, spread out, and migrate through the endothelium (4). The T cells can then react to a chemical gradient of chemokines in the tissue and migrate into the lamina propria.

T cell subsets

In the lymphoid tissues, naïve CD4⁺ T cells, or T helper (Th) cells, become activated after they interact with their cognate antigens presented by APCs. Once activated, they divide and secrete cytokines that regulate or assist in the immune response. The T cells can differentiate into various effector subsets, a process that involves activation of transcription factors with different functions and cytokine production (Fig. 3). The maturation pathway is regulated by cytokines produced by the APC or present in the microenvironment by the interaction between surface molecules on the APC and T cells and by the strength of the interaction between the T cell receptor and the antigenic peptide [53].

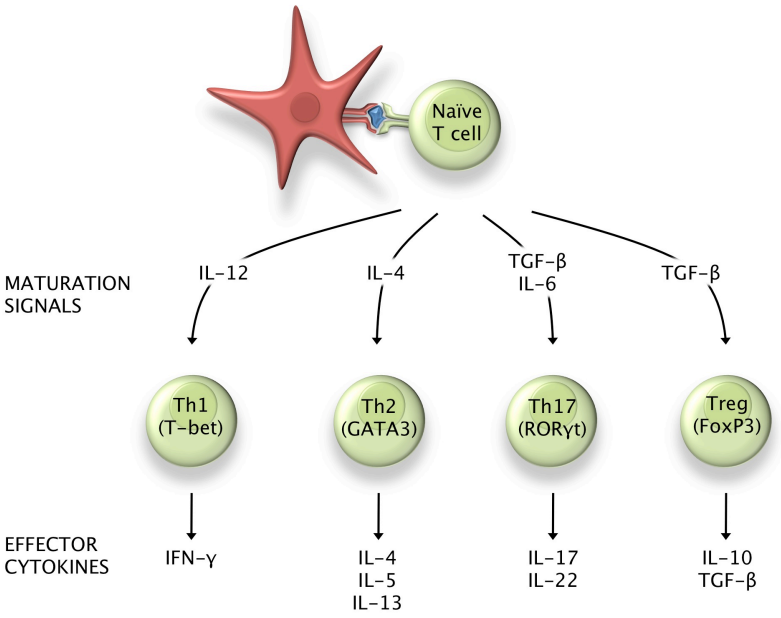

Figure 3. T cell subsets. A naïve CD4⁺ T cell can differentiate into diverse effector lineages (Th1, Th2, Th17 and Treg) upon activation, which are influenced by signals, such as cytokines from the antigen-presenting cell and the environment. The process involves the activation of transcription factors (in brackets) and results in different effector cells.

The various T cell subsets display different effector functions. Th1 cells produce large amounts of IFN-γ, which efficiently activates macrophages. IFN-γ also upregulates MHC molecules on various cells and, consequently, enhances their ability to present antigens to T cells. Th2 cells provide assistance with B cell activation and antibody production and produce IL-4, IL-5 and IL-13 that recruit and activate eosinophils and mast cells. Th2 cells are involved in the defense against helminths and the pathogenesis of allergic diseases by promoting the production of IgE antibodies. Th17 cells produce IL-17 and

IL-22 that recruit neutrophils and, thereby, promote tissue inflammation. Regulatory T cells (Tregs) downregulate immune responses and are described in more detail below.

Regulatory T cells

Tregs are characterized by the expression of the transcription factor FoxP3 (forkhead box transcription factor p3), high surface expression of the IL-2 receptor (whose α-chain is also referred to as CD25), and intracellular CTLA-4 (cytotoxic t lymphocyte antigen 4). One important characteristic of this cell type is its ability to downregulate several types of immune responses [54]. There are different subsets of regulatory T cells. Naturally occurring $CD4^{+}CD25^{++}$ regulatory T cells (nTregs) differentiate in the thymus and migrate to peripheral tissues with their regulatory function already in place [55]. However, it has been suggested that Tregs require IL-2 both during thymic development and for peripheral expansion and maintenance [56]. In addition to nTregs, naïve $CD4^{+}$ T cells can be induced in the periphery to differentiate into Tregs, so-called induced regulatory T cells (iTregs). This preferably occurs in the GALT [57] and in response to oral antigens [54, 58]. Tregs mediate their immunoregulatory function by cell contact dependent inhibition [59, 60] or via secretion of the suppressive cytokines IL-10 [61] and TGF-β [62].

Dendritic cell- T cell interactions

The interplay between DCs and the naïve T cell is fundamental for the T cell maturation pathway. DCs can present an antigen to a naïve T cell in such a manner that it is converted to a Treg [63-65]. $CD103^{+}$ DCs in the gut appear to be especially effective in converting T cells into iTregs [57, 66, 67]. On the other hand, DCs are targets for suppression by regulatory T cells, which downregulate co-stimulatory molecule expression on the DC. This results in the conversion of the DC into a "tolerogenic" DC [68-70]. Tregs constitutively express CTLA-4, which is an inhibitory T cell molecule that interacts with CD80 and CD86 on the APC in competition with the co-stimulatory molecule CD28. Interaction between T cell CTLA-4 and DC CD80/CD86 leads to the disappearance of the latter from the APC surface (Qureshi et al., presented at World Immune Regulation Meeting, Davos, Schweiz, 2010). DCs that do not express CD80 and CD86 induce poor T cell proliferation [70].

The role of retinoic acid

Vitamin A (retinol) is a fat-soluble vitamin that is essential for the formation and maintenance of skin, bone, and vasculature. However, it is also a key regulator of intestinal immunity [71]. By the action of retinaldehyde dehydrogenases (RALDHs), vitamin A is converted from retinal to retinoic acid (RA), the biologically active form of vitamin A that binds to specific retinoic acid receptors (RARs) and mediate effects on gene transcription and cell differentiation. The small intestine is the primary site of absorption and enzymatic processing of vitamin A to RA, and the GALT may experience high concentrations of retinoids [72]. Small intestinal and MLN CD103⁺ DCs express high levels of RALDHs [40, 67, 73]. The presence of RA during T cell activation induces the expression of integrin α4β7 and CCR9 (Fig. 4) [74].

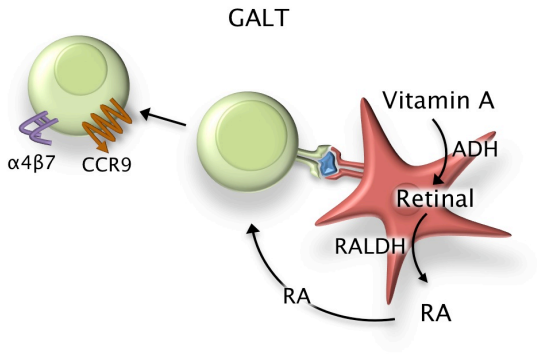

Figure 4. Generation of gut-tropic T cells by RA-producing CD103⁺ DCs. T cells stimulated by antigens presented by CD103⁺ DCs in the GALT upregulate the gut-migratory markers CCR9 (chemokine receptor 9) and integrin α4β7. This action depends on retinoic acid, which is generated from the vitamin A metabolite retinal by the enzyme RALDH.

RA production in gut-associated CD103⁺ DCs also enhances the TGF-β-dependent conversion of naïve T cells into Tregs (Fig. 5) [57, 66, 67]. In addition, RA has been implicated in the modulation of B cell tropism as well as the promotion of IgA production [75]. These findings suggest that CD103⁺ DCs play a critical role in the maintenance of gut homeostasis.

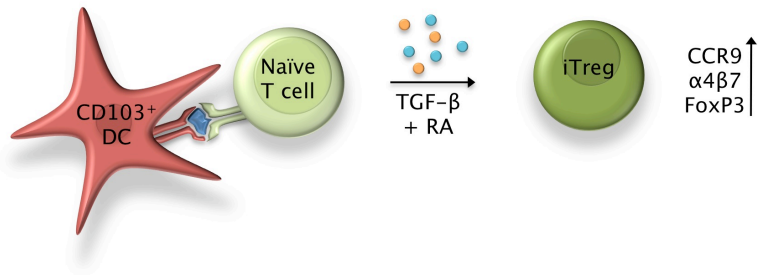

Figure 5. Retinoic acid and the generation of regulatory T cells. Upon activation in MLN, RA enhances the TGF-β-dependent conversion of naïve $CD4^+$ T cells into Tregs. In addition, these iTregs are imprinted with gut-migratory potential.

Mechanisms of oral tolerance

The manner in which dietary antigens are processed and presented to the immune system to induce tolerance, rather than immunity, is unclear. However, Tregs, DCs, and antigen processing by intestinal epithelial cells have been shown to be of importance for oral tolerance.

Regulatory T cells

There are two primary effector mechanisms of oral tolerance: the induction of Tregs that mediate active suppression and the induction of clonal anergy or deletion of antigen-specific T cells. The induction of oral tolerance is believed to be dependent upon the dose of antigen administered. Lower doses favor the generation of antigen-specific Tregs and active suppression, whereas higher doses also lead to clonal anergy/deletion [76, 77]. Low doses of tolerizing antigen are taken up and presented, preferentially by APCs in the GALT where the local environment may favor the generation of Tregs [78]. Higher doses of orally administered antigen result in anergy/deletion of specific T cells in the gut and in systemic antigen presentation after the antigen passes through the gut [79, 80]. Tregs are important in promoting oral tolerance. However, despite extensive research, the underlying mechanism of oral tolerance, including the induction of Tregs, remains elusive.

Dendritic cells

Different DC subsets have been suggested to be important for oral tolerance. Semi-mature DCs express lower levels of co-stimulatory molecules, resulting in diminished

T cell stimulatory properties. In addition, the $CD103^+$ DCs in the GALT are believed to have an impact on oral tolerance by enhancing the TGF-β-dependent conversion of naïve T cells into Tregs. The importance of DCs for oral tolerance has been demonstrated by *in vivo* expansion of the DC population in mice by injection with Flt3 (Fms-like tyrosine kinase 3) ligand [81]. In addition, oral tolerance can, for example, not be induced in CCR7-deficient mice displaying an impaired migration of DCs from the intestine to the MLN [82].

Intestinal epithelial cells and tolerogenic processing

It has been known since the 1980s that a tolerogenic factor appears in the circulation of mice shortly after antigen feeding and that this factor induces tolerance upon transfer into naïve recipients [83]. The phenomenon can be demonstrated by passive transfer of serum from a fed animal into a naïve recipient injected with this serum [83]. This serum factor has been indentified as exosomes or tolerosomes [34], small membranous vesicles secreted by intestinal epithelial cells as a consequence of fusion of multi-vesicular late endosomes/lysosomes with the plasma membrane. Oral tolerance has been suggested to be dependent upon generation of such exosomes. It is possible that these exosomes spread via the lymph and blood to reach lymphoid tissues, where they merge with APCs and convey their tolerogenic message.

The gut flora and immune maturation

The commensal gut flora strongly influences the maturation and function of the immune system. In germ-free mice, the GALT is underdeveloped, but expand upon colonization with conventional flora [84]. For example, the GALT in germ-free mice has reduced numbers of IgA-producing plasma cells [85, 86], fewer $CD4^+$ cells in the lamina propria [87], fewer intraepithelial lymphocytes [88, 89]. In addition, it lacks MHC class II expression in intestinal epithelial cells [90, 91]. All of these abnormalities can be reversed within a few weeks after colonization with a commensal microflora. Colonization with a single gut commensal or a simple mixture is not as effective in developing the immune system as conventionalization of animals with a full flora [85, 92, 93].

Bacteria promptly translocate across the intestinal epithelial barrier, reach the underlining mucosa, and are taken up by intestinal DCs. These DCs migrate to the MLN

and activate T cells, which induce IgA-producing cells [94]. Bacteria that colonize the gut for extended periods only stimulate the immune system upon initial colonization. Once a specific secretory IgA response has developed, the strain is prevented from translocating and stimulating the immune system [95]. Thus, the immune system is further matured every time a new bacterial strain manages to come in contact with the mucosal lymphoid tissues, but not by the presence of the same strain for extended periods of time. A full flora generally provides the required stimuli for the maturation of the intestinal immune system.

Gut flora and oral tolerance

It has been shown in animal models that the mechanism of oral tolerance is not fully functional in the absence of a gut flora [90, 91, 96]. Germ-free mice develop an incomplete and more temporary oral tolerance compared to conventionally raised animals [90]. In addition, the tolerogenic processing in germ-free mice is less active [97], and their Tregs have reduced functional suppressive capacity compared to conventional Tregs [98]. The manner in which the microflora acts in promoting tolerance remains unknown.

Establishment of the intestinal microflora

The establishment of a normal flora starts immediately after birth and provides a constant and continuous stimulation of the immune system. The gut flora normally establishes in an ordered fashion. The intestine of a newborn infant is rich in oxygen, which favors the expansion of facultative bacteria, such as *Escherichia coli* and other enterobacteria, enterococci, and staphylococci. As the facultative bacteria consume the oxygen, anaerobic bacteria including bifidobacteria, bacteriodes, clostridia, and lactobacilli, start to colonize. Microorganisms acquired from the maternal microbiota during delivery and from food and environmental exposure can colonize the newborn infant. However, with increasing complexity and competition in the microbiota, only bacteria adapted to this special niche may survive. Thus, several microbes from the vaginal microbiota of the mother can be present in the gut of the newborn infant, although they disappear promptly [99].

An altered colonization pattern in Western industrialized countries

Colonization by many traditional gut bacteria occurs late today in Western countries [100, 101]. In addition, the strain turnover is slow [102]. These findings suggest that many infants today are colonized by a commensal flora that provides inadequate stimulation to the developing immune system. In predisposed individuals, this can result in failure to become tolerant to harmless antigens, hence, allergy development. Indeed, a microflora containing few bacterial groups early in life is associated with increased risk of developing allergy [103].

Staphylococcus aureus

S. aureus is a typical member of the skin microflora, foremost colonizing the anterior nares [104-106]. More recently, *S. aureus* has become common in the gut flora of Swedish infants [107, 108], probably as a result of decreased competition by traditional fecal bacteria whose circulation has decreased in parallel with improved sanitary conditions [100]. Thus, 75% of Swedish infants have *S. aureus* in at least one stool culture during the first year of life [108]. The strains usually persist for several months and commonly originate from the parents' skin flora [107]. *S. aureus* counts are high in colonized newborn infants (10^7 colony-forming units (CFU)/g of stools on average), but decrease successively (down to 10^4 CFU/g at one year of age). This demonstrates that *S. aureus* lacks the ability to withstand the competition from an increasingly complex anaerobic microbiota that develops during the first year(s) of life [109].

Staphylococcal enterotoxins

The strongest known T cell activators are the superantigens, exotoxins produced by certain bacteria, such as *S. aureus.* To date, 20 different enterotoxins and related toxins have been described in *S. aureus*, with some differences in structure and biological activity [110, 111]. The classical superantigens include staphylococcal enterotoxin (SE) A, B, C, D, and E, as well as toxic shock syndrome toxin-1 (TSST-1). The non-classical toxins are SEG-J and the staphylococcal enterotoxin-like toxins (SEls) K-R and U-V [110, 111].

Unlike conventional antigens, superantigens bypass the classical route of antigen processing and presentation and cross-link outside domains of MHC class II molecules with the variable part (Vβ) of the T cell receptor (TCR)[112-114] (Fig. 6). This leads to a

massive activation of all T cells with a specific Vβ. The initial activation can be followed by anergy or depletion of the specific Vβ T cells [115-117]. The TCR Vβ specificity of the classical superantigens in mice is presented in table 1.

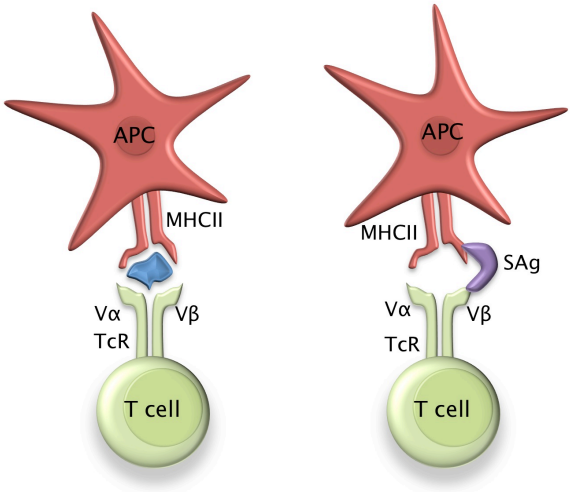

Figure 6. T cell activation via conventional antigen presentation versus activation via the cross-linking of MHC class II and the T cell receptor (TcR) by superantigen (SAg).

Table 1. The classical staphylococcal enterotoxins and their TCR Vβ specificity in mice.

Superantigen	Mouse TCR
SEA	Vβ1, 3, 10, 11, 12, 17
SEB	Vβ7, 8.1-8.3
SEC	Vβ7, 8.2-3, 10, 11
SED	Vβ3, 7, 8.3, 11, 17
SEE	Vβ11, 15, 17
TSST-1	Vβ15, 16

As many as 10-30% of all T cells can become activated by a certain superantigen, as compared to < 0.1% for normal antigens [118]. Superantigens are extremely potent, and the presence of a small amount of superantigen in the bloodstream rapidly elevates many cytokines to toxic levels, with IL-2, IFN-γ, and TNF-α generally believed to be the cause of the toxicity [119]. Approximately four to five times less superantigen is required to stimulate human peripheral blood lymphocytes than mouse peripheral blood lymphocytes, probably due to a slightly higher affinity toward human MHC class II. For example, mice that are transgenic for human MHC class II are more sensitive in their

T cell proliferation responses, cytokine release, and toxicity to injected superantigens compared to their non-transgenic littermates [120, 121].

Superantigen activation of T cells *in vivo* first requires that the superantigen bind to the MHC class II molecule and to concentrate onto the surface of APCs. Studies have shown that binding of SEA to APCs is extremely stable, with the superantigen remaining on the APC surface for up to 40 hours without any evidence of depletion [122]. After APC binding, the surface concentration is sufficient to successively engage and cross-link multiple T cell receptors, resulting in strong TCR signaling and activation and rapid cytokine production. Facilitation of APC-T cell interaction by superantigens activates the same signal transduction pathway as conventional antigens, ultimately leading to T cell division and cytokine production by both cell types. The massive cell activation induced by superantigens results in the release of various cytokines at high levels from both APCs and T cells. T cells produce IL-2, TNF-α, and IFN-γ, and IL-1. In addition, IL-1 and TNF-α are produced by the APCs [123-126].

All superantigens have a similar three-dimensional structure. However, they can be presented by MHC class II in distinct ways [127-131] and can be divided into three groups according to their ability to bind MHC class II. They are either single α-chain binding superantigen (e.g. SEB), or single β-chain binding (e.g. SEH) or they are capable of binding to two MHC class II molecules [128, 131]. In the latter case, where the superantigen cross-link two MHC class molecules, they bind one α-chain and one β-chain (e.g. SEA), two α-chains (e.g. SED) or two β-chains (e.g. SPE-C, streptococcal pyrogenic exotoxins C) [127, 129, 132-135]. Cross-linking of MHC class II molecules is believed to lead to more potent cell activation [133, 136]. In accordance, SEA has been proposed to be the most potent superantigen [137].

All bacterial strains do not produce all different types of superantigens [138, 139]. However, it has been demonstrated that most human *S. aureus* isolates harbor at least one gene encoding for these toxins. Eighty percent of the strains colonizing Swedish infants possess genes for production of one or more of the superantigens, the most common toxin type being the non-classical superantigens SEM and SEO, which are encoded by the *egc* gene cluster (Nowrouzian *et al*, in manuscript). Regarding the classical superantigen

are TSST-1 produced by 22%, SEC by 19% and SEA by 18% of the strains. Other superantigens are relatively uncommon (Nowrouzian *et al*, in manuscript).

Staphylococcal enterotoxins are the causative agents of staphylococcal food poisoning [140, 141] and induce vomiting and diarrhea within hours after digestion. The enterotoxins withstand heat, enzymatic degradation, and low pH and pass intact into the small intestine where they traverse the epithelium [142] and strongly activate intraepithelial lymphocytes (IELs)[143]. Superantigens induce substantial production of IFN-γ [144-146] by the IEL in the intestinal epithelium [147]. In response to the locally produced IFN-γ, the intestinal epithelium upregulates MHC class II [148]. TSST-1 does not induce diarrhea and vomiting due to inactivation in the digestive system. Instead, it is responsible for toxic shock syndrome that can occur in women using highly absorptive tampons.

The immunomodulatory effect by *S. aureus* colonization in infants

In birth cohort studies established to follow intestinal colonization pattern, immune development and début of allergies in childhood it was found that none of the infants that were colonized in the gut by *S. aureus* during the first two weeks of life developed food allergy within the first 18 months of life [149]. The incidence of food allergy in the rest of the infants in the cohort was 9% (Lindberg *et al*, unpublished). Food allergy is usually the first manifestation of an atopic disposition in a child and is strongly connected to later development of respiratory and skin allergy [150, 151].

Other signs of immune activation were seen in infants who were neonatally colonized with *S. aureus*, such as increased levels of the immunoregulatory molecule sCD14 [149]. Further, early colonization by *S. aureus* was associated with a more rapid increase in serum IgA levels [152]. No other bacterial groups in the gut flora provided this type of immune stimulation leading to allergy protection [149, 152]. Thus, infants colonized by *S. aureus* in the gut during the first weeks of life showed signs of both immune activation and functional tolerance development. Infants colonized by superantigen producing *S. aureus* were apparently healthy and did not have more gastrointestinal problems than other children [108].

Aims of the study

The main objective of this thesis was to examine the immune responses elicited by *S. aureus* colonization or staphylococcal superantigen exposure to the immature immune system in animal models.

The specific aims were:

Paper I and II

To investigate whether superantigen produced by *S. aureus* could prime the neonatal immune system and affect development of tolerance towards an innocuous antigen and/or influence sensitization in experimental allergy models.

To investigate the possible mechanisms behind the improvement in oral tolerance after priming the neonatal immune system with superantigen, by examining the effect on Tregs and DCs in the gut.

Paper III

To study $CD103^+$ DCs and the role of the vitamin A metabolism in the enhanced development of oral tolerance observed after neonatal superantigen treatment.

Paper IV

To examine the ability of superantigen-producing *S. aureus* to colonize the gut of germ-free mice and investigate whether this colonization affects the induction of oral tolerance.

Materials and methods

Animals and administration of *S.aureus* or superantigen

Animals

BALB/c mice, DO11.10 transgenic mice with T cells harboring TCR specific for ovalbumin (OVA) and Sprague-Dawley rats were maintained under conventional housing conditions and provided with food and water *ad libitum*. Germ-free Swiss-Webster mice originated from in-house breeding at the gnotobiotic department at the animal facility. Permission for all experiments was obtained from the regional Ethics Committee in Gothenburg (permission number 238-2006 and 408-2008).

Administration of superantigen (papers I-III)

Neonatal pups were given SEA (Sigma-Aldrich), starting when the pups were 4-5 d old. Treatment was carried out every other day, for a total of 6 times for peroral (p.o.) and intranasal (i.n.) administration or three times for intraperitoneal (i.p.) injections. Control mice (SHAM) were given saline. SEA treatment to adult mice (paper I) was performed via gastric intubation through a feeding needle.

Bacterial strains (paper IV)

Four different *S. aureus* strains, which were isolated from the gut flora of infants in the ALLERGYFLORA cohort, were used for the *in vivo* colonization of mice. The strains produced the SEA, SEC, and TSST-1 superantigens, which are referred to as "classical" superantigens, as shown both by production of toxin *in vitro* (SET-RPLA kit, Oxoid/Thermo Fisher Scientific) and the presence of the respective genes by multiplex PCR (Nowrouzian *et al*. in manuscript). Each of the three strains also carried the *egc* cluster encoding the "non-classical" superantigens SEM and SEO. A non toxin-producing strain (NON-TOX) that had none of the 13 genes encoding *S. aureus* superantigens was included. For *in vitro* experiments, strains of *S. aureus* capable of producing SEB, SEA/TSST-1 and SEM/SEO were also included. The bacteria were cultured on Colombia blood agar, harvested, washed, and adjusted to 10^9 bacteria/ml. For *in vitro* use, the bacteria were inactivated by exposure to UV light for 25 min, and inactivation was confirmed by negative viable count.

S. aureus colonization of germ-free mice (paper IV)

Germ-free mice were monocolonized with one of the four *S. aureus* strains. Three strains produced different superantigens and one was non toxin-producing. Approximately 3×10^8 CFU of bacteria were administered intragastrically using sterile feeding needles. The mice were subsequently kept under conventional housing conditions with the ability to spontaneously acquire microbes from the environment. Fecal samples were collected prior to colonization and cultured both aerobically and anaerobically to ascertain that the animals were germ-free. In addition, fecal samples were collected 48 h as well as 6 and 10 weeks after colonization and cultured quantitatively for aerobic and anaerobic bacteria.

Oral tolerization

Four weeks after neonatal SEA treatment or six weeks after colonization of germ-free mice, one dose of 5 mg of OVA (grade V; Sigma-Aldrich) or saline, was given by intragastric feeding. The dose was selected to induce partial oral tolerance (supplementary data, paper I), enabling the possibility to study improvement in tolerance induction.

In vivo inhibition of the vitamin A metabolism by Citral (paper III)

To investigate the role of the vitamin A metabolism for oral tolerance induction, groups of mice were given the RALDH inhibitor Citral (Sigma-Aldrich) in the drinking water (2 mg/mL) for 2 weeks, beginning 10 d prior tolerization (paper III). The Citral treatment started directly after weaning at 4 weeks of age. The daily dose of Citral was approximately 8 mg per mouse.

Allergy models

The degree of OVA tolerization was tested in an OVA-induced airway allergy model or OVA-induced food allergy model (Fig. 7). Nine days after oral tolerization, mice were sensitized twice 10 d apart by i.p. injection of 10 μg OVA adsorbed onto 2 mg of Al(OH)3 gel (Alum; Sigma-Aldrich) in order to trigger an IgE response to OVA.

Eight days after the second immunization, the animals were challenged with OVA. In the airway allergy model, the mice were briefly anaesthetized and given OVA i.n. on five consecutive days. In the food allergy model, the mice were challenged by intragastric feeding of OVA repeated every other day. They were deprived of food for 2 h prior to each challenge.

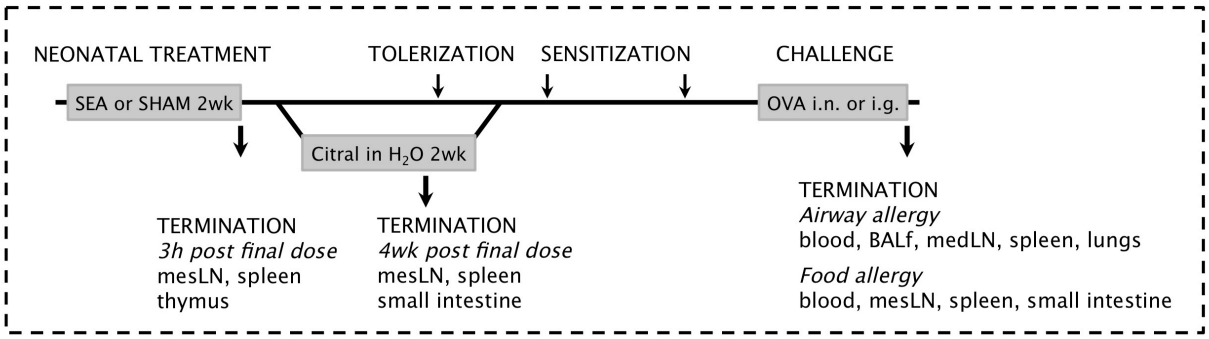

Figure 7. Experimental layout: the models of experimental airway allergy and food allergy.

Airway allergy model: sample collection and preparation (paper I)

Sensitization in the airway allergy model was measured as eosinophilia in the lungs, presence of OVA-specific IgE in serum and *in vitro* cytokine production of lung cells stimulated by OVA. In addition, the IgE concentration in serum was measured.

Twenty-four hours after the last OVA challenge, bronchoalveolar lavage (BAL) fluid, blood, mediastinal LN and lungs were collected. The mice were deeply anesthetized by i.p. injection of a mixture of xylazine (130 mg/kg) and ketamine (670 mg/kg). The chest was opened, blood was drawn by heart puncture, and serum was collected after clotting and centrifugation (3,000 × g) for 15 min. BAL was performed by instilling 0.4 mL of PBS through the trachea by gentle aspiration and then repeated a second time with 0.4 mL of PBS. The proportion of eosinophils in the BAL fluid was evaluated in cytospin preparations stained with May–Grünwald/Giemsa. Single cell suspensions were prepared from mediastinal LN and analyzed for the presence of regulatory T cells ($CD4^+FoxP3^+$) by flow cytometry.

The lungs was cut into small pieces, incubated with collagenase (10 mg/mL, type IV; Sigma-Aldrich) and deoxyribonuclease I from bovine pancreas (0.1 mg/mL; Sigma-Aldrich) for 20 min at 37°C, and gently mashed through a cell strainer. The lung cells

were stimulated with OVA for *in vitro* proliferation assay. Supernatants were collected after 48 h of culture and assayed for IL-5, IL-10 and IL-13 by cytometric bead array (CBA Flex sets; BD Biosciences).

The left lung was taken from sets of mice that did not undergo BAL lavage. The lung was embedded in freeze medium (Tissue-Tek O.C.T compound; Sakura) and instantly frozen in isopentane cooled with liquid nitrogen and stored at -70°C. Tissue sections (6 µm) were prepared using a cryostat. The sections were dried at 37°C for 1 h before staining with May–Grünwald/Giemsa and examination in the microscope (Leica DMR).

Food allergy model: sample collection and preparation (Papers II-IV)

Sensitization in the food allergy model was measured as total and OVA-specific IgE in serum, OVA-induced diarrhea, blood eosinophilia, and mast cell infiltration in the gut, as well as *in vitro* cytokine production of spleen cells stimulated by OVA. Diarrhea was assessed by visually monitoring mice for up to 1 h after challenge, with mice demonstrating profuse liquid stool were recorded as positive.

One hour after the last OVA challenge, the mice were sacrificed and blood, mesenteric lymph nodes, and pieces of the small intestine was collected. The mice were deeply anesthetized by i.p. injection of a mixture of xylazine (130 mg/kg) and ketamine (670 mg/kg). The chest was opened and blood was drawn by heart puncture. The blood was divided into eppendorf tubes for serum preparation and EDTA tubes for enumeration of eosinophils in the blood. The tubes were centrifuged (3,000 × g) for 15 min, and serum were collected and frozen at -20°C. Cell pellets in the EDTA tubes were treated with ammonium chloride buffer for lysis of red blood cells. Remaining cells were centrifuged onto glass slides using Cytospin and stained with May Grünwald/Giemsa to visualize eosinophils. A piece of the intestine (~10 cm) was fixed in formaldehyde for 24 h, washed in PBS overnight and stored in 70% ethanol before being embedded in paraffin for histological analysis.

After the blood was drawn, 20 mL 0.1% heparine/PBS was perfused into the heart of the anaesthetized animals. The small intestine was taken out and trimmed, and 5 cm of the proximal small intestine was dissected into small pieces for quantification of IgA. The pieces were kept in PBS with 0.1% BSA (bovine serum albumin; Sigma-Aldrich), 0.1

mg/mL soybean trypsin inhibitor (Sigma-Aldrich), 5 mM EDTA and 0.35 mg/mL Pefablock (Coatech) on ice until frozen and stored at -20°C. For histological analysis, intestinal pieces were fixed in formaldehyde for 24 h, washed in PBS overnight and stored in 70% ethanol before being embedded in paraffin.

The mesenteric lymph nodes were prepared into single cell suspensions. The cells were stimulated with OVA for *in vitro* proliferation assay. In addition, the mesenteric lymph node cells were analyzed by flow cytometry.

Immune status post SEA treatment (papers I -III)

To investigate the immune status at the time point for oral tolerization, the mice were sacrificed at 5-6 weeks of age (4 wk after SEA treatment/after 10 d of Citral intake). In addition, to study the direct effect of SEA exposure on the neonatal immune system, pups were sacrificed 3 h after the final dose. Spleen (3 h, 4 wk), thymus (3 h) and mesenteric lymph nodes (4 wk) were collected and prepared into single cell suspensions. Spleen cell suspensions were incubated with ammonium chloride buffer (pH 7.3) for 5 min at 37°C for red blood cell lysis. The cells were examined by *in vitro* assays or analyzed by flow cytometry. For immunohistochemical analysis, intestinal pieces (4 wk) were fixed in formaldehyde for 24 h, washed in PBS overnight and stored in 70% ethanol before being embedded in paraffin.

In vitro experiments

In vitro **proliferation assay (Papers I-II, IV)**

For *in vitro* proliferation assay, cells were suspended in supplemented medium (Iscove´s with 2 mM L-glutamine, 50 µM mercaptoethanol, 50 µg/ml gentamycin, and 10% FCS). Cells were aliquoted at 1-10×10^5 cells/well in microtiter plates. The stimuli used in the experiments were the model antigen OVA, various superantigens (SEA, SEB, SEC, TSST-1), the mitogen Concavalin A (ConA) or UV-inactivated *S. aureus*. After 48 h incubation (5% CO_2, 37ºC), supernatant was collected for cytokine analysis. After 4-7 d of culture, cell proliferation was assessed either by ^{3}H-thymidine incorporation in the last 8-16 h of culture or by flow cytometry assayed by CFSE staining.

In vitro suppression assay (paper I)

For *in vitro* suppression assay, $CD4^+$ cells from the mesenteric lymph nodes were fractionated into $CD25^+$ and $CD25^{neg}$ subpopulations using magnetic beads (Miltenyi Biotec). Further, APCs were isolated with $CD11c^+$ beads (Miltenyi Biotec). $CD4^+CD25^+$ T cells from SEA- or SHAM-treated mice were serially diluted in threefold steps and co-cultured together with a fixed number of $CD4^+CD25^{neg}$ responder T cells. The responder cells were from either the $CD4^+CD25^+$ T cell donor or a pool of responder cells isolated from SHAM-treated mice, yielding different ratios of $CD4^+CD25^+$: $CD4^+CD25^{neg}$ (1:1, 1:3, 1:9 etc.). The cells were cultured for 7 d together with $CD11c^+$ APCs and stimulated with 0.5 μg ConA. Proliferation was assessed by [^{3}H]thymidine (Amersham Biosciences) uptake during the last 16 h of culture.

DC-T cultures: antigen presentation assay (papers II-III)

$CD11c^+$ cells were separated from MLN and spleen using magnetic beads (Miltenyi Biotec). $CD11c^+$ cells were pulsed with OVA (250 μg/mL) for 2 h at 37°C. $CD4^+$ T cells from spleen and MLN from DO11.10 mice were separated by negative separation using magnetic beads (Miltenyi Biotec). A fraction of the $CD4^+$ T cells where subsequently stained with CFSE (5μM; Molecular probes, Invitrogen). Antigen-pulsed $CD11c^+$ cells (5×10^4) where co-cultured with either CFSE-stained $CD4^+$ T cells (5×10^4) or unstained T cells (5×10^4). Supernatants were collected after 48 h for cytokine production assay. Cells were analyzed by flow cytometry prior to OVA pulse as well as after 4-5 d of culture. To measure cell division, CFSE-stained $CD4^+$ cells were analyzed by flow cytometry after 5 days of culture.

In vitro block of retinoic acid (Paper III)

To inhibit the production or action of retinoic acid, the RALDH inhibitor Citral (10 μM; Sigma-Aldrich) or the RAR antagonist LE-135 (1 μM; Axon medchem) was added to the DC-T cultures.

Flow cytometry

Phenotypic analysis of cell subsets was performed by flow cytometry. Flow cytometry analysis was performed on single cell suspensions from spleen, thymus or MLN. To examine the expression of cell surface molecules, cells were pre-incubated with FcγR-

blocking mAb and stained with antibodies towards the different surface molecules. For intracellular staining, cells were fixed and permeabilized with Fix/Perm (eBioscience) for 30 min followed by staining. Cells were acquired using FACSCantoII (BD Biosciences) equipped with FACSDiva software and data was analyzed with FlowJo software (Treestar).

The following anti-mouse monoclonal antibodies were used: CD4 (clone RM4-5), CD8 (clone 53-6.7), CD19 (clone 1D3), CD11c (clone HL3), TCRVβ panel, CD62L (clone MEL-14), CD25 (clone PC61), CD69 (clone H1.2F3), CD103 (clone M290), α4β7 (clone DATK32) CTLA-4 (CD152, clone UC10-4F10-11), CD40 (clone 3/23), CD80 (B7.1, clone 16-10A1), CD86 (B7.2, clone GL1) and MHC class II (IA^d, clone AMS-32.1) (all purchased from BD Biosciences). CCR9 (clone 242503; R&D Systems), CCR7 (clone 4B12; eBioscience) and FoxP3 (clone FKJ-16s; eBioscience) were also used. For estimation of live/apoptotic/dead cells, cells were stained with 7-AAD (BD Biosciences) and Annexin V (BD Biosciences).

Quantification of immune mediators

Determination of specific and total IgE

OVA-specific IgE levels in mouse serum were assayed by passive cutaneous anaphylaxis (PCA) in Sprague-Dawley rats. Rats were anaesthetized by i.p. injection of xylazine (8 mg/kg) and ketamine (40 mg/kg). Then, 50 µL of mouse sera serially diluted in twofold steps with PBS were injected intradermally into the shaved dorsal skin. After 72 h, the rats were challenged intravenously with 5 mg OVA in 1 mL PBS with 1% Evan's blue (Sigma-Aldrich). Half an hour later, the rats were sacrificed, and the skin was examined for the appearance of blue spots. The PCA titers were defined as the reciprocal of the highest dilution of serum resulting in a spot of ≥ 5 mm in diameter.

The concentration of serum IgE was determined by sandwich ELISA. Costar plates were coated with capture antibody (mouse anti-IgE antibody, 1 µg/mL; BD Biosciences). Serum samples were diluted threefold in four steps. Mouse IgE (BD Biosciences) was used as the standard and serially diluted twofold in seven steps. Biotinylated anti-mouse

IgE (2 μg/mL; BD Biosciences) was used as the detection antibody with TMB (3,3′,5,5′-Tetramethylbenzidine) as the substrate.

Cytokine analysis in supernatants

Secretion of cytokines in cell culture supernatants was measured by Cytometric Bead Array (CBA; BD Biosciences). CBA Flex set (IL-4, IL-5, IL-13) and Mouse Th1/Th2/Th17 Kit were used. Samples were acquired using FACSCantoII and analyzed with FCAP software (Soft Flow Inc).

Determination of intestinal IgA (paper IV)

Intestinal pieces, treated with protease inhibitor (as described earlier) and stored at -20°C, were thawed, and 2% saponin was added. The tubes were incubated overnight at 4°C followed by centrifugation at 12,000 rpm for 10 min. The supernatant was collected and stored at -20°C until analysis. Levels of IgA were determined by sandwich ELISA. Costar plates were coated with mouse anti-IgA (1 μg/mL; Southern Biotech) overnight at room temperature. Mouse IgA (Southern Biotech) was used as the standard and serially diluted threefold in seven steps. HRP anti-mouse IgA (diluted 1:1000; Southern Biotech) was used as the detection antibody with TMB as substrate.

Histology/ Immunohistochemistry

Pieces from the proximal small intestine was collected and directly fixated in 4% formaldehyde for 24 h, washed in PBS overnight and stored in 70% ethanol prior to being embedded in paraffin for histological/immunohistochemical analysis. Tissue sections (4 μm) were prepared and stained with antibodies for Foxp3 (ebioscience), CD3 (Abcam), TGF-β (Abcam), and RALDH2 (gift from Prof. P McCaffrey, University of Aberdeen, Scotland). Sections were also stained with toluidine blue for visualization of total cell infiltration and May Grünwald/Giemsa for enumeration of mast cells and eosinophils.

Collection, culture and identification of fecal samples (paper IV)

Fecal samples were suspended in 1 ml of sterile peptone water, diluted serially, and plated on selective and non-selective media (see below) that were incubated under aerobic or anaerobic conditions for enumeration of various facultative and anaerobic bacteria. In brief, the total number of facultative bacteria was quantified on Colombia blood agar plates cultivated aerobically at 37°C for 24 h. *Enterobacteriaceae* were quantified on Drigalski agar and speciated using the API20E biotyping system (bioMérieux). Staphylococci were quantified on staphylococcus agar, identified by Gram-stained appearance and a positive catalase reaction. *S. aureus* was distinguished from coagulase-negative staphylococci by the coagulase reaction. Enterococci were quantified on enterococcosel agar, where they caused esculine hydrolysis and showed typical Gram-stained appearance.

Anaerobes were quantified after growth on Brucella blood agar incubated anaerobically at 37°C for 48-72 h. Each colony type was enumerated separately and checked individually for inability to grow under aerobic conditions and discarded if able to do so. However, scant aerobic growth of gram-positive rods was permitted, as many bifidobacteria and lactobacilli are able to replicate aerobically. Anaerobic strains were speciated by Rapid ID 32A biotyping (bioMérieux). The total counts of anaerobes were quantified on Brucella blood agar, after the numbers of facultative bacteria growing on the plates were subtracted.

Statistical analysis

Statistical analysis was conducted using the nonparametric Mann-Whitney U-test or survival analysis (diarrhea onset in the food allergy model). P values < 0.05 were considered to be statistically significant.

Results and comments

Papers I and II

Can neonatal exposure to superantigen affect oral tolerance induction and allergy development?

In paper I, the ability to develop oral tolerance to an innocuous antigen was investigated experimentally in a model of airway allergy. Mice that were fed OVA developed less IgE antibodies against OVA and had lower eosinophil infiltration into the lung upon challenge than mice fed PBS. That is, the former group of mice developed oral tolerance. In addition, mice treated with SEA neonatally developed more profound oral tolerance than control mice (SHAM), showing even lower levels of OVA-specific IgE and lung eosinophilia (Fig. 8). Improvement in oral tolerance was not seen when administering the superantigen at adult age, suggesting that SEA only had this impact on immune regulation when given neonatally (i.e. during the maturation of the immune system). Moreover, neonatal SEA treatment did not lead to any general protective effect on allergy development in non-tolerized mice, indicating that neonatal SEA treatment primarily affects the mechanism of oral tolerance.

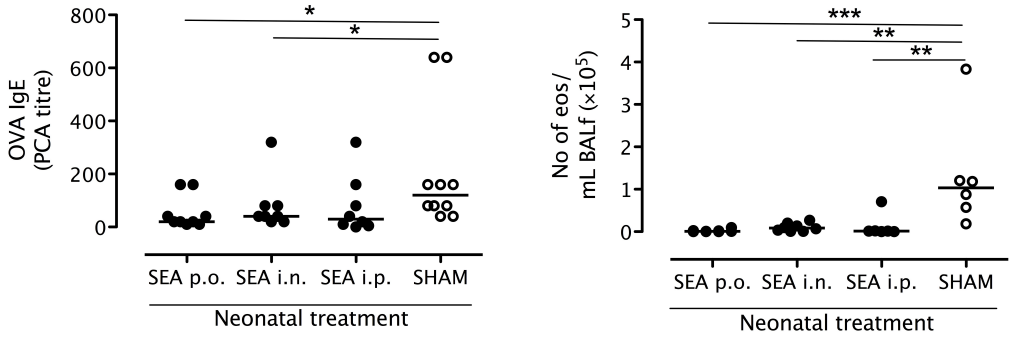

Figure 8. Neonatal SEA exposure leads to an increased ability to develop oral tolerance in a mouse model of airway allergy, as evidenced by lower levels of antigen-specific IgE and less eosinophils in bronchoalveolar lavage fluid (BALf).

In paper II, the ability to enhance oral tolerance by neonatal exposure to SEA was examined in a food allergy model. Consistent with the findings in paper I, neonatal peroral SEA treatment improved oral tolerance induction in this model, as evidenced by

lower blood eosinophilia and a tendency toward lower mast cell infiltration in the gut after repeated feeding of the allergen to which the mice had been sensitized (Fig. 9).

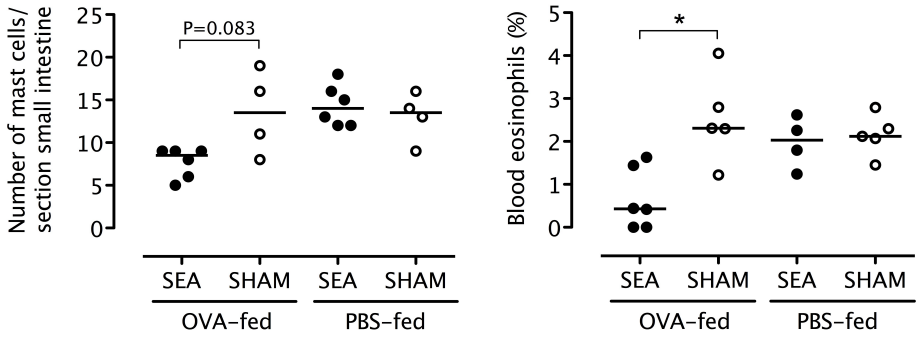

Figure 9. Neonatal SEA exposure leads to increased ability to develop oral tolerance in a mouse model of food allergy, as evidenced by fewer mast cells in the small intestine and less blood eosinophilia.

What is the mechanism behind the improved ability for oral tolerance following neonatal exposure to superantigen?

In order to investigate the mechanism behind the ability of SEA to enhance oral tolerance, we examined the immune status of the mice at the time for OVA feeding. We wanted to exclude the possibility that the observed improvement in oral tolerance would be due to the elimination of certain cell subsets or certain TCRVβ types or T cell unresponsiveness. When investigating lymphocytes and their activation markers four weeks after SEA treatment (at the time of OVA feeding), we found no abnormalities in the mice that were neonatally treated with SEA, confirming the absence of ongoing inflammation. For example, the numbers of B cells, DCs, and $CD4^+$ and $CD8^+$ T cells were similar. In addition, the activation status of the T cells in SEA-treated mice compared to control mice was comparable. Further, no long-term alterations of the TCRVβ repertoire or any persistent T cell anergy was noted.

Because the generation of Tregs is one of the major mechanisms behind oral tolerance, the presence and function of this T cell subset was studied. We were not able to find any increase in the proportion or numbers of Tregs ($CD4^+FoxP3^+$) in the MLNs four weeks after SEA treatment. Moreover, the suppressive capacity of $CD4^+CD25^+$ T cells was similar in SEA-treated mice as control mice. However, we noted that a higher proportion

of Tregs, as well as a higher proportion of all T and B cells expressed the gut-homing molecules α4β7 and CCR9 in mice that had been treated neonatally with SEA (Fig. 10). In agreement, the number of FoxP3-expressing cells in the lamina propria of the small intestine was higher at the time of OVA feeding (Fig. 10).

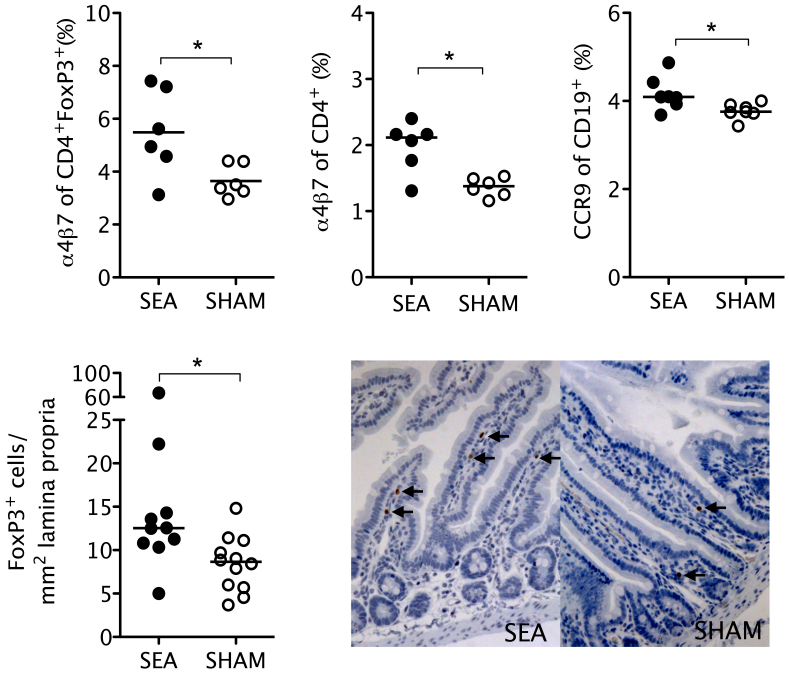

Figure 10. Neonatal SEA treatment increases the gut migratory potential of lymphocytes and leads to the accumulation of FoxP3⁺ T cells in the small intestinal lamina propria.

Given that DCs are important for the induction of oral tolerance, generation of induced Tregs, and imprinting of gut tropism on T cells, we investigated the CD11c⁺ DC population in the MLN at the time for oral tolerization. SEA treatment did not have any major long-term impact on the proportion or phenotype of the CD11c⁺ DCs in the mesenteric lymph nodes. However, we found that SEA exposure decreased the proportion of CD40-expressing DCs to some degree. Moreover, lower antigen-presenting ability of the CD11c⁺ DCs after SEA exposure was demonstrated, both in the MLN and the spleen, suggesting that neonatal SEA treatment favor tolerogenic DCs.

In conclusion, the findings of these two papers demonstrate that neonatal SEA treatment result in an increased ability for oral tolerance several weeks after treatment. We also found that this improvement is likely dependent upon an interaction between gut-

residing DCs and gut-migrating lymphocytes, particularly FoxP3$^+$ Tregs. Neonatal SEA treatment appears to create a more tolerogenic environment in the gut that persists long after treatment.

Paper III

Can blocking the vitamin A metabolism *in vivo* abrogate the improvement in oral tolerance seen after neonatal SEA treatment?

CD103$^+$ DCs in the gut induce gut tropism in a retinoic acid-dependent manner. In addition, retinoic acid together with TGF-β are involved in the conversion of naïve T cells into induced regulatory T cells. CD103$^+$ DCs and the production of retinoic acid have consequently been suggested to be important for the development of oral tolerance [153]. We wanted to investigate whether blocking the vitamin A metabolism *in vivo* would affect the ability to induce oral tolerance in the food allergy model. Interestingly, the improvement of oral tolerization offered by SEA treatment was abolished in mice that were given the RALDH inhibitor Citral during the oral tolerization phase. This was demonstrated by increased infiltration of mast cells and a tendency toward increased blood eosinophilia in SEA-treated OVA-fed mice given Citral compared to SEA-treated OVA-fed mice not given Citral (Fig. 11). Moreover, Citral did not affect the sensitization of non-tolerized mice, showing that Citral has no general immunogenic effect in this model. We conclude that the improved oral tolerance to OVA observed in mice that had received SEA in the neonatal period was dependent upon an intact vitamin A metabolism.

The increased gut-migratory potential noted after neonatal SEA treatment (seen in paper II) combined with the importance of a functional vitamin A metabolism for the improvement in oral tolerance suggest that the CD103$^+$ DCs have a significant role in our models. When investigating the CD11c$^+$ DCs in the MLN four weeks after SEA treatment, no increased proportion of CD103$^+$ DCs was observed. However, an accumulation of RALDH2-expressing cells in the small intestinal lamina propria was seen in SEA-treated mice.

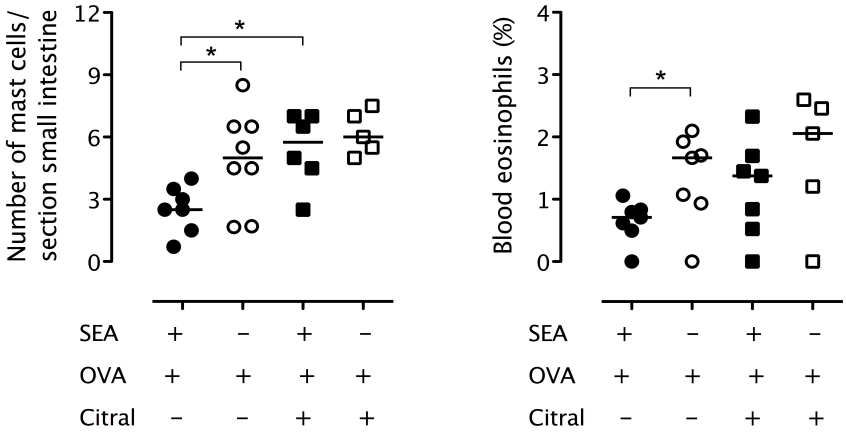

Figure 11. Inhibiting the vitamin A metabolism *in vivo* abrogates the improved oral tolerance observed following neonatal SEA treatment, as evidenced by increased sensitization (mast cell infiltration in the gut and blood eosinophilia) in SEA-treated OVA-fed mice after Citral intake prior to tolerization and sensitization in the food allergy model.

Flow cytometric analysis of $CD11c^+$ DC subsets ($CD103^+$ and $CD103^{neg}$) in the MLN showed that SEA exposure resulted in lower proportion of $CD103^{neg}$ DCs expressing CD40 and CD86. In addition, an increased expression of MHC class II on $CD103^{neg}$ DCs was observed. The phenotype of $CD103^+$ DCs was not affected by SEA treatment. Further, Citral intake resulted in decreased MHC class II expression, both on $CD103^+$ and $CD103^{neg}$ DCs, regardless of prior SEA treatment.

To investigate whether DCs isolated from SEA-treated mice were more prone to imprint gut-homing potential to T cells compared to DCs from control mice and whether this function was dependent upon the vitamin A metabolism, *in vitro* DC-T co-cultures were performed. $CD11c^+$ DCs were separated from MLNs four weeks after SEA exposure, pulsed with OVA, and co-cultured with DO11.10 T cells. Expression of the gut-homing marker CCR9 on the T cells was investigated. Cultures with DCs from SEA-treated mice induced a higher expression of CCR9 than cultures with DCs from SHAM-treated mice (Fig. 12). The capacity of the DCs to enhance CCR9 expression was suppressed by the RALDH inhibitor Citral or the RAR antagonist LE135 (Fig. 12). These results show that neonatal SEA treatment promotes DCs with an increased ability to induce gut-homing receptors on T cells. In addition, this function is dependent upon a functional vitamin A-

converting system in the DCs. Thus, the increased gut-homing potential of T cells observed *in vivo* was replicated *in vitro*.

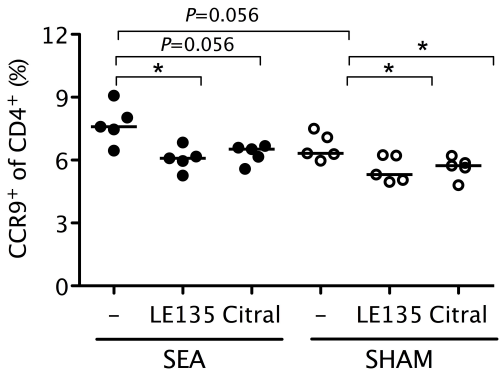

Figure 12. Neonatal SEA treatment generates DCs with an increased capacity to imprint gut- homing potential to T cells and this function is dependent upon the vitamin A metabolism.

When investigating the occurrence of FoxP3-expressing cells in the small intestine after Citral intake, the increased number of regulatory T cells in the gut observed after SEA treatment was abrogated (Fig. 13). As the RA-conversion of naïve T cells into regulatory T cells is TGF-β dependent, we also stained the small intestine for TGF-β expression and found an increase of TGF-β $^{+}$ cells in the lamina propria after neonatal SEA treatment which also was diminished by Citral intake (Fig. 13). These results demonstrate that the observed increase in both FoxP3^{+} and TGF-β^{+} cells after neonatal SEA treatment, depend on functional RALDH activity.

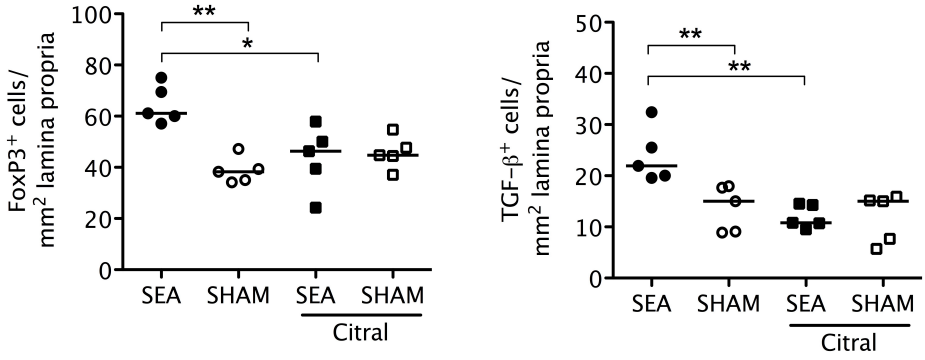

Figure 13. The accumulation of FoxP3^{+} and TGF-β^{+} cells in the small intestinal lamina propria following neonatal SEA treatment is diminished when blocking the vitamin A metabolism.

In conclusion, the findings of this paper demonstrate that neonatal SEA treatment affects gut DCs, inducing prolonged capacity in this subset to evoke gut-homing potential to T cells. Further, this effect is vitamin A-dependent. In addition, the improved oral tolerance and accumulation of putative regulatory T cells in the gut observed after neonatal SEA treatment is dependent upon the vitamin A metabolism.

Paper IV

Do *S. aureus* colonize the gut differently depending upon their ability to produce superantigen?

In paper IV, the capacity of different *S. aureus* strains to colonize the gut of germ-free mice was examined. *S. aureus* is not a specialized gut colonizer; it is a skin bacterium that can establish in the gut flora in the absence of competition from more professional gut bacteria. *S. aureus* was administered to germ-free mice by gavage. The mice were then taken out of the isolators to spontaneously acquire microbes from the environment. Shortly after colonization, all mice had *S. aureus* in high numbers. However, after a few weeks under conventional housing conditions, the mice acquired a variety of facultative and anaerobic bacteria. At the same time, the population counts of *S. aureus* decreased, suggesting that *S. aureus* is less fit for competition with an increasingly complex anaerobic flora. The four examined strains had fairly equal colonizing capacity. Thus, the capacity of the strains to produce different superantigens did not affect the ability to colonize the gut in this model. However, each animal was only colonized by a single *S. aureus* strain, and differences might have been revealed if the *S. aureus* strains were allowed to compete directly with one another.

Does colonization with superantigen-producing *S. aureus* affect oral tolerance?

Although the different superantigen-producing strains colonized the gut with similar efficiency, different immunological effects were noted. Mice were given a semi-tolerizing dose of OVA six weeks after colonization and then sensitized and challenged in the experimental food allergy model. Colonization with the *S. aureus* strain that was able to produce SEA was associated with decreased blood eosinophilia, increased intestinal IgA levels, and higher proportion of Tregs ($CD4^+FoxP3^+$) in the MLN, compared to colonization with a non toxin-producing strain (Fig. 14).

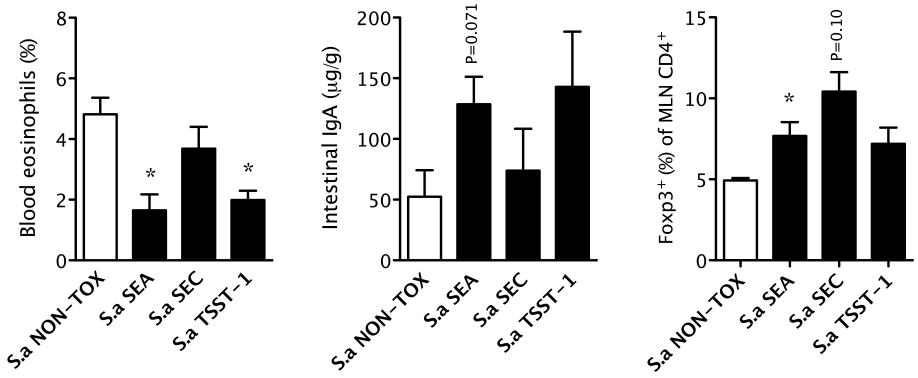

Figure 14. Colonization with superantigen-producing *S. aureus* improves oral tolerance, as evidenced by less blood eosinophilia after sensitization and challenge in a food allergy model. Further, mice colonized with the SEA-producing strain had higher levels of IgA in the intestine as well as higher proportion of Tregs in the MLN compared to mice colonized with a *S. aureus* strain without the capacity to produce any superantigen.

How do different *S. aureus* bacterial cells and pure superantigens affect cells from germ-free mice *in vitro*?

As superantigen-producing *S. aureus* strains tended to have different effects on the immune system than a non toxin-producing strain when colonizing germ-free mice, we examined the effect of pure superantigens as well as UV-inactivated *S. aureus* on immune cells *in vitro*. When stimulating splenocytes from germ-free mice with purified superantigens (SEA, SEB, SEC and TSST-1), various potency levels were found, with SEA and TSST-1 inducing the most vivid T cell proliferation. In addition, a dose-dependent increase in the proportion of FoxP3 was noted among the T cells when stimulated with SEA, and also SEC in high doses (Fig. 15). Notably, colonization with SEA- (P=0.036) and SEC- (P=0.10) producing strains also resulted in high proportion of FoxP3 expressing cells *in vivo* (Fig. 14). Stimulation with intact *S. aureus* bacterial cells resulted in poor T cell activation but induced strong B cell activation.

In conclusion, the findings of this paper demonstrate that colonization with superantigen-producing *S. aureus* can increase the capacity for oral tolerance, which is in accordance with our findings in human infants in whom early colonization by *S. aureus* is linked to protection from food allergy [149]. The uniquely strong T cell-activating capacity of *S. aureus* enterotoxins may provide the developing immune system with adequate activation and maturation signals.

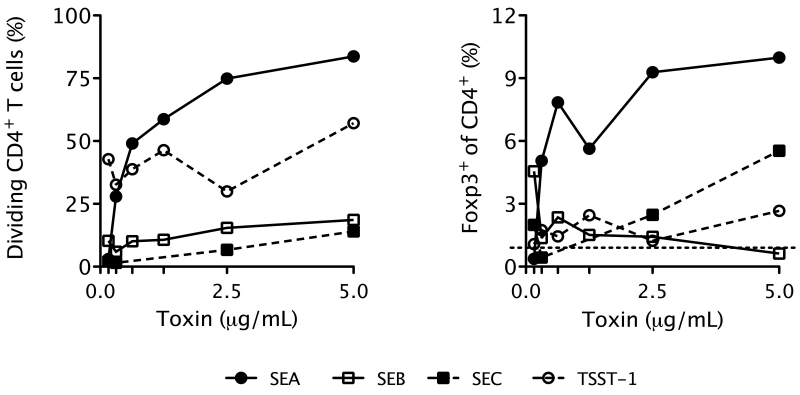

Figure 15. *In vitro* stimulation of splenocytes reveals differences in T cell stimulatory properties, with SEA being the most potent. In addition, *in vitro* stimulation with SEA and SEC generates FoxP3+ T cells.

Additional data

How does neonatal SEA treatment directly affect the thymus and the spleen?

Preliminary results show that mice treated with SEA early in life have increased thymus size and, consequently, higher numbers of CD4+ and CD8+ cells as well as CD4+CD8+ double positive cells shortly after SEA exposure (3 h after the final dose; Fig. 16). However, the proportion of double positive cells decreased after SEA treatment, whereas the proportions of CD4+ and CD8+ T cells increased (Fig. 16).

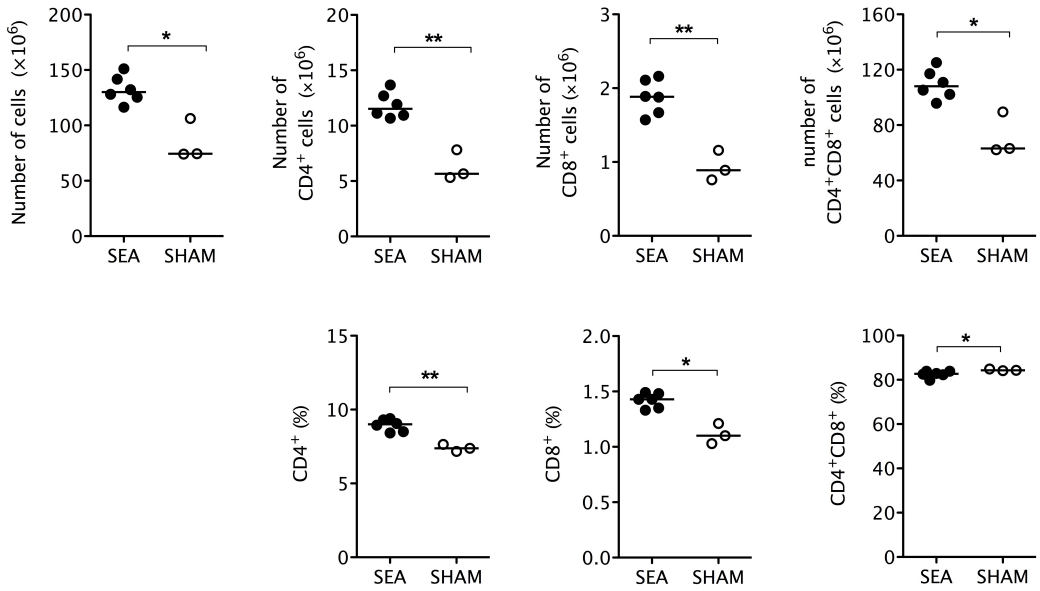

Figure 16. Neonatal SEA treatment results in increased thymus size and, consequently, leads to higher number and proportion of CD4+ and CD8+ T cells in the thymus.

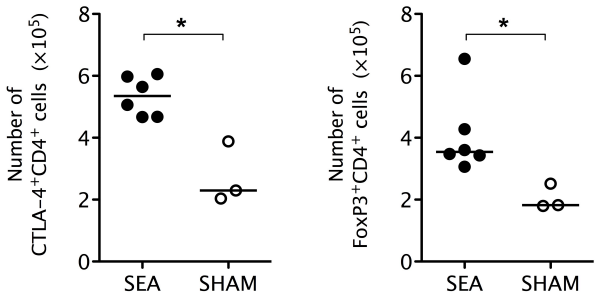

Figure 17. Neonatal SEA treatment results in higher numbers of putative regulatory T cells in the thymus.

Accordingly, a greater number of $CD4^+$ T cells in the thymus leads to a greater number of Tregs (CTLA-4 or FoxP3 expressing $CD4^+$ cells; Fig. 17), even though the proportion of Tregs did not differ between SEA- and SHAM-treated mice. At the same time point, an increased spleen size and number of T cells and Tregs was observed (Fig. 18).

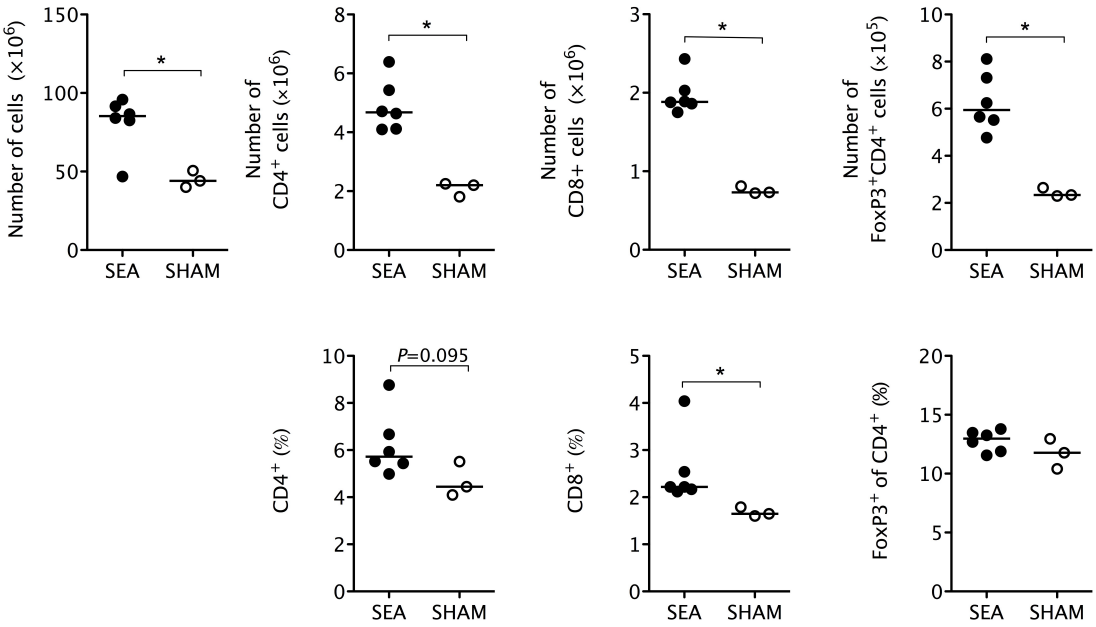

Figure 18. Neonatal SEA treatment results in increased spleen size and consequently leads to higher numbers of $CD4^+$, $CD8^+$ T cells and $FoxP3^+$ Tregs in the spleen.

These results suggest that there is a higher output of T cells from the thymus shortly after superantigen exposure, which might reflect the supposedly massive T cell activation and subsequent deletion and associated need to restore the T cell population.

Discussion

In these studies, we have shown that neonatal exposure to superantigen or colonization of germ-free mice by superantigen-producing *S. aureus* improves the ability to actively tolerize a fed protein antigen. The antigen dose selected for tolerization in these studies was optimized to induce partial tolerance, which enabled us to study potential improvement in oral tolerance induction. Oral tolerance manifested as reduced responses in experimental allergy models. Further, this tolerance was enhanced in mice that were treated neonatally with SEA. This improvement in oral tolerance following SEA treatment was abrogated when blocking the vitamin A metabolism.

The mechanism behind oral tolerance is unclear, as to understand how and where antigens are presented to the immune system to induce tolerance rather than immunity. Nevertheless, newborn pups that were given oral doses of SEA improved their ability to become orally tolerized. The enhanced capacity for oral tolerance must involve long-term changes to or priming of the immune system, as tolerization occurred four weeks after the last SEA dose was given. When studying the improvement in oral tolerance, one or more of the following aspects of the immune response was taken into consideration:

- APCs and antigen presentation in the gut
- Regulatory T cells
- The handling of antigens by epithelial cells

Superantigen and antigen-presenting cells

It is known that antigens that enter the body via the gut are accepted by the immune system and lead to development of tolerance. Further, APCs in the gut-associated immune system are primed to present antigens in a tolerogenic, rather than an immunogenic fashion [154]. It has been shown that DCs residing in the intestinal lamina propria and the MLN have tolerogenic properties and favor the development of Tregs [154]. Different DC subsets have been suggested to be important for tolerance induction. Semi-mature APCs express lower levels of co-stimulatory molecules, resulting in a decreased ability for antigen presentation and subsequent T cell activation. Another DC

subset in the GALT, CD103^{+} DCs, are believed to have an impact on oral tolerance by enhancing the TGF-β-dependent conversion of naïve T cells into Tregs and to imprint gut-homing phenotype to lymphocytes via the production of retinoic acid. These two DC subsets have both been suggested to be migrating under steady-state conditions in the GALT (i.e. during normal conditions in the absence of inflammation).

Although the impact of superantigens on T cells is well established, less is known about their direct effects on APCs, despite the fact that binding to MHC class II on APCs is fundamental for the action of superantigens. However, it has been shown that APCs produce high levels of cytokines and chemokines including IL-1α, IL-1β, TNF-α, IL-8, IL-10, IL-12, CCL3, and CCL5 shortly after exposure to superantigen [155-158]. Superantigens also induce migration and maturation of DC populations *in vivo*. For example, injection (i.p., i.v.) of a superantigen into mice induces DC maturation, shown by the upregulation of MHC class II, CD40, CD80 and CD86 [159, 160]. In addition, superantigens fail to induce DC maturation in RAG mice (lacking both T and B cells) or MHC class II-deficient mice, suggesting that T cell activation is a prerequisite for this matter [159].

In our experiments, the DC population of adult mice that had been treated with SEA neonatally was more "tolerogenic" than DCs from control mice, since less antigen-specific T cell proliferation was induced. These functional changes were accompanied by some phenotypical alterations, such as increased expression of MHC class II and decreased expression of CD86 and CD40 that are typical characteristics for semi-mature DCs. Higher expression of antigens via MHC class II, in combination with absence of full co-stimulation, would favor antigen-specific tolerance. The changes were confined to the CD103^{neg} subset, but inhibition of RALDH markedly altered the phenotype in both the CD103^{neg} and CD103^{+} DCs. Thus, stating a clear dependency of RALDH for DC phenotype in the gut. As RALDH is highly expressed by the CD103^{+} DC subset, these findings might be explained by a chain of interactions: CD103^{+} DCs induce gut-homing regulatory T cells in a TGF-β and RA-dependent manner, and the regulatory T cells that circulate through the gut induce functional and phenotypic alterations of DCs in the GALT. In this way, the phenotypic changes on CD103^{neg} DCs will be dependent on RA *in vivo*.

In agreement, neonatal SEA treatment resulted in higher RALDH expression in the lamina propria in adult mice. This suggests an expansion of the $CD103^+$ DC subset in the gut. The decreased *in vitro* T cell stimulatory properties of the $CD11c^+$ DC population isolated from SEA-treated mice, could depend on the functionality of the $CD103^+$, $CD103^{neg}$, or both subsets. In addition, we found higher CCR9 expression on T cells stimulated with MLN-isolated DCs from SEA-treated than from control mice, which implies a better activity of the gut-imprinting $CD103^+$ DC population. In conclusion, we believe that both the $CD103^+$ and $CD103^{neg}$ DC population are primed, directly or indirectly, by neonatal SEA treatment to improve tolerogenic antigen-presenting ability.

Superantigen and T cells

Staphylococcal superantigens bind to and activate specific T cells with regards to their TCRVβ region. The TCRVβ repertoire of mature T cells in mice is mainly formed under the influence of endogenous retroviral superantigens [161-163]. Clonal deletion of particular Vβ is caused by superantigens encoded by endogenous MMTV (mouse mammary tumor virus) [162, 164-167]. MMTV is transmitted via maternal milk [168], and infections result in endogenous proviruses incorporated in the genome of most mouse strains [169]. The elimination of certain Vβ subsets has been shown to occur during T cell maturation in the thymus, as mature single-positive CD4 and CD8 T cells display an altered Vβ repertoire compared to double-positive T cells [170]. BALB/c mice harbor the proviruses *Mtv*-6, *Mtv*-8, and *Mtv*-9 [169], leading to partial or complete elimination of the Vβ3, Vβ5.1, Vβ5.2, Vβ11 and Vβ12 subsets on mature T cells [170]. This natural deletion of specific Vβ subsets in BALB/c mice might explain the minor differences of the Vβ repertoire between SEA- and SHAM-treated BALB/c mice in our studies.

Superantigen and regulatory T cells

Tregs located in the gut play a central role to maintain immune homeostasis in the gut [171], and the interplay between DCs and Tregs is important for oral tolerance. Tolerogenic DCs favor the development of Tregs. In addition, Tregs are responsible for providing DCs with tolerogenic signals, thus keeping them in a tolerogenic state.

Experimental studies have indicated that exposure to superantigen favors the development and functional activity of Tregs. For example, repeated i.v. injections of SEB to mice result in the development of T cells with a regulatory function [172, 173], an increased *in vitro* suppressive capacity of isolated Tregs [174], and increased serum levels of IL-10 [173], which is known to counteract T cell proliferation and IFN-γ production. In addition, pre-activation of Tregs with superantigen has been shown to enhance their suppressive potency [175].

Still, we have not been able to show that neonatal SEA treatment generates increased numbers of or functionally more suppressive Tregs ($CD4^+FoxP3^+$) in the MLN of adult mice. However, SEA exposure increases the density of FoxP3-expressing cells in the lamina propria of the small intestine, which is consistent with the finding of more lymphocytes in the MLN with gut-homing potential (i.e. expression of CCR9 and α4β7). Despite the increased gut-homing potential of all lymphocytes, we found no general increase in cell infiltration in the intestinal mucosa after SEA treatment apart from higher numbers of $FoxP3^+$ cells. This might depend upon the intensity of the expression of the gut-homing molecules, given that the $FoxP3^{neg}$ T cells expressed lower intensity of the molecules compared to $FoxP3^+$ T cells. These $FoxP3^+$ cells could contribute to the tolerogenic environment in the gut by rendering gut DCs more tolerogenic before they migrate to the MLN and, in turn, generate more Tregs. Because DCs are more short-lived than T cells, we speculate that Tregs and their influence on APCs, thus providing a self-sufficient system of Tregs and tolerogenic DCs in the GALT, mediate the long-term tolerance-improving effect of SEA.

Superantigen and the handling of antigens by intestinal epithelial cells

Intestinal epithelial cells actively participate in the establishment of the tolerogenic environment in the gut. It has been known since the 1980s that a fed protein acquires a tolerogenic phenotype by passing through the intestinal epithelium, a process termed "gut processing" or "tolerogenic processing" [83]. Epithelial cells produce small membranous vesicles carrying MHC class II molecules loaded with peptides from a dietary antigen on their surface [34]. These "tolerosomes" convey a tolerogenic message (i.e. recipients of tolerosomes develop antigen-specific tolerance) [34]. It can be

anticipated that tolerosomes reach lymphoid organs via the lymph and/or blood stream where they may merge with APCs, which present the contained dietary peptides to naïve re-circulating T cells in a tolerogenic fashion.

One possible way in which neonatal SEA treatment could regulate tolerance induction is by increasing tolerogenic processing. SEA feeding is known to induce vigorous activation of the IEL population [176], producing large quantities of IFN-γ [144-146]. IFN- γ, in turn, facilitates tolerogenic processing [177]. Interestingly, in collaboration with another research group, we investigated whether mucosal exposure to SEA could influences the tolerogenic processing by the intestinal epithelium. The enhanced ability for oral tolerance after SEA treatment was transferable to naïve recipient mice by the adoptive transfer of serum, suggesting that the exosome fraction produced by SEA-exposed epithelium more efficiently modulates the immune system into a tolerogenic response to the fed antigen (Lindgren *et al*, in manuscript). SEA-exposed mice had significantly higher numbers of IEL compared to control mice three days after superantigen exposure for five days via the drinking water. Four weeks after neonatal SEA treatment, IEL expansion was not observed. Moreover, it is unlikely that they would still be activated to secrete high levels of IFN-γ. Nevertheless, it remains to be investigated if tolerogenic processing is improved after neonatal SEA treatment and whether our observed improvement in oral tolerance depends upon priming of the gut mucosa by IEL and IFN-γ.

Connection between experimental and clinical observations

Neonatal exposure to staphylococcal superantigen or colonization of germ-free mice with commensal *S. aureus* gave rise to immune modulation that in many aspects resembled that seen in newborn human infants colonized with superantigen-producing *S. aureus*. Infants colonized in the gut by *S. aureus* during the first week(s) of life are protected from development of food allergies [149], and have increased numbers of putative Tregs in the blood at four months of age (Östman *et al*, in manuscript). Further, higher levels of serum IgA in infants colonized by superantigen-producing *S. aureus*, but not by colonization by other *S. aureus* strains, have been observed [152]. The uniquely strong T cell-activating capacity of *S. aureus* enterotoxins may provide the developing

immune system with the adequate activation and maturation signals that may be lacking in infants with a poorly developed intestinal microflora.

Whether *S. aureus* colonizing the gut in fact produce their toxins *in situ* and in quantities sufficient to afford immune activation is unknown. Although the temperature in the intestinal lumen is optimal for toxin production [178], the oxygen level might be too low [179]. Moreover, products secreted from other bacteria may dampen the ability of *S. aureus* to produce toxins. For example, lactic acid, produced by many intestinal bacteria under anaerobic conditions, is a strong inhibitor of enterotoxin production [180]. However, during the first days of life, when the oxygen content still is high in the gut and more or less devoid of lactic acid-producing anaerobes, the intestinal microenvironment may be favorable for superantigen production. The increased capability of *S. aureus* to produce superantigens during these first weeks might explain why colonization by *S. aureus* has to occur early in life to confer protection against allergy development, given that infants colonized by *S. aureus* after two weeks of age are not protected from food allergy. However, this does not exclude effects mediated by the bacterium itself, which may provide other maturation signals. For example, almost all *S. aureus* strains have protein A on their surface, a potent B cell mitogen [181].

Further, mice treated with SEA at adult age do not show enhanced ability for oral tolerance suggesting a greater impact of the superantigen on immune regulation during maturation of the immune system. Tregs are central in oral tolerance, and an important "window" during Treg development in early life has been suggested. Thymectomy of mice at three days after birth leads to multiorgan autoimmune disease, which can be restored by the adoptive transfer of Tregs [182]. This is not observed if the thymus is removed at a later stage. Therefore, the first days of life seems to be important for establishing a sufficient Treg repertoire. It has also been observed in infants that the number of putative Tregs ($CD4^+CD25^+FoxP3^+$) expands during the first postnatal days of life [183]. A high number of Tregs in the blood at four months of age is positively correlated with colonization by *S. aureus* as early as day three of life, whereas later colonization is not associated with Treg counts in the blood (Östman *et al*, in manuscript). Preliminary results show that mice treated with SEA early in life have increased thymus size and a higher number of $CD4^+$ and $CD8^+$ cells as well as $CD4^+CD8^+$ double-positive cells shortly

after SEA exposure, suggesting a higher output of T cell, and, consequently, higher number of Tregs.

Summary: Superantigen and the regulatory immune response

We believe that the largest factor contributing to the enhanced tolerance observed after neonatal superantigen treatment is the Tregs. As the APC population of the gut mucosa is renewed within a few days, the impact on APCs cannot be caused by a direct effect of neonatal superantigen treatment. It is also unlikely that the superantigen induce permanent changes in the gut epithelium that also regenerate within days. Instead, neonatal exposure to superantigen must expand or mature a long-lived cell subset that can imprint a tolerogenic phenotype upon the gut APCs. Tregs are a candidate cell population that could exert these properties. These Tregs can be generated by one or more of the following mechanisms. Further, a possible hypothesis regarding the direct and long-term effects of superantigens on the immune system is depicted in Figure 19.

Mechanism I: Generation of Tregs by increased tolerogenic processing

In neonatal mice, the gut flora triggers the expression of MHC class II in the intestinal epithelium, a process that takes a few weeks. By administering superantigen to neonatal mice, this process will be enhanced as the superantigen traverses the epithelium and activates IEL to produce IFN-γ, which upregulates MHC class II in the intestinal epithelial cells. Expression of MHC class II is a prerequisite for tolerogenic processing, a mechanism leading to the generation of Tregs. Consequently, superantigen-treated neonates may have extended tolerogenic processing and thereby increased generation of Tregs.

Mechanism II: Increased output of natural Tregs from the thymus

The generation of nTregs in the thymus of mice may take place during the first days of life, as demonstrated experimentally. Neonatal exposure to superantigen leads to increased thymus size and increased numbers of Tregs in the thymus. If the nTreg development is limited to a certain time period, the superantigen activation may actually increase the number of circulating nTregs. The nTregs, mainly specific for self-antigens, could also favor tolerance towards oral antigen by downregulating the

immunostimulatory capacity of APCs, presenting self-antigens at the same time as presenting oral antigens.

Mechanism III: Tregs produced as a negative feedback mechanism of T cell activation

The superantigen binds to MHC class II on APCs present in the lamina propria. Those APCs migrate to the MLN, where they vigorously activate T cells. In addition, the cross-linking of APCs and T cells by a superantigen also leads to APC maturation with higher expression of MHC class II and co-stimulatory molecules, in favor of antigen presentation and co-stimulatory signals to naïve T cells. The T cell activation is followed by clonal deletion of most activated T cells, whereas a few become memory T cells. It is likely that Tregs will become induced as a negative feedback mechanism of the massive T cell proliferation. The Tregs then act by dampening the APCs in the MLN and lower the immune response. In addition, Tregs generated in the MLN migrate to the site of inflammation, where they also dampen the immune response by acting on effector T cells and on APCs. In turn, this gives a self-sustained system where Tregs keep APCs in a tolerogenic state, and presentation of antigens by tolerogenic APCs leads to more Tregs homing to the gut and so on.

In summary, we believe that neonatal exposure to superantigen leads to a faster and more potent immune maturation of the neonatal gut that favor the development of Tregs by any, or all of the aforementioned mechanisms. These Tregs then sustain a prolonged immunoregulatory effect by promoting tolerogenic signals to the APCs and the local environment in the gut, in favor of the development of oral tolerance.

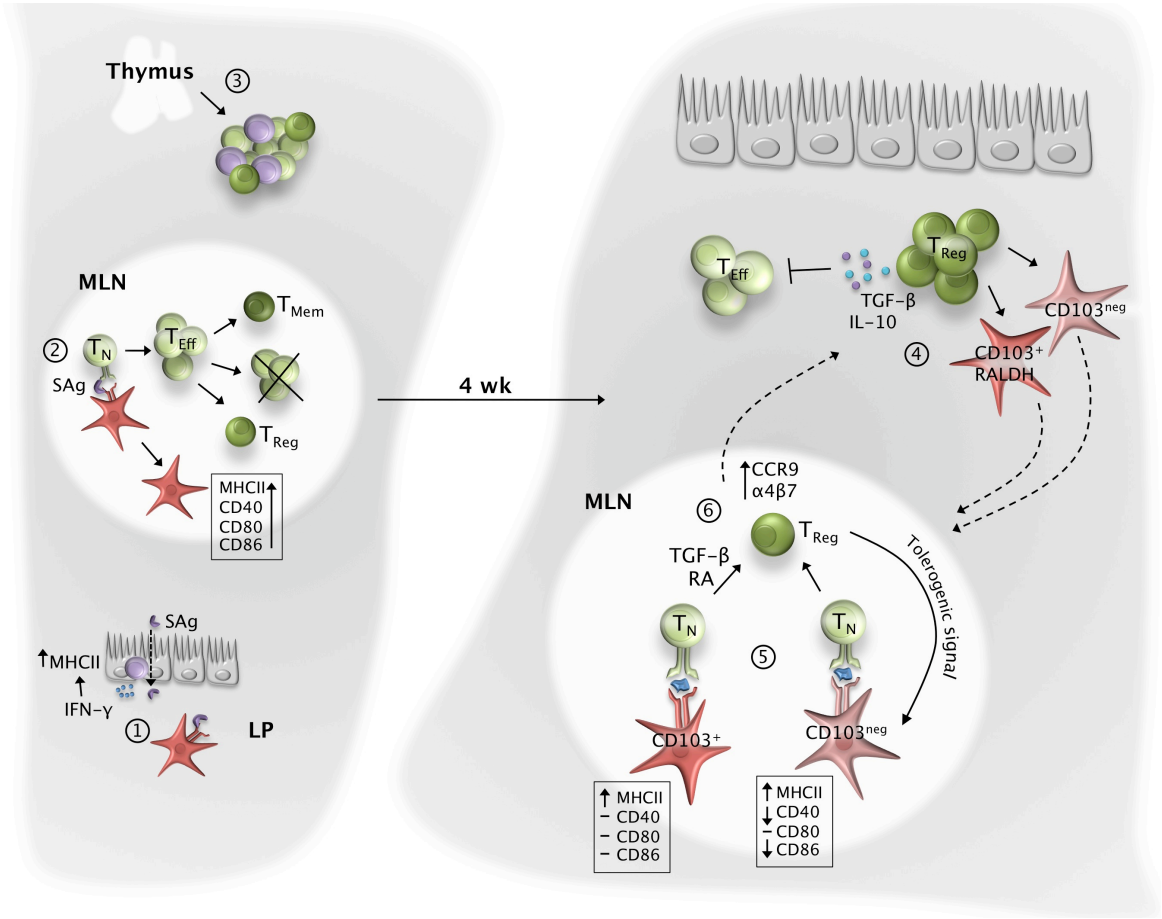

Figure 19. Hypothesis of the direct and long-term effects on the immune system by superantigen. ① Superantigen (SAg) produced by *S. aureus* colonizing the gut traverses the gut epithelium and activates IEL to produce IFN-γ. Production of IFN-γ upregulates MHC class II in intestinal epithelial cells in favor of tolerogenic processing and, in turn, generation of Tregs. The superantigen bind to MHC class II on APCs in the lamina propria, which migrate to the MLN where they vigorously activate T cells. ② The massive T cell activation is followed by clonal deletion and the generation of memory T cells and Tregs. In addition, superantigen also upregulate MHC class II and co-stimulatory molecules on the APC. ③ To compensate for the massive T cell depletion, the thymic output of T cells is enhanced, resulting in a larger T cell population and, consequently, more Tregs. ④ In the long-term, the increased density of Tregs in the lamina propria and their production of IL-10 and TGF-β control effector T cells in the gut, and condition dendritic cells in the lamina propria giving them a tolerogenic phenotype. Tolerogenic DCs migrate during steady-state to the MLN, where they interact with naïve T cells. ⑤ Tolerogenic DCs may convert naïve T cells into Tregs, either by $CD103^+$ DCs in a TGF-β and RA-dependent manner or via antigen presentation by semi-mature DCs. In turn, regulatory T cells might deactivate dendritic cells with which they interact via CTLA-4, generating tolerogenic DCs. ⑥ T cells activated in the MLN are imprinted with a gut-homing phenotype, which enables migration to the lamina propria. This results in a self-sustained system where Tregs keep APCs in a tolerogenic state, and presentation of antigens by tolerogenic APCs leads to more Tregs homing to the gut.

Concluding remarks

The hygiene hypothesis states that exposure to microbes during early infancy protects against later development of allergy. So far, no particular microbe has been shown to possess a full immunomodulatory effect. It still remains elusive in what way microbes can promote the developing immune system. This thesis provides insights into how bacterial superantigens influence the development of tolerance to fed antigens and reveal evidence for important mechanisms acting to prevent allergy development.

Populärvetenskaplig sammanfattning

Vårt immunförsvar har till främsta uppgift att skydda oss från infektioner som orsakas av bakterier, virus och parasiter. När en mikrob lyckas invadera kroppen reagerar olika immunceller och kraftfulla reaktioner leder till att mikroberna elimineras. Det är dock avgörande att immunsystemet inte reagerar emot ofarliga ämnen från den egna kroppen eller från omgivningen via födan, huden eller luften. Angrepp på kroppens egna ämnen orsakar autoimmuna sjukdomar och allergier uppstår när försvaret riktas mot exempelvis pollen eller födoämnen. För att undvika sådana oönskade reaktioner har immunsystemet utvecklat effektiva regleringsmekanismer. En viktig del i denna reglering förmedlas av speciella immunceller som kallas regulatoriska T-celler. Dessa har förmåga att stänga av och/eller dämpa andra immunceller och är avgörande för att upprätthålla balans inom immunsystemet. Varje gång ett nytt födoämne passerar genom mag-tarmkanalen bildas regulatoriska T-celler som förhindrar uppkomsten av allergi emot det nya födoämnet.

Under de senaste decennierna har man kunnat se en kraftig ökning av sjukdomar som orsakas av att immunsystemet reagerar fel. Det finns olika teorier till varför sjukdomar av det här slaget har ökat så mycket. En tänkbar förklaring ges av den s.k. hygienhypotesen, vilken föreslår att ökningen av autoimmuna sjukdomar och allergier som skett i västvälden beror på att vi numera är mindre utsatta för infektioner och överlag har färre bakterier i vår omgivning. Enligt hygienhypotesen behöver immunsystemet en viss grad av mikrobiell stimulering för att det ska utvecklas och mogna ordentligt och fungera korrekt.

Vår forskargrupp har funnit att barn som mycket tidigt koloniseras i tarmen med stafylokocker (*Staphylococcus aureus*) löper lägre risk att utveckla födoämnesallergi. Varför just dessa bakterier kan ge skydd mot allergiutveckling är inte helt klarlagt. Dock producerar de flesta *S. aureus* toxiner, så kallade ”superantigen”, som är mycket kraftfulla. De kan orsaka matförgiftning om man får i sig för mycket, men en liten mängd som produceras av bakterier i tarmen skulle kunna stimulera immunsystemet på ett

gynnsamt sätt. För att testa denna hypotes har vi koloniserat möss med olika *S. aureus* bakterier och även testat att ge nyfödda möss små doser av *S.aureus* superantigen.

I det första arbetet visade vi att musungar som fått superantigen hade en förbättrad förmåga att acceptera främmande födoämnesantigen dvs. en förbättrad oral tolerans. Vuxna möss som fick samma behandling med superantigen fick dock ingen förbättrad tolerans och detta visar att immunstimuleringen måste ske tidigt i livet för att ha en positiv inverkan på immunsystemet.

I det andra arbetet ville vi undersöka mekanismen bakom den förbättrade orala toleransen. Vi fann bland annat att musungar som fått superantigen hade fler regulatoriska T-celler i tarmen vid vuxen ålder. Dessa regulatoriska T-celler kan påverka andra immunceller att reagera mindre. En viktig celltyp är så kallade antigen-presenterande celler. Dessa plockar upp och presenterar antigen för andra immunceller som då aktiveras och startar immunreaktioner. När vi testade de antigen-presenterande cellernas förmåga att stimulera andra celler fann vi att de var mindre effektiva och detta är troligen en bidragande faktor till den förbättrade orala toleransen som vi ser hos möss som behandlats med superantigen.

Vi vet att antigen-presenterande celler i tarmen har förmåga att bryta ner vitamin A till retinolsyra och att denna mekanism ger regulatoriska T-celler med förmåga att ta sig till tarmen. Eftersom vi funnit fler regulatoriska T-celler i tarmen hos möss som behandlats med superantigen ville vi i det tredje arbetet undersöka om vitamin A metabolismen påverkades av superantigen. Vi fann fler celler med förmåga att omvandla vitamin A till retinolsyra i tarmen hos superantigen-behandlade möss och när vi sedan blockerade vitamin A metabolismen, genom att ge en blockerande substans i dricksvattnet, fann vi att den förbättrade förmågan till oral tolerans hos möss som behandlats med superantigen försvann.

I det sista arbetet ville vi studera om kolonisering med olika *S. aureus* bakterier kunde påverka förmågan att utveckla oral tolerans. Vi fann att *S. aureus* bakterier som hade förmågan att producera vissa superantigen var mer effektiva till att förbättra toleransförmågan än *S. aureus* bakterier som inte kan producera superantigen.

Sammanfattningsvis, hygienhypotesen föreslår att tidig exponering för mikrober skyddar mot allergiutveckling senare i livet. Än så länge har ingen specifik mikroorganism identifierats som innehar immunreglerande effekt och det är fortfarande oklart hur mikrober kan gynna utvecklingen och mognaden av immunsystemet. Denna avhandling ger en inblick i hur bakteriella superantigen kan påverka utvecklingen av oral tolerans och ger även belägg för att tidig stimulering av immunsystemet för att kompensera för den minskade mängden bakterier i vår omgivning följaktligen kan skydda mot allergi.

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