On the regulation of immune responses to dietary and self antigens

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Finding out, in balance

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Till min familj

ABSTRACT

Regulatory mechanisms are necessary to avoid the misdirected aggressive immune responses responsible for the pathology seen in autoimmunity and allergy. Thymic-derived $CD4^+CD25^+$ regulatory T cells are indispensible for this regulation. We investigated if $CD4^+CD25^+$ Treg prevents auto-reactive responses in adult peripheral blood and cord blood. Mononuclear cells, as well as $CD4^+$ T cells isolated from peripheral blood of adults or from cord blood, were stimulated with self-antigens and recall antigens in the absence or presence of $CD25^+$ cells. We demonstrate that adult human $CD25^+$ cells regulate the response to myelin oligodendrocyte glycoprotein (MOG), while cord blood $CD25^+$ cells are not equally efficient in the inhibition of responses to self-antigens. We conclude that activation of self-reactive T cells in normal healthy individuals is prevented by the presence of self-antigen-specific $CD25^+$ regulatory T cells and that the majority of these cells mature after birth.

T cells with regulatory properties can also be induced in the periphery, for example in response to a fed antigen. The physiological requirements and localization of the tolerance induction are largely unknown. We studied the antigen-specific activation and induction of regulatory T cells from naïve $CD4^+$ T cells in different lymphoid compartments following oral administration of a protein antigen. A significantly higher proportion of antigen-specific $CD4^+$ T cells developed into the putative regulatory phenotype in the liver-draining celiac lymph node (CLN), compared to other sites. This suggests that induction of regulatory T cells in the CLN may be relevant for the generation of tolerance to dietary antigens.

Oral tolerance is impaired in germfree animals, which indicates a role of the enteric flora. Using a mouse model of allergic airway inflammation, we investigated how a natural adjuvant from the commensal microflora, *Staphylococcus aureus* enterotoxin A (SEA), aids in the tolerogenic processing of antigens. We found that recipients of serum from SEA-treated and ovalbumin-fed donors were better protected against allergic airway inflammation with diminished influx of eosinophils into the lungs and reduced antigen-induced production of interleukin-5 and interleukin-13 compared to controls. This was associated with increased density of $CD8\alpha^+$ intraepithelial lymphocytes in gut-sections from SEA treated donors. Our results suggest that SEA promotes oral tolerance by facilitating tolerogenic processing of dietary antigens, possibly via activation of intraepithelial lymphocytes acting on the absorptive intestinal epithelium.

Intestinal epithelial cells have the capacity to sample and package environmental antigens into exosomes, which are found in the serum-fraction that mediates antigen-specific tolerance when transferred to naïve recipients. Exosomes isolated from the murine small intestinal epithelial cell line IEC4.1 were characterized by flow cytometry, electron microscopy and their immunomodulatory capacity was explored in a mouse model of ovalbumin-induced allergic airway inflammation. The exosomes were found to carry MHC class I, MHC class II, CD9 and MFGE-8. When antigen-pulsed exosomes from IEC4.1 cells stimulated with low level of IFN-γ were given to naïve mice they were able to partly prevent the allergic sensitisation.

Keywords: Tolerance, regulatory T cells, self antigens, oral tolerance, dietary antigens, intestinal epithelial cells, exosomes, Staphylococcus aureus enterotoxin A.

ORIGINAL PAPERS

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

I. Kajsa Wing, Susanne Lindgren, Gittan Kollberg, Anna Lundgren, Robert A Harris, Anna Rudin, Samuel Lundin, Elisabeth Suri-Payer. CD4 T cell activation by myelin oligodendrocyte glycoprotein is suppressed by adult but not cord blood CD25+ T cells. *Eur J Immunol. 2003 Mar;33(3):579-87*[1].

II. Susanne Hultkrantz, Sofia Östman, Esbjörn Telemo. Induction of antigen-specific regulatory T cells in the liver-draining celiac lymph node following oral antigen administration. *Immunology. 2005 Nov;116(3):362-72*[2].

III. Susanne Lindgren, Nina Almqvist, Anna Lönnkvist, Sofia Östman, Carola Rask, Esbjörn Telemo *, Agnes E Wold*. Oral exposure to *Staphylococcus aureus* enterotoxin A promotes tolerogenic processing of a fed antigen. *In manuscript.*

IV. Susanne Lindgren*, Nina Almqvist*, Ulf Gehrmann and Esbjörn Telemo. Characterization and immunomodulatory role of intestinal epithelial cell derived exosomes. *In manuscript.*

* these authors contributed equally to the study

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Sensing self, non-self and the very foreign[3] – an introduction

While keeping up an effective defence to protect the host from infections, aggressive immune response to harmless antigens must be avoided to maintain health. The immune system reacts vividly to bacterial and viral components, but generally not to self-structures that makes up each individual or to structures in e.g. the food we eat. Some very foreign patterns found on pathogens, and indeed some produced by self-tissue when in alarm, are recognised by cells of the innate immune system that starts an immune response. However, the specific distinction of the immune response is not made on major differences between various molecules. Self-components are tolerated, while those from a genetically different individual of the same species are rejected. A dietary antigen is tolerated once it has been fed and will not evoke aggressive immune responses upon challenge. In contrary, if a challenge with the same antigen is performed prior to feeding, the immune system will readily respond to it.

This tells us that the immune system learns what to tolerate. It learns to tolerate self-structures, and it learns to tolerate harmless non-self antigens as in the food we eat. It is also becoming increasingly clear that the absence of an aggressive response is not a lack of response. It is a response, but of a different kind. There are intricate effector mechanisms that carry out the tolerance to self and harmless non-self antigens, which exist in parallel with the potential to respond aggressively to these antigens. A shift in the balance might cause a break in the tolerance leading to diseases such as autoimmunity, allergy or inflammatory bowel disease.

The homeostasis in health is the focus of this essay, and some mechanisms of tolerance to self and non-self antigens are addressed.

[3] Inspired by Polly Matzinger, "Friendly and dangerous signals: is the tissue in control?", Nature Immunology 2007.

Aims

Paper I. It was previously shown that immune responses to self-antigens were found in healthy individuals, when mononuclear cells from peripheral blood were stimulated *in vitro*. As these cells also included regulatory T cells, we wanted to study the reactivity to self-antigens in the absence of regulatory T cells, and to investigate the potential role of these cells in the control of autoreactive T cells. We also wanted to compare the role of regulatory T cells in adults and newborn.

Paper II. A fed protein antigen induces a state of active tolerance, known as oral tolerance, that involves the induction of regulatory T cells. The immune system associated with the intestines has naturally been in focus for the study of these cells, but the liver is closely connected to the gut through the portal vein and has unique qualities for tolerance induction. We therefore wanted to study antigen-specific T cell responses in the liver draining celiac lymph node, compared to responses at other sites.

Paper III. In the process of oral tolerance induction, a tolerogenic serum-factor is produced that we believe has the form of exosomes produced by the intestinal epithelial cells, carrying the processed antigen. It has been shown that stimuli from the gut flora are important for the induction of oral tolerance and a reduced microbial exposure is associated with an increase in allergy. Early gut colonization of *Staphylococcus aureus* seems to be protective against the development of food allergy in children, and many of the gut-colonizing *S. aureus* strains produce enterotoxins. We therefore wanted to study the impact of *S. aureus* enterotoxin A on the production of a tolerogenic serum factor.

Paper IV. In serum from fed animals, the tolerogenic factor is confined to a fraction pelletted by ultracentrifugation that contain exosomes partly derived from intestinal epithelial cells (IEC). Two earlier studies on exosomes produced by antigen-pulsed IEC cultured *in vitro* have reached conflicting results regarding their ability to modulate immune responses and we wanted to investigate this further.

APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
CCR	CC chemokine receptor
CD	Cluster of differentiation
CLIP	Class II-associated invariant chain peptide
CLN	Celiac lymph node
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
d3Tx	Thymectomy on day 3 after birth
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DTH	Delayed-type hypersensitivity
EM	Electron microscopy
FoxP3	Forkhead box P3
IBD	Inflammatory bowel disease
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICOSL	Inducible costimulator ligand
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
ILT-3, 4	Immunoglobulin-like transcript
IDO	Indoleamine 2,3-dioxygenase
IDDM	Insulin-dependent diabetes mellitus
IEC	Intestinal epithelial cell
IFN	Interferon
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
GAD	Glutamate decarboxylase
GALT	Gut-associated lymphoid tissue
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HEV	High endothelial venules
LAG-3	Lymphocyte-activation gene 3
LAMP-1	Lysosomal-associated membrane protein 1
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
KO	Knock-out
mAb	Monoclonal antibody
MAd-CAM	Mucosal vascular addressin cell adhesion molecule 1
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MFG-E8	Milk fat globule-EGF factor 8
MLN	Mesenteric lymph node
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MVB	Multivesicular body
NLR	NOD-like receptor
NOD	Non-obese diabetic mice
Nrpl	Neuropilin
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PLN	Peripheral lymph node
PP	Peyer's patch
RA	Retinoic acid *or* Rheumatoid arthritis
RAG	Recombination activating gene
SE (A)	Staphylococcus aureus enterotoxin (A)
SCID	Severe combined immunodeficiency
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TECK	Thymus-Expressed ChemoKine
TIM	T-cell immunoglobulin- & mucin-domain-containing molecule
TLR	Toll-like receptor
TSST-1	Toxic shock syndrome toxin-1

Pattern recognition and rearranged receptors. How can we respond against a nearly infinite number of pathogens, and still remain tolerant to self-proteins, proteins of the microbial flora and the diet and other proteins in the surroundings? The question is fundamental, yet there is no final answer to it.

The cells of the *innate* immune system use receptors that recognise conserved structures found on microbes. The pattern recognition enables a fast and homogenous response by cells that can be mobilised very quickly. This first line defence is crucial for the survival of the host and is seen in almost all animal species.

In addition, the immune system of most vertebrates is extended by the *adaptive* immunity with a memory function, associated with a change in the number and quality of the antigen specific cells in an individual. T and B lymphocytes maintain this acquired protection. $CD4^+$ T cells are central in directing the adaptive immune response, by activating the effector cells best suited to handle a certain type of infection. Among the $CD4^+$ T cells, subsets with the potential to mediate autoimmune diseases can be found, but also those involved in the prevention of such diseases. This role of the $CD4^+$ T cells will be the focus of this essay.

T lymphocytes recognises their antigens through the T cell receptors (TCRs). The TCRs have an enormous diversity and can interact with almost any peptide. This is due to a random gene rearrangement of the gene segments that code for the receptor, caused by the site-directed recombinases RAG-1 and RAG-2 and additions of nucleotides by the TdT enzyme during lymphocyte maturation. Thus, each lymphocyte has a unique receptor with the ability to respond selectively to a particular antigen (1).

MHC interaction. Proteins to be presented to $CD4^+$ T cells are picked up from the surroundings by antigen presenting cells (APCs). They are then cut into peptides, which are allowed to bind to MHC class II molecules. The MHC class II/peptide-complex is transported to the surface of the APC where the peptide is exposed to the $CD4^+$ T cells. Each individual have a set of MHCs class II molecules, resulting from the possible combinations of α- and β-chains each expressed by alleles at 3 different loci. From the randomly created T cell receptor repertoire, receptors that recognise the specific set of MHCs expressed by an individual must be favoured, as receptors unable to interact with *self-MHC* with bound peptides are useless. On the other hand, TCRs that interact with *self-peptides* bound to self-MHC can be dangerous, unless the interactions are limited and the reactions controlled. There are different strategies for controlling inappropriate T cell activation, as will be outlined below.

The thymus is central in the establishment of the self-MHC restriction (2-6) and self-tolerance (7, 8) of the T-cell population. T cells originate from the bone marrow but the progenitors migrate at a very early stage to the thymus for further differentiation. The thymus provides a microenvironment essential for the developing T cells, where cell-cell contact with other cell types plays an important role. Here, a useful repertoire of T cells is selected. The T cell precursors that enter the thymus do not have a T-cell receptor (TCR) and neither of the two co-receptor molecules CD4 and CD8 - thus they are called double-negative (DN). After differentiation to double positive (DP) cells, that also express a low level of TCR, the selection takes place in two steps, namely the positive and negative selection (Figure 1).

Positive selection. From the full register of possible TCRs that originate from their combinatorial generation, T cells with TCRs able to bind self MHC are rescued in a process called positive selection. The majority of T cells receive no recognition signal and are lost to "death by neglect". The selected T cells are said to be self-restricted as they are able to interact with the certain set of MHCs expressed by an individual. The process takes place in the cortex of the thymus and depends on the influence from the stromal cells, the cortical thymic epithelial cells (cTECs). The positive selection allows for the lymphocyte's transition from an immature DP cell to a single positive ($CD4^+$ or $CD8^+$) stage. At this step the selection of either T helper cells ($CD4^+$) or cytotoxic cells ($CD8^+$) is made.

Negative selection. The positively selected cells then move on towards the medulla of the thymus and are subject for the second selective step, i.e. the negative selection, originally postulated by F M Burnet. In this step any cell that binds too strongly to a peptide presented in the thymus are eliminated. The negative selection can be mediated by several cell types, but is most efficiently driven by the bone marrow derived dendritic cells (DCs) and macrophages (1). It was also discovered that the medullary epithelial cells (mTECs) in the thymus express a wide variety of organ-specific proteins, such as glutamic acid decarboxylase (GAD67), insulin and tyrosinase (9). This promiscuous gene expression is under the control of the transcription factor Aire (10). Patients with a mutation of *aire* suffer from a variety of symptoms, caused by autoimmunity to various organs (APECED, autoimmune polyendocrinopathy-canidiasis-ectodermal dystrophy, reviewed in (11)). Thus, the thymic self - nonself discrimination is directly linked to the influence on autoimmunity.

As outlined above, both positive selection and central tolerance, in the context of elimination of self-reactive cells, imply recognition of self-structures. Several models that try to explain this apparent paradox have been proposed. One hypothesis states that the "avidity" of the interaction determines the fate of the lymphocyte – i.e. a low avidity is

enough for positive selection while a too high avidity leads to negative selection (12, 13). Others suggest a qualitative difference between the signals driving positive and negative selection (14, 15). Despite great efforts in this field, there is still no definite explanation. Crucial for this entire selective model is that positive selection must include more receptor specificities than those leading to negative selection. If not, no T cells would ever leave the thymus.

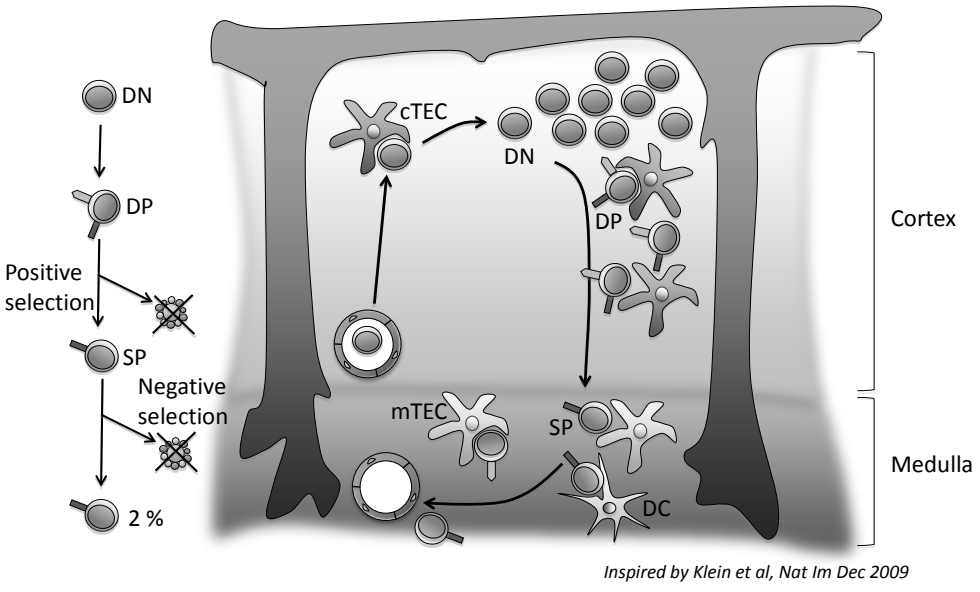

Figure 1. Development of T cells in the thymus. T cell progenitors from the bone marrow enter the thymus through a blood vessel in a double-negative (DN) state, lacking the co-receptors CD4 and CD8, as well as the T cell receptor (TCR). Successful recombination of the TCR β chain results in the expression of a pre-T-cell receptor and the progression to a double-positive (DP) state, with expression of both CD4 and CD8. The TCR α chain is then rearranged and the complete TCR is expressed in low levels. The DP cells will interact with cortical thymic epithelial cells (cTEC) in order to get positively selected. Most of the TCRs are not able to interact with self-MHC and will therefore fail positive selection. Thymocytes bearing those receptors will die. The thymocytes that are able to recognise self-peptide/MHC complexes mature and cease to express one of the co-receptors, to become either $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes. The thymocytes will then also undergo negative selection through the interaction with dendritic cells (DCs) and medullary thymic epithelial cells (mTEC). By negative selection, those cells that are responding to strongly to self-antigens are eliminated. Only about 2% of the DP thymocytes survive both positive and negative selection and are exported to the periphery. The time from the entry of the progenitor T cell, to the export of a mature, naïve T cell takes about 3 weeks in the mouse.

Consequences of cross-reactivity. The models for a recessive tolerance resulting from elimination or inactivation of auto-reactive clones in the thymus do not cover all characteristics of natural tolerance. While they can explain tolerance to self-antigens expressed intrathymically, they are insufficient to explain tolerance induction to tissue-specific antigens not expressed in the thymus or to harmless antigens in our environment. The impossibility of an elimination of every potentially self-reactive T cell during thymic selection is further pointed out by Mason in a mathematical analysis concluding that a high degree of cross-reactivity is a necessary property of antigen recognition by T cells (16). There are far more foreign peptides in the environment to which the repertoire can potentially react than one has T cells. Furthermore, the frequency of naïve T cells that can recognise an individual foreign peptide must be sufficient to ensure a relatively rapid response to the foreign antigen. If the reactivity to foreign antigens, rather than the avoidance of self-reactivity, is the factor that determines how cross-reactive the TCR can be, there must be a limitation in the number of self-antigens that mediate deletion. Mason concludes that if self-tolerance required clonal deletion or anergy of every T cell potentially reactive to self-peptides, virtually all T cells would be deleted or non-functional.

Ignorance and anergy. As could be expected from the previous reasoning, it has been demonstrated that healthy individuals are hosts for potentially pathogenic, auto-reactive T cells. Accordingly, autoimmune disease can be induced in normal adult animals (17) and T cells from healthy human subjects respond to a variety of tissue-specific antigens associated with autoimmune disease (18-20). Still, autoimmune diseases are relatively uncommon, together affecting about 5% of the population. There are probably multiple mechanisms working together to keep potentially dangerous cells in a harmless, inactive state. First, naïve $CD4^+$ T cells lack homing receptors for most tissues and show a limited recirculation pattern restricted to blood vessels and secondary lymph tissue. This limits the exposure of tissue-specific self-antigen to the T cells. Second, the state of ignorance by the immune system is further augmented by the fact that activation of a T cell requires 60-200 copies of a relevant peptide/MHC complex (21, 22). A self-antigen that fails to achieve this level of expression in the periphery will be ignored. Third, in addition to specific engagement of the TCR, a naïve T cell requires co-stimulatory signals for activation. These signals can only be provided by activated APCs in the lymph nodes. Recognition of an antigen in the absence of this second stimulus drives the cell to an anergic state. The anergic cell will not respond if it is anew exposed to its antigen, even if co-stimulation is provided this time. Recently, it has also been demonstrated that stromal cells in the lymph nodes express Aire, similar to mTEC in the thymus (23). The resulting expression of tissue-specific antigens mediates peripheral deletion $CD8^+$ T cells specific for the antigens (24).

Nevertheless, passive mechanisms as clonal deletion, ignorance or anergy are insufficient to explain all features of tolerance. Certain T cell subsets that mediate active suppression to limit misdirected immune responses and maintain immune homeostasis have attracted enormous interest during the last decade.

Identification of an active mechanism for tolerance. The protective significance of a certain population of thymus-derived lymphocytes that are developed early in life was first observed in female mice subjected to thymectomy on their third day of life (d3Tx) (25). These mice became infertile due to ovarian atrophy. The syndrome of post-d3Tx-induced organ specific autoimmunity was further investigated and it was demonstrated that disease could be prevented by the injection of thymocytes from 7-day-old or adult mice or of a spleen cell suspension from adult mice (26). The cells of significance appeared to be generated in the thymus of the neonate, yet did not spread to the peripheral lymphoid organs during the first days of life. A direct evidence of the presence of a regulatory T cell population was that the removal of a certain subset of T cells from an otherwise normal animal resulted in disease, while reconstitution of the same cells re-established self-tolerance and prevented autoimmunity (27). Tolerance clearly involve an active dominant mechanism, further proved by the fact that the tolerant state can be transferred from tolerant donors to naïve animals with $CD4^+$ T cells, as shown by several groups (28, 29). These regulatory T cells were later defined as a minor (≈10%) subset of $CD4^+$ T cells which continuously express the CD25 (IL-2R alpha-chain) surface marker (30). A similar cell population was later isolated from human thymus, tonsils, blood and cord blood. The discovery of the transcription factor FoxP3 as specific marker of regulatory T cells and in control of their suppressive function raised an enormous interest (31, 32).

The existence of regulatory T cells is now well established, and they have been in focus for intensive investigation during recent years. It has become clear that there are different subsets of regulatory T cells rather than one homogenous population. The origin of T cells with regulatory abilities has been under much debate. It is now widely accepted that in addition to regulatory T cells derived from precommitted precursors in the thymus, naive T cells in the periphery can be induced to differentiate into regulatory T cells. Fully differentiated regulatory T cells, able to inhibit proliferation of other T cells *in vitro* and prevent development of autoimmune disease are found in the thymus, where they represent 5-10% of $CD4^+CD8^-$ thymocytes in mice (33) and humans (34). This population of regulatory T cells that originate from the thymus are referred to as natural regulatory T cells (nTreg), while $CD4^+CD25^+$ regulatory T cells induced in the periphery by different mechanisms are sometimes referred to as inducible regulatory T cells (iTreg).

A model describing both mechanisms in cooperation was proposed several years ago to explain tolerance induction to antigens present in the thymus, as well as to those only found in the periphery (35). It comprised a role for the thymus-derived regulatory T cells in educating naïve T cells in the periphery to in turn become suppressive. Experimental evidence later supported this model, as it was demonstrated that thymus-derived natural Treg could convert naïve $CD4^+$ T cells into suppressive cells *in vitro* (36, 37). The suppressive cells generated in this way are not contact-dependent as the natural Treg (se

below), but depend on the production of suppressive cytokines, IL-10 (36) or TGF-ß (37) to be able to suppress. In this, they resemble other *in vitro* induced suppressive cells. Repetitive TCR-stimulation of naive $CD4^+$ T cells in the presence of IL-10 generates IL-10 producing T regulatory 1 (Tr1) cells (38). Other strategies to engender suppressive cells *in vitro* include antigenic presentation by immature or tolerogenic dendritic cells (reviewed in (39)). It is becoming clear that the most probable situation for maintaining tolerance *in vivo* is a co-operation of different subsets of regulatory T cells, each with specialised mechanisms of action.

THYMIC-DEPENDENT NATURAL $CD4^+CD25^+$ REGULATORY T CELLS

Characterisation. In humans, $CD4^+CD25^+$ T cells exists as both $CD25^{int}$ and $CD25^{++}$ and it is only the bright $CD25^{++}$ T cells that have the regulatory function (40), while the $CD25^{int}$ represent activated cells. In a normal laboratory mouse, the majority of $CD4^+CD25^+$ T cells belong to the regulatory T cell population. However, if the murine immune system is activated, the identification of regulatory T cells will become more difficult.

Natural regulatory T cells constitutively express the glucocortico-induced tumour necrosis factor receptor family-related gene, GITR (41), and the co-inhibitory molecule CTLA-4 (42), considered to have a relevance for the suppressive function (se below). However, these markers, as well as CD25, are also upregulated by activated conventional $CD4^+$ T cells, and do not provide a specific marker for regulatory T cells.

Thymus-derived $CD25^+$ Treg in adults have a memory phenotype ($CD45RB^{low}$ in mice and CD45RO in humans), hence they are believed to be in a late stage of differentiation (42). However, the majority of the $CD25^+$ Treg are positive for CD62L, L-selectin (42, 43) and the chemokine receptor CCR7 (43), which together enables the cells to leave the bloodstream and home into the lymph nodes. This indicates that the regulatory T cells have a migration pattern similar to naïve $CD4^+$ T cells that share this phenotype, and recirculate the secondary lymphoid compartments.

In addition, a connection between the homing molecule CD103 ($\alpha E\beta 7$) and a population of regulatory T cells has been described (44-46). The CD103 integrin mediates adhesion to epithelial cells through its binding to E-cadherin, which is expressed selectively on epithelial cells (47, 48). CD103 may therefore be important for the localization of regulatory T cells to the skin and intestine. The subpopulation of regulatory T cells that express CD103 has been showed to have a preferential capacity to prevent IBD (49).

Neuropilin-1 (Nrp1), a receptor involved in axon guidance and angiogenesis, was suggested as a specific surface marker for $CD4^+CD25^+$ Treg cells. The expression is co-regulated with that of FoxP3 in mice and murine Treg constitutively express a high level

of Nrp1, while the expression is down-regulated in conventional T cells upon activation (50). By contrast, human FoxP3$^+$ T cells do not specifically express Nrp1 (51).

No surface marker specific for regulatory T cells has been identified so far. Although useful for characterisation of regulatory T cells in a nonactivated immune system, activated conventional T cells also express the markers currently used for characterization. The lack of a specific surface marker is a disadvantage in the study of these cells. At present, Foxp3 is the most specific molecule for Treg cells but as it is located intracellularly, it cannot be used for the isolation of viable Treg.

A more accurate identification of Treg based on surface staining can be achieved by the combination high expression of CD25 and low expression of CD127, the IL-7R (52, 53). While IL-7 is very important for most T cell subsets (54) and activated T cells express high levels of CD127, Treg rely on IL-2 for their maintenance and may not need IL-7. In humans, Treg are therefore probably best characterised as CD4$^+$CD25^{high}CD127^{low} lymphocytes.

Thymic selection. At least a portion of the CD25$^+$ regulatory T cells originate from the thymus, and are dependent on thymic function for their development (30), but it is not known precisely how they are selected as compared to conventional CD4$^+$ T cells. Unlike the naïve T cells, the FoxP3 natural Tregs are already antigen-primed and functionally mature when they leave the thymus (33). Thymocytes expressing FoxP3 are detectable already in the late CD4$^+$CD8$^+$ stage and constitute about 5% of mature CD4$^+$CD8$^+$ thymocytes (55).

Evidence suggests that the development of Treg requires a strong TCR-signal in the thymus. It is believed that CD4$^+$CD8$^+$ thymocytes with increased affinity to self-MHC/peptide complexes are positively selected toward regulatory CD4$^+$CD25$^+$ T cells, thus representing the subset with the highest avidity for self of the selected T cells (56, 57) as originally proposed by Coutinho and colleagues (35). Analysis of the TCR repertoires has revealed that Treg and conventional T cells have distinct TCR repertoires, although there is a degree of overlap that is not yet fully determined (58, 59). In addition, sufficient co-stimulation must be provided by the thymic stromal cells for adequate generation of regulatory T cells, as deficiency of CD28, CD40, CD11a/LFA-1 or CD80 and CD86 results in substantial reduction of Tregs in the thymus and in the periphery (reviewed in (60)). Both epithelial cells and dendritic cells in the thymus contribute to the generation of regulatory T cells.

It has been suggested that the gene expression by mTECs might drive clonal selection of regulatory T cells by the expression of various self-antigens under the control of AIRE. However, *aire* deficient mice have normal numbers of CD4$^+$CD25$^+$ T cells in lymphoid organs (10, 61) and they appear normal in their regulatory function *in vitro* and *in vivo* (62). By, contrast, it has been reported that patients with established APECED have a reduced number of circulating regulatory T cells (63) and a recent study suggested that Aire$^+$

mTECs expressing tissue-specific antigens may facilitate development of Treg specific for tissue-specific antigens (64).

Still, the main function of the expression of peripheral organ-specific proteins in the thymus appears to be the promotion of clonal deletion of self-reactive thymocytes (61, 65, 66). Any particular tissue-specific protein is expressed in only a small fraction (ca 1%) of the relatively rare mTECs (9) and it was suggested that so few cells would be ineffective in purging the entire emerging T-cell repertoire. But the mature thymocytes spend almost two weeks in the thymic medulla and they are very motile. In addition, the tissue-specific antigens are expressed by pre-apoptotic mTEC and are therefore also presented by dendritic cells after phagocytosis of apoptotic mTECs.

One study has demonstrated that efficient Treg development only occurs when the precursors are present in very low frequencies (67). The mechanism that limits the niche for regulatory T cells is unclear, but the results indicate that thymic Treg development may not be easily studied using TCR transgenic mice. A very limited niche for the regulatory T cells is also supported by another study that suggests that the development is instructed by the TCR (68). The molecular mechanisms guiding thymic selection of regulatory T cells are still unclear, despite enormous efforts during the last decade.

Lineage commitment. The transcription factor FoxP3 has a key role in the differentiation and function of regulatory T cells. FoxP3 is expressed by thymic-derived $CD4^+CD25^+$ regulatory T cells and is also acquired by naïve $CD4^+$ T cells by their conversion into regulatory T cells in the periphery. Retroviral transduction of naïve $CD4^+CD25^-$ T cells with FoxP3 converts them into suppressive cells expressing CD25, CTLA-4, CD103, and GITR. (31,32). The discovery of FoxP3 has allowed better understanding of the development and function of regulatory T cells. However, not even FoxP3 is a reliable specific marker for regulatory T cells, as it is transiently induced by activation of conventional T cells at least in humans (69, 70).

The mouse strain "Scurfy" has a mutation in the FoxP3 gene and $CD4^+CD25^+$ cells from scurfy mice lack suppressive activity (71). They develop a syndrome characterised by an uncontrolled activation and expansion of $CD4^+$ T cells, autoimmunity and uncontrolled inflammation. The symptoms are similar to those seen in mice lacking CTLA-4 or TGF-ß. In humans, mutation of the gene FoxP3 is the cause of an X-linked syndrome termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)(72). Infants born with this syndrome develop several organ-specific autoimmune diseases, inflammatory bowel disease as well as atopic dermatitis that are fatal if these children are not transplanted with bone marrow from a healthy donor . This indicates a common etiology of these diseases in the absence of proper control of immune responses.

Recently, it was demonstrated that thymocytes with the FoxP3 protein destroyed by an insert of a green fluorescent protein nevertheless acquired some characteristics of Treg cells. Although lacking suppressive activity, they had a high transcriptional activity at the *Foxp3* locus and expressed *Il2ra*, *Nrp1*, *Ctla4* and *Icos* at a high rate (73, 74). Furthermore,

a closer analysis of cells that had been transduced with Foxp3 or induced to express Foxp3 by TGF-ß showed that at the most one third of Treg cell signature transcripts were restored by FoxP3-expression (75, 76). A group of genes were found to be co-regulated with, but not induced directly by FoxP3. It is possible to distinguish genes that are influenced individually by FoxP3, TGF-ß or activation through TCR/IL-2R triggers and act together to generate the Treg signature (76). This indicates that Foxp3 is just one of several transcriptional regulators that can be complementary and synergistic, and that there might be a higher order of regulation. Thus, FoxP3 is probably not the "master regulator" for lineage commitment of regulatory T cells as have been stated previously.

In parallel with the discovery of FoxP3, the role of corresponding transcription factors essential for differentiation of other subsets of $CD4^+$ T cells have emerged. When a naïve T cell interacts with its cognate peptide displayed by an antigen-presenting cell in a peripheral lymph node, the $CD4^+$ T cell can differentiate into various effector subsets (Figure 2). The decision is mainly governed by the cytokines in the microenvironment, influenced by the strength of the interaction between TCR and antigen (77).

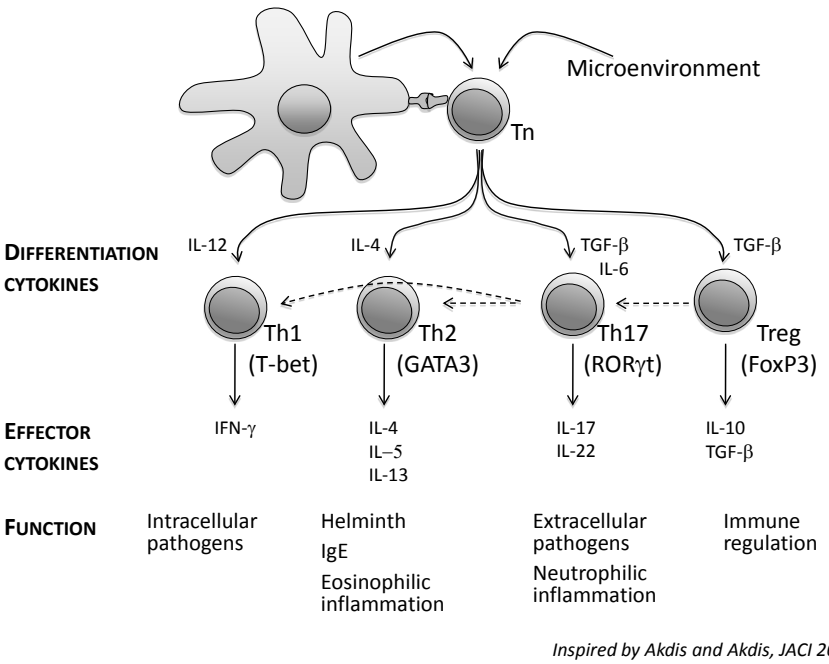

Figure 2. A naïve $CD4^+$ T cell (Tn) can differentiate into diverse effector lineages (Th1, Th2, Th17 and Treg) upon activation, influenced by signals as differentiation cytokines from the antigen presenting cell and the microenvironment. The process involves activation of transcription factors (in brackets) and results in cell types with different effector cytokine production and function. The phenotypes are not entirely stable and one lineage can be converted into another, as indicated by the dashed arrows.

TGF-β induces expression of FoxP3 and conversion of naïve T cells into regulatory T cells in the periphery (78). IL-12 and IFN-γ polarize cells toward the Th1 lineage, characterised by the production of IFN-γ, that activates cytotoxic $CD8^+$ T cells and macrophages in the defence of intracellular pathogens. The differentiation program towards Th1 is initiated by the signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet. Differentiation of Th2 cells is mediated by the transcription factor GATA3 that is induced by IL-4. Th2 cells produce IL-4, IL-5 and IL-13 and are required for humoral immunity in control of extracellular pathogens including helminths. Th17 cells produce IL-17 and IL-22 and are important in the defence against extracellular bacteria and fungi, especially at mucosal surfaces. Their differentiation requires the transcription factor retinoid-related orphan receptor (ROR)γt that is induced by TGF-β in combination with the proinflammatory cytokines IL-6, IL-21 and IL-23 that activate phosphorylation of Stat3 (79).

Initially the differentiation into different effector lineages was thought to involve stable programs of gene expression, with epigenetic changes of cytokine genes. However, it has become clear that the T cell nature is much more plastic than originally thought. For example, it has been shown that regulatory T cells have a tendency to differentiate into Th17 or Tfh cells, and Th17 cells can be converted into Th1 or Th2 cells (reviewed in (80)). The implications of the plasticity and unstable phenotypes of the Treg and Th17 T cell subsets are necessary to take into account in the design of new therapeutical strategies for the treatment of infections or autoimmunity.

Proliferation. Because of their constitutive expression of CD25, the IL-2R α-chain, regulatory T cells have a high affinity for IL-2. However, they do not produce IL-2 by themselves and are dependent on exogenous IL-2 for proliferation *in vitro* and *in vivo*. Natural regulatory T cells are anergic to *in vitro* antigenic stimulation, but the anergic state can be broken by the addition of exogenous IL-2. Once IL-2 is removed, they revert to their original anergic state and remain suppressive, being even more effective on a per cells basis than before activation (81). In contrast to their anergic behaviour *in vitro*, they proliferate actively upon antigenic stimulation *in vivo* (82, 83). It has also been reported that the nature of the APC might be a determining factor for their proliferation. Yamazaki et al found that $CD4^+CD25^+$ regulatory T cells can proliferate *in vitro* in the absence of added cytokines, when stimulated with mature, bone marrow derived DCs, loaded with antigen (84).

Contact-dependent suppression. In order to be suppressive, $CD4^+CD25^+$ T cells need to be specifically activated *via* their T cell receptor, but once activated, they are capable of antigen non-specific suppression of any $CD4^+$ or $CD8^+$ T cell (85). They can suppress in an APC-independent manner by direct T cell - T cell interaction (86). The suppression by natural $CD4^+CD25^+$ regulatory T cells is strictly cell-cell contact dependent *in vitro* but many questions concerning the effector mechanisms remain open. Despite intense

research, no molecular pathway for this cell contact-dependent inhibition has yet to be found. However, the ultimate result of the suppression is the inhibition of IL-2 and IFN-γ transcription in the responder T cells (87, 88). Membrane-bound TGF-ß has been postulated to be responsible for the contact-dependent suppression exerted by natural regulatory T cells (89). However, the results have been difficult to reproduce by others (88, 90, 91). Furthermore, membrane-bound TGF-ß is predominantly expressed on resting $CD4^+CD25^+$ T cells. Upon activation, it is downregulated on regulatory T cells while it is upregulated by conventional $CD4^+CD25^-$ T cells (37). In addition, $CD4^+CD25^+$ T cells from neonatal TGB-β $1^{-/-}$ mice are as suppressive as $CD4^+CD25^+$ from wild type mice, and $CD4^+CD25^+$ T cells are also able to suppress conventional $CD25^-$ T cells expressing dominant-negative TGF-ß receptor II (90). Thus, the potential role of membrane-bound TGF-ß in contact-dependent suppression remains controversial. It has been suggested that CTLA-4, constitutively expressed by regulatory T cells (see below), may interact with CD80/CD86 on effector T cells (92, 93) in order to down-regulate T cell functions but the relative contribution of this mechanism is still unclear.

Suppression by targeting APC. In addition to the effect mediated directly on the responder T cells, APCs are also targets for Treg suppression (Figure 3). Different mechanisms have been proposed that primarily affect the function of the APC. A number of studies demonstrate that regulatory T cells can down-regulate the expression of co-stimulatory ligands on dendritic cells in co-culture (94-96). Several molecules have been proposed to participate in the suppressive function. CTLA-4 (CD152) is an inhibitory T cell molecule that interacts with CD80 and CD86 on the APC in competition with the co-stimulatory molecule CD28, but with a much higher relative affinity for CD80 and CD86. Mice deficient in CTLA-4 develop lymphoproliferative disorders associated with lethal infiltration polyclonal T cells in many organs (97, 98). Regulatory T cells constitutively express CTLA-4 and signalling through this molecule may contribute to suppression. This mechanism is supported by *in vivo* experiments, showing that inoculation of anti-CTLA-4 antibody in a normal mouse elicited autoimmune disease similar to that caused by the depletion of $CD4^+CD25^+$ T cells (99). In addition, the protective function of $CD4^+CD25^+$ T cells in a murine model of IBD was abolished by the administration of anti-CTLA-4 mAb (100, 101). Takahashi et al also found that anti-CTLA-4 mAb reversed the suppressive activity of $CD4^+CD25^+$ T cells *in vitro* (99). In contrast, others could not find an inhibitory effect of the anti-CTLA-4 mAb *in vitro* (102) and $CD4^+CD25^+$ cells from CTLA-4 deficient mice do exhibit some suppressive activity *in vitro*, although weaker than $CD4^+CD25^+$ cells from normal mice (99). But DCs conditioned with regulatory T cells to down-regulate CD80 and CD86 induce poor T-cell proliferation responses and down-modulation of CD80/86 was inhibited by blocking CTLA-4 (96). Recently, Wing et al demonstrated that mice with a selective deletion of the expression of CTLA-4 in regulatory T cells develop systemic autoimmunity at 7 weeks of age. This deletion does not alter the development or homeostasis of regulatory T cells, and they remain anergic. Nevertheless, the selective deficiency in Treg alone is sufficient to cause fatal disease

(103). The CTLA-4 deficient Treg were also less suppressive in vitro in a system with DCs as stimulator cells by a mechanism that at least partly were due to abrogated down-regulation of CD80/CD86. By interaction of CTLA-4 with CD80/86, regulatory T cells can also condition DCs to express indoleamine 2,3-dioxygenase (IDO), which results in induction of catabolism of tryptophan into proapoptotic metabolites and abrogated activation of effector T cells (104). The different candidate molecules known today do not appear to provide a complete explanation for the contact-dependent suppression. Further studies of FoxP3 controlling the transcription of suppressive genes in regulatory T cells may reveal new candidates. Deficiencies in other molecules expressed by regulatory T cells, such as LAG-3, granzymes and the cytokine IL-35 can impair the function of regulatory T cells in vitro but do not cause autoimmunity as other mechanisms compensate for the deficiencies (105).

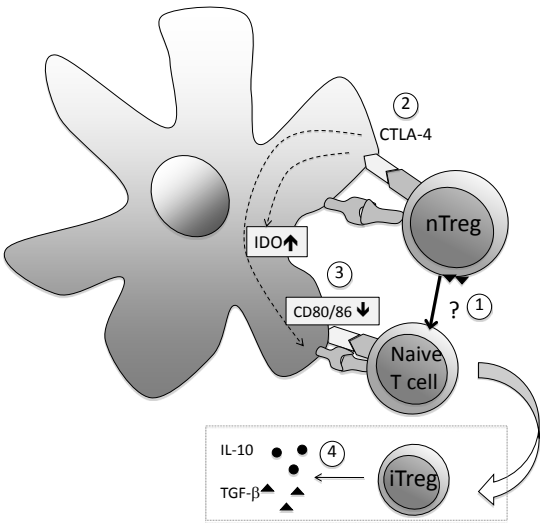

Figure 3. Suppression by regulatory T cells. (1). In vitro, regulatory T cells can suppress effector T cells by a contact dependent mechanism that is independent of the APC, but the mechanism involved is not clear. (2). By the interaction of CTLA-4 on the regulatory T cell and CD80/CD86 on the APC, the function of the APC can be modified. This results in (3) down-regulation of co-stimulatory molecules as CD80/CD86 and upregulation of IDO causing tryptophan deprivation, that both abrogate activation of effector T cells. In addition, (4) signals from the APC and possibly from the regulatory T cells can induce other subtypes of regulatory T cells that suppress in a cytokine-dependent manner (more on this below).

Cytokines in suppression. Although the *in vitro* suppression largely depends on a cell contact-dependent mechanism, secretion of TGF-ß, IL-10 and other cytokines may contribute to the effector function of thymic-derived regulatory T cells. TGF-ß is deeply involved in the regulation of the immune system and mice deficient of this cytokine die shortly after loosing access to TGF-ß from mother's milk, as a result of severe and widespread inflammation. TGF-ß is also important for suppression in models of intestinal inflammation, but results differ whether it must be produced by the regulatory T cells themselves (106) or if it can be provided by other cell sources (107). However, Treg were not able to suppress colitis caused by effector cells with a defect TGF-ß receptor type II, as they escaped the control of regulatory T cells (107).

Treg-expansion. Stimulation via other accessory molecules expressed by regulatory T cells lead to their expansion. GITR is expressed at low levels by various lymphocyte subsets, DCs and macrophages and the expression is increased upon activation but high surface expression of GITR is confined to resting regulatory T cells in the thymus and the periphery. Stimulation of GITR in the presence of IL-2 induces vigorous proliferation of regulatory T cells (41, 108). The GITR/GITRL system potentiates immune responses by effects on innate immune cells, co-activation of effector T cells and inhibition of regulatory T cells (109). GITR-signalling may prevent the induction of suppressor activity in resting $CD4^+CD25^+$ T cells and blocked GITR-GITRL signalling improved Treg function and graft survival in a model of transplantation (110). Regulatory T cells can also expand in the response to stimulation via Toll-like receptors (TLRs), independent of specific antigen recognition via the TCR and therefore modify their activity directly in response to pathogens (111).

As mentioned, regulatory T cells express a high density of the high affinity receptor for IL-2 and they also require a much lower antigen-concentration than naïve T cells for activation (112). This, in combination with the synergistic effect by GITR-ligation and possibly TLRs gives the regulatory T cells an advantage compared to naïve T cells in immune responses. Nrp-1 that is expressed by murine regulatory T cells promotes the interaction between Treg cells and immature dendritic cells, which also may give regulatory T cells a head start over naïve T cells under antigen-limiting conditions (113). After expansion the regulatory T cells can retain their suppressive function (83, 84, 114), but the overall number of regulatory T cells are kept relatively constant at around 10-15% in normal animals, which indicates that they die after having exerted their suppressive function.

nTreg in health and autoimmunity. As mentioned earlier, the thymic clonal deletion of self-antigen specific, potentially dangerous T cells is not complete, and self-antigen specific T cells circulate in the periphery.

Much work has been performed to survey the auto-reactive pattern in groups of patients affected by autoimmune diseases such as IDDM and MS where auto-reactive T cells

specific for certain pancreatic ß-cell antigens or myelin antigens are thought to play an important role in the pathogenesis. In several of the studies, antigen-specific responses against the myelin self-antigens myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) (19, 115, 116) and against the ß-cell antigens insulin and glutamic acid decarboxylase (GAD) (117-119) were seen not only in patients, but also in healthy controls, although to a low extent and with low frequency. This means that self-aggressive T cells easily can be detected in healthy individuals. Still, overt autoimmune diseases are relatively infrequent, indicating that these cells normally are kept under control by an active suppressive mechanism *in vivo*. In paper I, our aim was to investigate the role of $CD25^+$ regulatory T cells in the control of immune responses directed against organ-specific self antigens. As total PBMCs were used in all the earlier studies, this meant that also the regulatory population was included. We therefore wanted to study the reactivity to self-antigens in healthy individuals in the absence of regulatory $CD25^+$ T cells, to investigate the potential role of these cells in the control of auto-reactive T cells in healthy subjects. We hypothesised that stronger and more frequent responses should be seen in the absence of the $CD25^+$ regulatory T cell population.

We found frequent responses to the myelin self-antigen MOG in whole PBMC (12/18) and when $CD25^+$ cells were depleted the responses were significantly higher and could be detected in every subject tested. Thus, self-aggressive T cells specific for MOG are present but the balance is in favour for the regulatory T cells in the normal situation.

Interestingly, responses to the recall antigen tetanus toxoid were not enhanced by the depletion of $CD25^+$ cells. This indicates antigen specificity in the regulatory T cell subset. Indeed, $CD25^+$ T cells proliferated vigorously when stimulated with MOG in the presence of IL-2, and pure $CD25^+$ cells could suppress the proliferation of $CD25^-$ cells to MOG if added in a 1:1 ratio. It is likely that $CD25^+$ cells are stimulated by MOG in this culture in order to be suppressive and this suggests that there are many MOG specific $CD25^+$ cells present in healthy individuals. Furthermore, the fact that almost all $CD25^+$ cells are of memory phenotype indicates that they are activated by self-antigen in the host.

MOG elicited proliferative responses in both naïve and memory T cell subsets (paper I), which suggest that MOG specific T cells are indeed primed and activated *in vivo,* even in healthy individuals. It is likely that regulatory T cells are central for maintaining immunological self-tolerance, and therefore they will serve as an obvious target in future treatment of autoimmune disease. However, the role and function of Treg in autoimmunity in humans is not yet very well defined and data obtained are contradictive. Different studies have observed decreased, unchanged or increased number of regulatory T cells in peripheral blood of patients with organs-specific or systemic autoimmune diseases (120-125). The suppressive function of regulatory T cells have alternately been reported to be normal or decreased (126-128). Whether this inconsistency reflects a true qualitative difference of the suppressive function, or simply differences in the degree of contamination of activated effector T cells remains unclear. It is difficult to study the function of the regulatory T cells after the outbreak of the disease and perhaps this is also not a relevant point of time to do so. While the regulatory T cells repeatedly have been

shown to be effective in the prevention of disease, their odds to reverse the course when the disease is established are more limited. Thus, their major role may be to maintain immune homeostasis. Once the balance is already shifted, they don't easily regain control. The study of the Treg function just prior to the onset of disease might provide relevant information, but is difficult in human disease. Not only is the point of time hard or impossible to catch; it is also impossible to study the local conditions in the regional lymph nodes where the most relevant shift in function may occur. In addition, there is a lack of specific markers that safely separate Tregs from activated T cells.

Certain genetic factors are strongly associated with autoimmune disease. IL-2-deficincy was early associated with autoimmunity (129) and the IL-2 was subsequently found to be necessary for the development and expansion of regulatory T cells (30, 87, 130). Mice defective in IL-2, IL-2Rα or IL-2Rβ all die early in life of severe lymphoproliferation and autoimmunity (131-133). One of the most obvious examples is mutation in the gene encoding for FoxP3. Mutations of this gene are responsible for the uncontrolled activation and expansion of $CD4^+$ T cells associated with autoimmunity and inflammation in the disease syndromes IPEX and scurfy in humans and mice respectively, due to lack of suppressive function of Treg. The lack of CTLA-4 results in a very similar disease (97, 98), even if the deficiency is restricted to regulatory T cells (103). Even more dramatic is the complete lack of the regulatory cytokine TGB-β in TGF-ß KO mice that die as a result of widespread inflammation shortly after weaning, when they lose access to the TGB-β in their mothers milk (134, 135). When the TGB-β deficiency is restricted to T cells, the mice remain healthy until 4 months of age, and thereafter they develop a wasting disease. Furthermore, T cell specific ablation of TGB-β signalling by the dnTGFβRII results in an early-onset mortal autoimmune-associated inflammation (136). The results added together indicate a central role for the regulatory T cells in control of autoimmunity and the critical role of TGB-β. Although not necessarily produced by the regulatory T cells, TGF-β is needed in the control of effector T cells. Many autoimmune diseases are also associated with MHC class II polymorphisms, which may relate to presentation of distinct sets of peptides or differences in the T cell repertoire selected in the thymus (137, 138).

Clearly, expansion of the natural regulatory T cells by IL-2 is essential for immune homeostasis. There is also an increasing amount of evidence for the significance of external stimuli in the generation and/or expansion of regulatory T cell subsets. As discussed in more detail below, microbial stimuli from the commensal microbiota, especially the intestinal flora, provides signals for the maturation of the immune system and are relevant for peripheral tolerance induction. Expansion might be accomplished through the direct stimulation via TLRs, specifically expressed by regulatory T cells, or indirectly e.g. by the upregulation of GITRL on antigen-presenting cells. The commensal flora probably influences the complex and finely tuned interactions between the epithelial, stromal and dendritic cells and different subsets of T cells that form the base for immune homeostasis by numerous pathways not yet fully recognised. The association between reduced microbial stimuli in Western societies, and the observed increase in autoimmune

as well as allergic diseases in the same regions, led to the postulation of the hygiene hypothesis.

Although relatively uncommon in the total population, autoimmune diseases cause considerable suffering for the affected individuals. As several studies have found dysfunction of the *in vitro* suppression by Tregs isolated from patients with autoimmune diseases, treatment regimens aimed to restore function or increase frequency of regulatory T cells have been investigated. Interestingly, regulatory T cells from RA patients had lost their ability to convey naïve $CD4^+CD25^-$ into suppressor cells (126) by the mechanism known as infectious tolerance. However, the ability was restored in anti-TNF-α-treated patients, as was the suppression of cytokine production. In addition, a rise of the number of Treg in peripheral blood was found in patients treated with anti-TNF-α, correlating with the reduction of CRP. It is unclear if the restoration of Treg function after anti-TNF-α treatment is a direct effect on Treg, i.e. that proinflammatory cytokines hinders Treg function. Treatment with anti-TNF-α is one possibility, but disfavoured by the increased susceptibility to infections followed by this regimen. Anti-CD3 monoclonal antibodies have been used as a therapy in models of autoimmune diseases (139, 140). This treatment induces anergy in pathogenic effector cells and has also been shown to induce regulatory T cells in a TGB-β dependent manner. A recent study reported that treatment with CD3 mAb induced TGF-β production by immature DCs and macrophages upon exposure to apoptotic cells, leading to the induction of regulatory T cells as well as reducing the number of Th17-cells (141). Transfer of competent regulatory T cells is a possible future therapeutic approach. Tregs can be successfully expanded *in vitro* without loosing their suppressive properties (84, 142). Expanded $CD25^+$ regulatory T cells have been shown to be effective in a model of autoimmune diabetes (143) and collagen-induced arthritis (144) in mice. However, in the case of a defective Treg population in patients with an autoimmune disease, the absence of functional regulatory T cells to expand may compromise this method. In addition, the recently acknowledged plasticity of the T cell subsets, with a tendency of regulatory T cells to differentiate into Th17-cells, and the relative instability of the FoxP3 expression in regulatory T cells converted in vitro must be taken into account (80). A rational immunotherapy for autoimmune diseases clearly requires a thorough understanding of the regulation of autoimmune reactivity in the homeostatic situation. Others and our results (paper I) indicate a substantial degree of self-recognition in healthy subjects, a situation that require a complex network of regulation to avoid immune responses directed against self. A more detailed understanding of the factors that regulate our immune system might reveal novel strategies in the treatment of autoimmune diseases.

Treg in the newborn. In adult humans a large portion of the circulating $CD4^+$ T cells express CD25 (40%), but it is only the few percent with the highest CD25-expression that co-express intracellular CTLA-4 and CD122 (42), and are suppressive *in vitro* (40). In

contrast, the majority of the thymic and cord $CD4^+CD25^+$ cells have a high-level of CD25 expression, combined with expression of CTLA-4 and CD122 (42) as well as FoxP3 (145). The percentage of $CD4^+CD25^+FoxP3^+$ cells has been observed to increase during the first days of life (146). Thus, while the bulk of $CD4^+CD25^+$ T cells in adults probably are memory cells, resulting from the encounter with foreign antigens, the thymic and cord $CD4^+CD25^+$ mainly consists of the putative regulatory phenotype.

In contrast to $CD4^+CD25^+$ Treg in adult peripheral blood, that have a memory phenotype ($CD45RA^-$ $CD45RO^+$), cord $CD4^+CD25^+$ T cells have a naïve phenotype ($CD45RA^+$ $CD45RO^-$). The thymic counterpart have a mixed phenotype, possibly explained by a non-complete conversion from $CD45RA^-$ $CD45RO^+$ thymocytes to mature but naive $CD45RA^+$ $CD45RO^-$ T cells (42). This suggests that the $CD4^+CD25^+$ regulatory T cells mature in the thymus, but leave the thymus in a naïve state and become activated in the periphery.

In paper I, we found that mononuclear cells from cord blood proliferated in response to multiple self-antigens, including MOG, myelin basic protein, and GAD. However, contrary to our findings with adult PBMCs, we could not detect an increase in proliferation of cord blood cells in the absence of $CD25^+$ cells. This implicates that cord blood $CD25^+$ cells are not suppressive in the natural 1:10 ratio. Furthermore, we were unable to detect a significant and consistent suppression also at a 1:1 ratio of cord blood naïve T cells and cord blood $CD25^+$ T cells. This implies that $CD25^+$ T cells in cord blood either consists of an immature population of Treg precursors, not yet fully suppressive, or that there is a low frequency of antigen-specific $CD25^+$ Treg in cord blood.

Thus, we further investigated if $CD25^+$ T cells from cord blood were able to suppress polyclonal antigen stimulation. As cord $CD25^-$ cells do not proliferate in response to anti-CD3, we used adult $CD25^-$ T cells as responder cells. With this system, we could conclude that cord blood $CD25^+$ cells do indeed function as Treg after polyclonal stimulation. Suppressive function of polyclonal antigen stimulation, but reduced compared to Treg from adults, has later been confirmed by another study (147).

Wing et al has later further developed the proliferation assay for detection of antigen-specific suppression by cord $CD25^+$ T cells. Using a higher T cell number to achieve increased sensitivity of the suppression assay, they could readily detect cord $CD25^+$ Treg suppression of both proliferation and cytokine-production induced by MOG (148). However, $CD25^+$ Treg from the thymus were not able to suppress MOG-induced proliferation, while they could suppress production of IFN-γ. This indicated that the frequency of MOG-specific Treg is lower in the newborn than in adults, but still higher in cord blood cells than in the thymic cells.

However, there are additional phenotypic differences between $CD25^+$ Treg isolated from adult peripheral blood and cord blood that may indicate additional functional differences. The majority of the Treg in cord blood express the gut-homing integrin $\alpha4\beta7$ but not CCR4, a chemokine receptor that is associated with homing to other tissues than the

intestines, while the opposite is observed by Treg from adults (149, 150). The switch in expression of homing receptors takes place during the first years of life (146). This implies that the majority of Treg in cord and infant blood will migrate to intestinal secondary lymphoid tissue, which points to the significance of the gut as the primary site of antigen-exposure early in life. Another difference is that while an average of 83% of the $CD25^+$ Treg from adult peripheral blood co-express GITR, only 14% of cord blood $CD25^+$ Treg expressed GITR (paper I). Furthermore, $CD25^+$ Treg from adult peripheral blood, but not from cord blood, express IL-10 on a mRNA-level (145).

The naïve $CD25^+$ Treg that leave the thymus are probably restimulated in the periphery by DCs presenting self-antigens in the draining lymph nodes. Upon this restimulation, they convert into a memory phenotype and are probably expanded to match the peripheral occurrence of self-antigens. Mason's laboratory has clearly demonstrated that thyroids need to be present for the survival of thyroid-specific Treg (151). It is possible that the Treg, during this process, also accomplish their suppressive capacity. The development of Treg during different stages of life, particularly in the early life, is a main focus of interest, and will certainly be explored once the general mechanisms underlying the suppression by $CD25^+$ Treg becomes better understood.

INDUCTION OF THYMIC-INDEPENDENT REGULATORY T CELLS

In vitro generation of suppressive T cells. As previously mentioned, natural $CD4^+CD25^+$ regulatory T cells, believed to be specific for self-antigens, are dependent on thymic function for their development (30). However, other subtypes of regulatory T cells can be induced from conventional naïve $CD4^+$ T cells in a thymus-independent manner. *In vitro*, T cells with regulatory properties can be generated in several different ways.

Groux and colleagues first showed that regulatory T cells could be generated *in vitro* by repetitive stimulation of naïve, antigen-specific T-cells, in the presence of exogenous IL-10 alone (38), or with IL-10 in the combination with TGF-ß (152). These T regulatory type 1 (Tr1) cells produce IL-10 in their turn, and are able to inhibit both Th1 and Th2 responses *in vivo*.

Furthermore, T cell receptor stimulation in the combination with TGF-ß convert naïve murine $CD4^+$ T cells into FoxP3-expressing $CD25^+$ regulatory T cells (153). Regulatory T cells very similar to natural Treg were also generated from naïve $CD4^+$ T cells activated in the presence of IL-2 and TGF-ß (154). However, TGF-ß has a dual role on T cell development and the addition of IL-6 instead programs TGF-ß-stimulated naïve cells to differentiate into pro-inflammatory IL-17-producing Th17 cells, at least in mice (155-157).

Data from *in vitro* experiments have shown that the activation status of the antigen-presenting cell is central in the priming of naïve T cells. Antigen-presentation by an

immature DC, which lack sufficient co-stimulation to prime effector T cells or a "differentially activated" DC can result in the generation of T cells with regulatory properties. Jonuleit et al showed that stimulation of naïve, allogenic $CD4^+$ T cells by immature $CD83^-$ DCs resulted in non-proliferating, IL-10-producing T cells, able to suppress the proliferation of Th1 cells in a contact-dependent manner (158). Production of IL-10 by dendritic cells can promote the induction of T cells with regulatory functions (159). It is also possible that the expression of certain alternative ligands on the APC induce regulatory T cells rather than effector T cells. For instance, DCs expressing molecules such as ICOS-L, DC-SIGN, ILT3 and ILT4 are prone to induce tolerance rather than aggressive immunity (160, 161).

Infectious tolerance, i.e., that regulatory T cells can transmit their regulatory properties to other T cells, was demonstrated some years ago in transplantation models. Transferred regulatory T cells were then found to submit suppressive properties to naïve T-cell subsets of host origin (162). More recently, two groups independently showed that $CD25^+$ regulatory T cells shown to be suppressive by cell-cell contact dependent mechanisms are able to induce naïve $CD4^+$ T cells to suppress *via* a cytokine-dependent mechanisms *in vitro*: TGF-ß (37) or IL-10 (36). A mechanism of infectious tolerance to explain some of the features of peripheral tolerance was suggested several years ago by Coutinho and co-workers (35), and the *in vitro* findings are now beginning to unravel how this might occur. Still, the specific mechanisms behind the transfer of suppressive properties from one T cell to another remain unclear.

Peripheral induction of regulatory T cells. The natural $CD4^+CD25^+$ T cells that develop early in life are believed to be specific for self-antigens that are present in the thymus at the time (163, 164). But how does the immune system deal with the regulation of the response to innocuous antigens not present in the thymus e.g. dietary antigens, antigens from our commensal flora, allergens or organ-specific self-antigens not expressed in the thymus? The *in vitro* experiments described above have pointed out different mechanisms for how induction of regulatory T cells might take place in the periphery and animal models have provided further insight. The induction of peripheral tolerance is vital to preserve immunological homeostasis. This way, tolerance to self-structures can be propagated and maintained, and new structures encountered from the environment can be embraced by the immunological tolerance. The acceptance of the growing fetus is perhaps the most fascinating feature of peripheral tolerance induction, though outside the scope of this essay.

In the steady state, dendritic cells continuously migrate from peripheral tissues to the draining lymph nodes, carrying self-antigens to be presented to the recirculating naïve T cells in the T cell-areas (165). Dendritic cells from the intestinal lamina propria also carry a variety of environmental antigens, derived from the diet and the microbial flora. In absence of inflammation, immature or alternatively activated, tolerogenic dendritic cells can convert naïve T cells to regulatory T cells (166-168). The message communicated to the naïve T cells will be influenced by the different signals that the dendritic cells has

received in the tissue. Local factors in the tissue, such as TGB-β that is abundant in the intestinal mucosa where it is produced by various cell types, will condition the dendritic cells during uptake of antigen and affect the expression of costimulatory molecules and cytokines by the dendritic cells. The signals received by the naïve T cell will guide differentiation as well as the following migration into the tissue. Subsets of regulatory T cells, induced by tolerogenic dendritic cells in the gut draining mesenteric lymph nodes under steady state conditions, can therefore home back to the intestinal mucosa. Their production of anti-inflammatory cytokines as IL-10 and TGB-β will then in turn contribute to the conditioning of new dendritic cells. The microenvironment during antigen uptake is likely to be a key factor to determine if a DC will be tolerogenic rather than immunogenic.

When an antigen is administered at mucosal surfaces, the general response is tolerance. In response to a fed protein antigen the tolerance is induced at both a local and a systemic level, which is a well-known phenomena known as oral tolerance. This form of tolerance is dependent on the induction of peripheral regulatory T cells. After feeding of an antigen, we found induction of T cells with the phenotype and suppressive function of regulatory T cells in the local mesenteric lymph nodes, as well as the liver draining lymph node and peripheral lymph nodes (paper II). Also nasal administration of antigen leads to induction of regulatory T cells in mice (169).

A low-dose intravenous antigen administration has also been shown to generate $CD25^+$ anergic T cells (170). Apostolou and Von Boehmer demonstrated that regulatory T cells expressing FoxP3 can be induced in naïve $CD4^+$ T cells in the periphery. In their model, peptides were continuously delivered to adult thymectomized TCR transgenic mice on a RAG-/- background (171).

Experience from organ transplantation shows that a short treatment of non-depleting anti-CD4 antibodies can induce tolerance to foreign protein antigens (172). This treatment enables long-term survival of MHC-mismatched cardiac grafts (173). Tolerance is further established by the addition of anti-CD8 and anti-CD154 (CD40L) antibodies, which results in the acceptance of a second donor graft at any later time, while the graft from a third party will always be rejected (174). The maintenance of the tolerance induced by anti-CD4 antibodies is dependent on $CD4^+$ regulatory T cells (175). Cobbold et al showed that the regulatory $CD4^+$ T cells induced in TCR-transgenic mice by non-depleting anti-CD4 antibodies expressed FoxP3, and that the induction of FoxP3 was TGF-ß dependent (176). Thus, it appears that regulatory T cells with a phenotype similar to natural $CD4^+CD25^+$ Treg can be induced in the periphery by this strategy. A recent transplantation study demonstrates that the treatment of non-depleting anti-$CD4^+$ antibodies selectively expands donor-specific regulatory T cells, while inhibiting the effector T cells (177). Induction of FoxP3 in $CD4^+GITR^{low}$ T cells could not be observed in this study. Thus, there is some disagreement about the relative contribution of *de novo* induction of regulatory T cells in the allograft tolerance induced under the cover of non-depleting anti-CD4 antibodies.

As already mentioned, anti-CD3 antibodies have also been applied as therapy in models of autoimmune disease, where the mechanism seems to involve induction of regulatory T cells in a TGF-β dependent manner (139-141, 178).

In conclusion, a wide variety of strategies have successfully induced regulatory T cells *in vivo*. While the results differ regarding the phenotype of the induced regulatory T-cell populations and their relative importance of cytokines in suppression, TGB-β is clearly a central cytokine in the maintenance of peripheral tolerance.

The many possible ways of experimental induction of regulatory T cells in the periphery probably mirrors the importance of the mechanism in maintaining peripheral tolerance. The anatomical location in the most dire need of extensive regulatory mechanisms under physiological conditions is the gastrointestinal tract, for reasons that will be outlined below. Antigen handling of luminal antigens and induction of regulatory T cells in lymphoid tissue associated with the gut will therefore be the focus of the following sections.

THE INTESTINAL ENVIRONMENT

The intestinal environment is challenging as the highest load of foreign antigens, microbial and dietary, is separated from the largest immunological tissue of the body by a single layer of columnar epithelium. While the main topic of this essay is focused on how the immune system avoids misdirected and uncontrolled immune reactions, the defence against infections, being the fundamental primary task of the immune system, must not be forgotten. The mucosal surfaces are the major entry sites for pathogens and mucosal infections are the major cause of death for children below the age of 5 years.

The mucosal surfaces are thin barriers that are particularly sensitive to infection. Their physiological functions, i.e. gas exchange and food uptake, require that they are permeable barriers between the environment and the interior of the body. Especially the gut is the entry point of a vast array of different foreign antigens derived from the environment and the food. In addition, the gut is colonised by about 10^{14} commensal microorganisms with beneficial effects. Thus, the intestinal immune system has to be able to discriminate between harmful and innocuous antigens. Since immunogenic material constantly cross the epithelial barriers the body must avoid vigorous immune responses directed against for example dietary proteins or commensal bacteria in order to prevent chronic inflammatory disorders at these sites. At the same time, invading pathogens have to be promptly detected and effectively killed. The mucosal adaptive immune system has therefore developed different mechanisms to handle the various kinds of antigens.

Organization of the intestinal immune system. A single cell layer of columnar epithelium forms the lining of the intestinal mucosa. The cells are connected by tight junctions to create a selective barrier to the luminal external environment. The surface area is enlarged by folds, crypta, villi and microvilli and covers about two hundred square meters in a human being (179) making it more than 100 times larger than the area of the skin. Underlying the epithelium is a thin layer of richly vascularised loose connective tissue, the lamina propria that contains both blood and lymph capillaries and effector cells of the immune system. The small intestine is the major site for digestion and the absorption of nutrients, water and electrolytes and the intestinal epithelial cells are responsible for the import of luminal contents for further transport by blood and lymph.

Lymphoid cells are located in different compartments in the gut (Figure 4). These are the organised structures of the GALT (gut-associated lymphoid tissue), the mesenteric lymph nodes and small foci of lymphocytes and plasma cells scattered throughout the lamina propria and the surface epithelium of the gut wall.

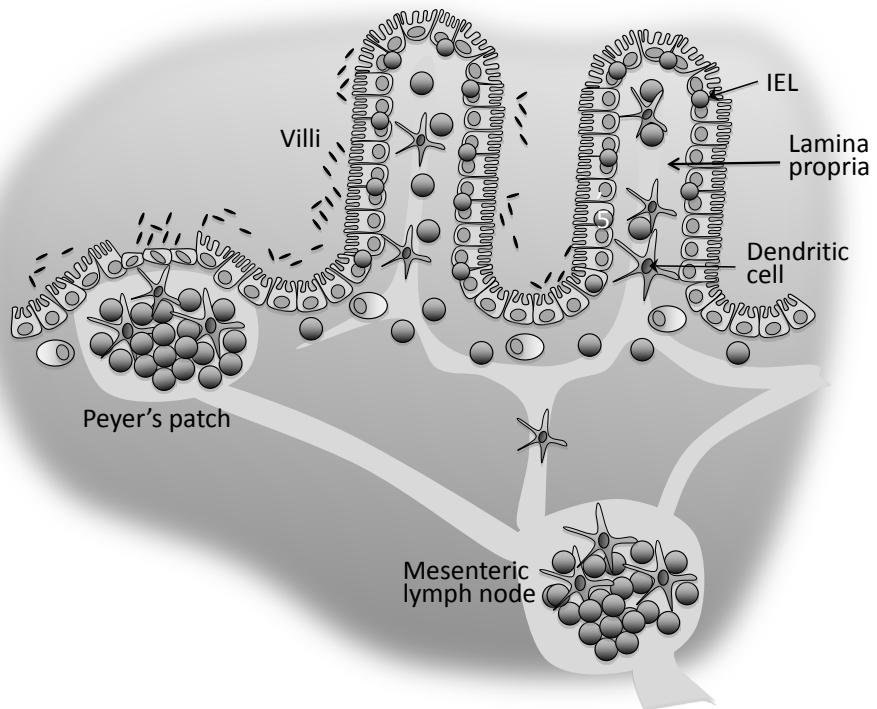

Figure 4. The lymphoid tissue of the small intestine. Diffusely scattered lymphocytes, dendritic cells and plasma cells in the lamina propria, intraepithelial lymphocytes (IEL) and organized lymphoid structures such as Peyer´s patches and draining mesenteric lymph nodes.

The GALT consists of the tonsils, the adenoids in the back of the mouth, the Peyer's patches of the small intestine, the appendix and the solitary lymphoid follicles of the large intestine and rectum. They are all located within the mucosa itself and have no afferent lymph flow. Together with the gut-draining mesenteric lymph nodes, these are the major sites for induction of immune responses to promote protective immunity. Lymphoid cells in the lamina propria and the epithelium are mainly effector cells.

The intestinal microflora. The intestinal lumen is the home of approximately 10^{14} commensal bacteria, 10 times the number of the combined eukaryotic cells of the human body. The microflora include several hundred species, and their collective genetic material is estimated to encode as much as 100 times the number of genes in the human genome (180).

The gut is sterile before birth but the colonization of bacteria from the surroundings starts immediately to create a rich and dynamic ecosystem, with the highest bacterial load in the colon. Initially, the flora is very variable and has a high turnover in species. The composition depends mainly on what bacterial species the infant happens to be exposed to and is dominated by facultative bacteria that tolerates the abundance of oxygen in the neonatal gut (181, 182). The composition then gradually changes as an increasing number of anaerobic species are established in the gut. The establishment of a full flora, dominated by obligate anaerobes, takes several years. The complex mature flora strongly prevents establishment of new species, but the flora continuously changes, even in adult life.

Evolution has formed a symbiotic relationship between the commensal bacteria and their mammalian hosts. The microbiota facilitate the digestion and absorption of nutrients and occupies the environmental niche, protecting the host from colonization by pathogenic microorganisms. Signals from the bacteria are essential for development of normal intestinal physiology (reviewed in (180)). The commensal flora also promotes angiogenesis, development of the intestinal epithelium and the development of Peyer's patches (180). Stimuli from the microbiota provide a basal level of activation necessary for normal function and homeostasis of the immune system, as will be discussed in more detail in later sections.

Antigen exclusion. The mucosal barrier provides a first line of protection against the external environment in the intestinal lumen. Lining the epithelial cells is a mucus layer formed by secretions from goblet cells. The mucus layer is rich in anti-bacterial peptides produced by the epithelial cells, including defensins, cathelicidins and calprotectins, with broad-spectrum antimicrobial action by the formation of pores in bacterial cell walls. The microvillar extensions on the apical surface of the epithelial cells form a brush border that impedes the attachment and invasion of pathogens while epithelial tight junctions prevents translocation between cells. Secretory IgA antibodies, produced by plasma cells in the lamina propria, inhibit or modulate colonisation and translocation of microorganisms and reduce the penetration of potentially dangerous soluble agents such

as bacterial toxins. At least 80% of the antibody production of the body takes place in the lamina propria, mainly in the form of dimeric IgA that is translocated to the gut lumen by polymeric Ig receptor mediated transport (183). Immune exclusion limits the interactions by pathogens and toxins with the epithelium and constitutes a first line in mucosal defence mechanisms. Mainly pathogens and particulate antigens taken up through the thin M-cells covering the Peyer's patches stimulate secretory immunity. The intestinal epithelial cells also play an active role in immunity, which will be further discussed below. Cytokines and other signal substances are produced in response to microbes after recognition by the various pattern recognition receptors expressed by the epithelial cells. The epithelium forms an active barrier in communication with immune cells nearby and contributes to the microenvironment that forms their maturation.

Inclusion of luminal antigens. Although the epithelium comprises an efficient barrier function of the mucosal lining, the luminal content is constantly sampled and basal stimuli from the commensal bacteria influence the intestinal homeostasis. The Peyer's patches are dome-shaped structures consisting mainly of B-cell follicles. T-cell areas are occupying the space between the follicles, and the structure is covered by an epithelium rich in specialised antigen sampling cells, termed M (microfold) cells. The M cells are particularly effective in the uptake of live and dead particulate antigens e.g. bacteria from the gut lumen. This material is collected by endocytosis and transferred in vesicles to the basolateral membrane, where they are released into the extracellular space. At the basal surface of the M cells, the material comes in contact with naive B cells and is also taken up and processed by local dendritic cells.

A specialized subset of dendritic cells in the lamina propria of the small intestine can form tight junctions with epithelial cells and extend their dendrites to directly sample the luminal content (184), a process that might be most frequent and significant at times of inflammation. The mechanism is dependent on the expression of the CX_3CR1, the receptor for the chemokine CX3CL1 (fractalkine) (185) and influenced by the composition of the commensal flora (186).

Dietary antigens are mainly taken up through the extensive epithelial surfaces rather than by the M cells. They are transferred through the cells by transcytosis to be released on the basolateral side. As will be discussed in more detail below, the epithelial cells are, similar to professional antigen presenting cells, equipped with structures that enable them to process protein antigens and load peptides on MHC class II (187). Soluble protein antigens are rapidly pinocytosed from the intestinal lumen and co-localised with MHC II in a vesicular compartment of the intestinal epithelial cells (IECs) (188, 189). The basal membrane prevents direct interaction of the epithelial cells with $CD4^+$ T. However, the epithelial cells release small MHC II-bearing vesicular structures, exosomes, which may influence antigen presentation at the mucosal or systemic level, independent of direct cellular contact with effector cells ((189-193) and discussed below).

Immune responses are mainly initiated in secondary lymphoid structures associated with the intestinal mucosa, i.e. the Peyer's patches and mesenteric lymph nodes. Here, circulating naïve lymphocytes are interacting with dendritic cells presenting their sampled antigens. The liver that receives the venous outflow from the intestines, and the lymph node draining the liver may be an important second level in this process (paper II and below).

Interactions in the mucosa. The specific microenvironment of the lamina propria is formed by the interaction between a variety of cells - among them the intestinal epithelial cells, macrophages, dendritic cells, numerous lymphocytes - and stimuli from the microbial flora.

The dendritic cells (here defined as MHC II^+CD11c^+ cells) constitute an unusually large proportion of the cells in lamina propria (about 10-15%), the majority that are $CD11b^+$ (194). A subpopulation expressing CD103 is preferentially migrating under steady-state conditions (194, 195) in a process depending on expression of the chemokine receptor CCR7 (196). The conditioning of dendritic cells in their local tissue environment has probably an important role in forming their function (Figure 4). TGB-β, produced by cells of both haematopoietic and non-haematopoietic origin as the epithelial cells, is abundant in the normal intestine. The epithelial cells also secrete other immunoregulatory molecules that might regulate DC function, including different chemokines and TSLP (thymic stromal lymphopoetin), prostaglandin E_2, IL-10 and IDO (indoleamine 2,3-dioxygenase) (recently reviewed in (180)). Dendritic cells that are co-cultured with epithelial cells produce TGB-β, express lower levels of MHC class II and co-stimulatory molecules and are less sensitive to stimulation via Toll-like receptors (167). The epithelial cells interact with the bacterial flora, and their expression of pattern recognition receptors are restricted to intracellular compartments (TLR3, TLR7, TLR8, TLR9 and NLRs) or the basolateral membrane (TLR5) (180) may aid them to distinguish between invasive and commensal bacteria. The lamina propria dendritic cells express a wide range of pattern recognition receptors and can acquire antigen from the gut lumen and respond to invasive pathogens. In a state of infection with invading pathogens, the intestinal epithelial cells will be activated through their intracellular and basolateral PPRs. This may induce their expression of pro-inflammatory cytokines and chemokines such as CXCL8 (197) that attracts neutrophils. The escalating inflammatory environment will then increase the infiltration of blood-borne DC precursors. These cells, that have not been conditioned in the lamina propria under steady state conditions, may be activated by pathogens and pro-inflammatory cytokines (198). Pathogen invasion and tissue damage causes increased exogenous and endogenous danger signalling, which results in increased activation of dendritic cells via a variety of PRR (pattern recognition receptors), such as TLRs, C-lectin-like receptors (CLRs) NOD-like receptors (NLRs), and RIG-I-like receptors

(RLRs) (199) during their maturation and antigen sampling. They can then migrate to the draining lymph nodes to drive inflammatory Th1/Th17 T cell responses.

Homeostatic regulation of DC activation in the LP seems to be dependent on peripherally induced regulatory $CD4^+$ T cells that home back to the lamina propria to produce IL-10 and TGB-β that contribute to the conditioning of dendritic cells. Thus, the direction of the resulting immune response is probably mainly controlled by the conditioning of the dendritic cell that in turn depends on the signals received in the tissue during antigen sampling. Under physiological, non-inflamed conditions, $CD11b^+CD103^+$ dendritic cells migrate from the mucosa to present antigens in the lymph nodes. As a result, dietary antigens, components from apoptotic epithelial cells and the commensal flora are presented in association with signals that drive a suppressive rather than aggressive immune response.

Induction of regulatory T cells. The main entrance route for foreign antigens under physiological conditions is from the intestinal lumen. The intestinal immune system tolerates repeated exposure to food antigens and the commensal flora, while maintaining the capacity to initiate strong immune responses against pathogens. This ability to tolerate ingested antigens is referred to as oral tolerance and relies on the induction of antigen-specific regulatory T cells.

It was early recognised that a fed protein antigen normally generates a state of antigen-specific hyporesponsiveness, which is known as oral tolerance. The phenomenon was already in 1911 described by Wells, who observed that systemic anaphylaxis in egg sensitised guinea pigs was prevented by previous feeding of hen egg protein (200). Once oral tolerance has been established, antigen-specific immune responses are suppressed both at local and systemic level. Various immune responses, such as T-cell mediated delayed type hypersensitivity (DTH) (201, 202) and production of antibodies (203, 204) are suppressed *in vivo* after oral antigen intake. *In vitro*, antigen-specific proliferation (204, 205) as well as cytokine production (206, 207) are suppressed by lymphocytes from tolerant animals.

One of the major mechanisms in oral tolerance is the induction of regulatory $CD4^+$ T cells, known as Th3 cells. Their active suppression is mediated by the production of cytokines such as IL-10 and TGB-β, and appears to depend particularly on TGB-β (208, 209). Characterisation of antigen-specific $CD4^+$ responses was enabled by the generation of the DO11.10 transgenic mouse that carries a gene coding for a TCR specific for a single peptide of OVA in the context of MHC II $I\text{-}A^d$ on most of the T cells (210). A more physiological level of antigen-specific T cells was achieved by adoptive transfer of transgenic cells into wild type BALB/c mice (211).

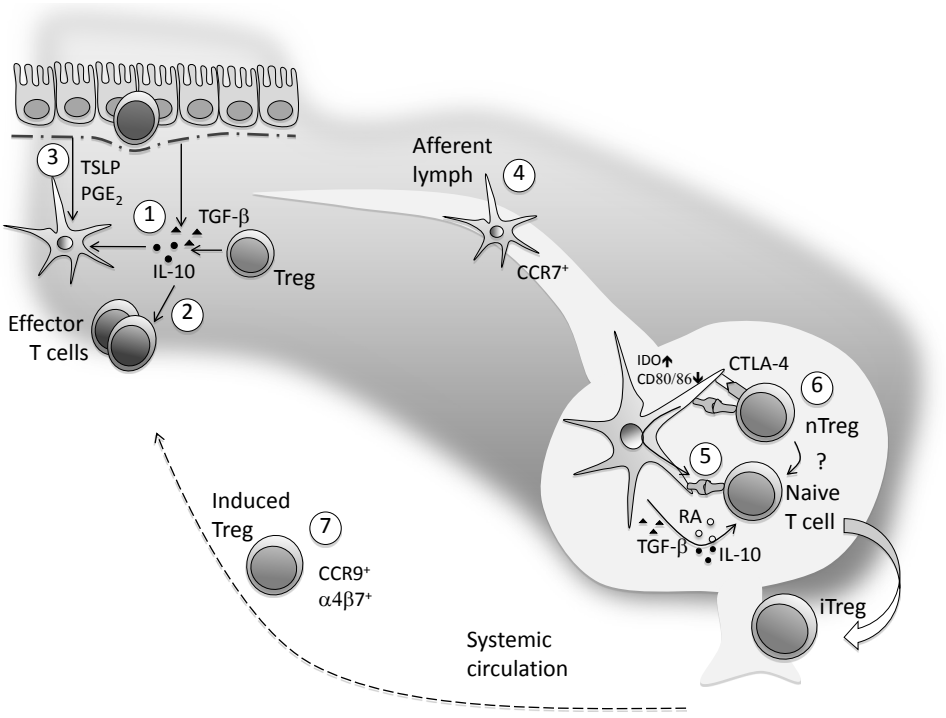

Figure 5. Hypothesis of the regulatory immune response in the lamina propria and mesenteric lymph node. (1) IL-10 and TGF-β are produced by $CD4^+$ regulatory T cells in the lamina propria, but also by other cells such as the epithelial cells. (2) These non-inflammatory cytokines control the effector T cells in the lamina propria. (3) TGF-β and IL-10, together with other immunomodulatory molecules such as TSLP and PGE_2 produced by the epithelium conditions the dendritic cells in the lamina propria during antigen uptake. (4) Dendritic cells migrate in the steady state to the mesenteric lymph nodes by a mechanism dependent on CCR7. (5) In the lymph node, interactions with naïve T cells can take place. Dendritic cells conditioned in the lamina propria may then induce new subsets of regulatory T cells by mechanisms dependent on TGF-β and retinoic acid (RA). (6) This process may also be influenced by natural regulatory T cells, directly acting on the naïve T cells or indirectly via the dendritic cells. (7) Induced regulatory T cells, suppressive by the secretion of IL-10 and TGF-β, are imprinted ($CCR9^+\alpha4\beta7^+$) to home back to the lamina propria where they contribute to the anti-inflammatory microenvironment.

The secondary lymphoid tissue associated to the intestine is probably the preferential site for conversion of regulatory T cells as a consequence of the high requirement for regulation (Figure 5). The dendritic cells can direct the adaptive immune response in favour for a tolerogenic or inflammatory phenotype by the delivery of co-stimulatory and cytokine signals at the time of antigen presentation. A subset of mucosal dendritic cells with a plasmacytoid phenotype, normally characterized as producers of type 1 interferons, have been shown to reduce their production of these cytokines in response to TGB-β and IL-10 (212). When isolated from the mesenteric lymph nodes, this subset was able to induce regulatory T cells (167).

Regulatory T cells have recently been shown to be induced by a mechanism dependent on the combined effect of TGF-ß and the vitamin A metabolite retinoic acid (RA) (194, 213-215). RA-synthesising enzymes are abundant in the intestinal epithelial cells and certain populations of dendritic cells (216) and RA can manipulate DC functions (217). The presence of RA also antagonizes the generation of Th17 cells that is mediated by TGB-β in the presence of IL-6 (215). It has been suggested that the simultaneous production of RA and TGB-β has evolved in the gut as a self-contained strategy to promote conversion of naïve T cells into regulatory T cells, which would explain some of the unique capacity of the gut to induce tolerogenic immune responses (194).

Bystander tolerance. It has been demonstrated that oral tolerance involves a "bystander effect", i.e. after induction of tolerance by oral administration of one protein, the response to other proteins are also suppressed when encountered together with the fed protein in the periphery (218-220). This indicates that regulatory T cells specific for the fed antigen can affect naïve T cells in their close proximity and presumably presented by the same dendritic cells (221). The induction of oral tolerance by oral administration of self-proteins is effective in suppressing pathology in animal models of autoimmune diseases (222), especially in a preventive approach. By the administration of an organ-specific protein, regulatory T cells can be directed to the lymph nodes draining this organ. Here, they may affect the priming of T cells with other antigen-specificities, so that the tolerance is extended to include new epitopes, and epitopes from other proteins. In some cases oral administration of a self-antigen has also been able to cure already established autoimmune disease (223).

Targeting of lymphocytes. To target lymphocytes to areas of infection or where they are needed for regulation, a system controlled by combinations of chemokines and adhesion molecules has evolved. During activation, the lymphocytes are imprinted with a specific expression of receptors so that they can respond to adhesion molecules and chemokines expressed in their target organs. Naïve lymphocytes express CD62L, so that they can recirculate secondary lymphoid organs by interaction with L-selectin binding GlyCAM-1 on high endothelial venules (HEV). Lymphocytes primed in Peyer's patches and MLN downregulate CD62L and upregulate the $\alpha 4\beta 7$ integrin. The activated lymphocytes,

travelling via the lymphatic system into the blood stream, can therefore re-enter the mucosal tissues from the capillaries by binding to the mucosal addressin MAd-CAM1. In parallel, they also upregulate the chemokine receptor CCR9, which allows them to respond to CCL25 (TECK), specifically expressed by intestinal epithelial cells (224, 225).

The mucosal imprinting is also dependent on retinoic acid produced by $CD103^+$ dendritic cells migrating from the intestinal mucosa. In rats deprived of vitamin A, IgA-producing plasma cells and $CD4^+$ T cells are virtually absent from the lamina propria (226). Activation of intracellular retinoid receptors in the lymphocytes leads to the transcription of genes encoding $\alpha4\beta7$ and CCR9 (216).

In parallel, dendritic cells from the skin metabolize vitamin D3 induced by the sunlight and program T cells to respond to chemokines expressed in the skin (227). T cells primed in the periphery express the $\alpha4\beta1$ integrin and CCR4 and can therefore not migrate to mucosal surfaces (228). As a result, mucosal vaccination is required to generate protection against mucosal infections.

Activated cells can spread all across the mucosal surfaces after the initiation of an immune response localised to a few Peyer's patches. However, there is also a regionalisation, so that primed immune cells selectively home back to effector sites corresponding to the inductive site where they first were triggered. The activated lymphocytes enter distinct compartments in the intestine. B-cell blasts mature into IgA-producing plasma cells and stay in the lamina propria. $CD4^+$ T cells also remain in the lamina propria, while $CD8^+$ T cells migrate preferentially to the epithelium (229).

It seems that RA has a very central function in the immune system associated with the intestine. In concert with TGB-β, RA is important for the induction of regulatory T cells in the mesenteric lymph nodes. It is also necessary for directing the following homing into the intestinal mucosa and for the support of the IgA-switch of B cells. Enzymes catalyzing the production of RA are expressed by subsets of dendritic cells in the lamina propria and MLN, and also by the intestinal epithelium. RA acts through receptors from the RAR and retinoic X families, working as transcription factors that directly or indirectly might affect hundreds of genes (198).

In conclusion, there are several distinct anatomical features and cell types of the mucosal immune system. They maintain the homeostasis in the gut, which is a sensitive and exposed organ, where accurate immune responses are necessary to avoid chronic inflammation.

Serum confers tolerance. The remarkable efficiency that tolerance is induced with after feeding of a protein antigen indicates how important this mechanism is in order to avoid damaging immune responses. The involvement of regulatory T cells is now well established and mechanisms for their induction are emerging. Despite this, it remains partly unclear how dietary antigens are processed and presented to the immune system to induce tolerance rather than an aggressive immune response.

Strobel et al showed in 1983 that serum of animals fed a protein antigen contained the antigen processed into a tolerogenic form (230). Transfer of serum from ovalbumin-fed donor mice to naïve recipients, who were then immunized with ovalbumin, suppressed DTH-responses in an antigen-specific way (Figure 6). By contrast, serum collected after parental administration of ovalbumin did not induce tolerance (230, 231).

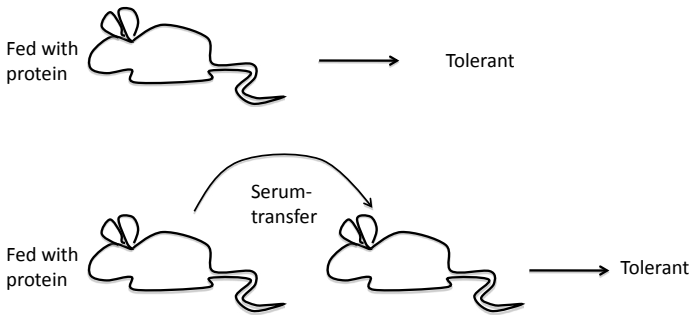

Figure 6. If a mouse is fed with a protein antigen, tolerance is induced by a mechanism known as oral tolerance. If serum is collected from the fed mouse shortly after feeding, and given to a new mouse, the tolerant state can be transferred.

Thus, a dietary antigen is altered as it crosses the intestinal mucosa and enters the circulation. The process is time-dependent but does not correlate to the serum level of the absorbed antigen, as an adjusted amount of serum collected at an earlier time point does not confer tolerance in the recipient (232). In addition, feeding of an irradiated animal, with a damaged intestinal barrier, does not result in a tolerogenic serum factor despite a higher serum concentration of the fed protein, indicating that irradiation damage allows for a higher proportion of non-processed immunogenic antigen to enter the bloodstream (233). These experiments illustrate the relevance of the digestive process in the regulation of immune responses to a fed antigen. That leads us on to consider the function of the intestinal epithelial cells (IEC) more closely.

Expression of MHC class II on enterocytes. Dietary antigens are taken up mainly through the extensive epithelial surfaces, rather than by M cells of the Peyer's patches. The columnar epithelium of the small intestine was shown to express MHC class II in rodents and man (234, 235) just as antigen-presenting cells of hematopoietic origin. MHC class II is expressed by mature enterocytes on the distal two-thirds of the villi in the small intestine (235). In rodents the epithelial expression of MHC II starts at weaning and is fully developed at about 6 weeks of age. The demonstration of MHC class II-expression raised the suggestion that they might act as antigen-presenting cells, activating regulatory T cells directly at the mucosal portal of antigen entry. Bland and Warren showed that isolated intestinal epithelial cells from the rat were able to activate T cells from immunized animals in vitro in an antigen-specific way that could be blocked by the addition of anti-MHC class II antibodies (236). T cells modified by co-cultures with IEC were further able to suppress the response of primed T cells (237). Intestinal epithelial cells express receptors for IFN-γ on the basolateral membrane(238) and in the presence of IFN-γ expression of MHC class II is strongly upregulated (239, 240).

Other components for antigen processing. Intestinal epithelial cells constitutively express MHC class II the expression can be further enhanced under inflammatory conditions. For a cell to efficiently process and present antigens to $CD4^+$ T cells, the invariant chain (Ii) is needed to prevent binding of endogenous peptides and direct the MHC class II molecule to an acidic intracellular compartment. Another important chaperon molecule is the HLA-DM(human)/H2-M(mice), which removes the last fragment of Ii (CLIP) from the peptide binding groove and replace it with an antigenic peptide, a process that also involves a number of proteases (241, 242). The intestinal epithelial cells express the Ii and HLA-DM in the normal intestine at steady state, (187) and process antigens by the same class II processing pathway as conventional antigen presenting cells (243).

Trafficking of MHC class II. Mayrhofer and Spargo demonstrated that the expression of MHC II was localised to the basolateral cell membrane and to intracellular multivesicular bodies, where MHC class II expression was found on the limiting membrane as well as on the membrane of the enclosed small vesicles (190). Zimmer et al later demonstrated that orally administered antigens are rapidly targeted to late endosomes containing the endosomal marker LAMP-1 (lysozome associated membrane protein-1) and MHC class II in enterocytes of normal mice (244). Antigen and MHC class II were colocalized in vacuoles filled with membrane sheets and vesicles that could be observed to fuse with the basolateral membrane. SCID mice, that have a defect recombination of the antigen-recognition receptors lack functional mature T and B cells and are unable to produce a tolerogenic serum factor after feeding. In these mice a fed antigen did not target to late endosomes of the IECs, but instead rapidly reached the

paracellular space transported by vacuoles lacking MHC class II and LAMP-1 (244). These data indicated that late endosomal compartments were important for antigen processing by enterocytes. Sorting of antigens into late endosomes is induced by IFN-γ in conventional APCs (245) and Büning et al demonstrated that co-localisation of antigen with MHC class II and LAMP-1 in SCID mice could be restored by IFN-γ treatment (246). Intraepithelial lymphocytes, stimulated by the commensal flora, are the main source of IFN-γ in the mucosa of normal mice (247) and these cells are absent in the SCID mice, and their IECs are therefore devoid of MHC II expression.

Key to the relevance of MHC class II expression. In vivo, direct interactions between epithelial cells and $CD4^+$ T cells are limited because of the basal membrane. In addition, naïve cells do not have access to the mucosal tissue in the normal situation, as they express CD62L rather than $\alpha4\beta7$ and therefore circulate secondary lymphoid organs. It was discovered that intestinal epithelial cells are secreting MHC class II/peptide–loaded exosomes (191, 192)and it was further shown that the tolerogenic component in serum was found in the fraction of serum that contains the exosomes derived from the epithelial cells. The exosomes are sedimented by ultracentrifugation at 70 000 x g and this fraction induced tolerance, while the supernatant had lost its ability to induce tolerance after centrifugation(191). The sedimented fraction was rich in MHC class II and the removal of MHC class II from the 70,000×g fraction eliminated its capacity to induce tolerance in the recipients. This means that MHC class II/peptide-complexes, derived from the intestinal epithelial cells and carried by exosomes, can reach local cells of the immune system as well as the systemic circulation.

The exosome-fraction of serum from fed donors has been shown to induce tolerance when administered intraperitoneally to naïve syngenic recipient mice, subsequently challenged systemically in a Th1-dominated (191, 248) as well as a Th2-dominated (249) model.

Characteristics of exosomes. Exosomes are 30-90 nm vesicles that are formed by inward budding in endosomal compartments called multivesicular bodies (MVBs). By fusion of the MVBs with the plasma membrane, the vesicles trapped inside are released into the extracellular space. The membrane orientation of the exosomes is therefore the same as it would be on the donor cell and they expose the luminal/extracellular side of their membrane. The release of exosomes was first demonstrated from reticulocytes during their maturation to erythrocytes (250, 251). The interest for their role in immunology started as it was discovered that they are also released from professional antigen presenting cells such as B cells (252) and dendritic cells (253), and were shown to be able to directly stimulate T cells in an antigen specific manner. They are also produced by placental trophoblasts during pregnancy (254) and appear to be involved in the regulation of the maternal immune response to the fetal tissue. Exosomes have been purified from body fluids as human plasma, serum, bronchoalveolar fluid, amniotic fluid and milk (255).

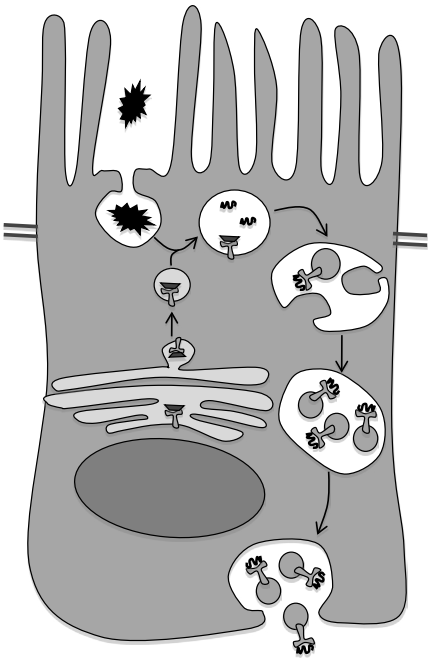

Figure 7. Formation of exosomes by intestinal epithelial cells. Dietary antigens in the gut lumen are endocytosed from the apical side of the intestinal epithelial cell and degraded. Early endosomes fuse with vesicles containing newly synthezised MHC class II molecules. The Invariant chain in the peptide binding groove of the MHC is exchanged by a peptide derived from the dietary antigen. Exosomes are formed by the inward budding of the endosome and multivesiclar bodies (MVB) are created. The MVB fuse with the basolateral cell membrane and exosomes with MHC class II/peptide complexes are released.

CD63 (LAMP-3), a marker of late endosomal compartments and other tetraspanins such as (CD9, LAMP-1) are enriched in exosomes, which confirms that they are not fragments from the plasma membrane but originate from an endosomal compartment (Figure 8). Moreover, the exosomes carry both MHC class I and MHC class II and tissue specific membrane proteins such as A33 from intestinal epithelial cells. They are frequently also equipped with CD54 (ICAM-1) and MFG-E8 that enable their interaction with e.g. dendritic cells. MFG-E8 binds to phosphatidylserine that is a component of the exosome membrane, and allow the interaction with integrins present on dendritic cells. In addition, the exosomes contains cytosolic proteins such as proteins from the cytoskeleton (actin, tubulin), enzymes (kinases, dehydrogenases) and heat shock proteins that may be trapped in the exosomal lumen during their formation. Reticulocytes, T cells and resting B cells secrete detectable levels of exosomes only after activation of cell surface receptors. Dendritic cells and macrophages on the other hand, as well as intestinal epithelial cells, constitutively secrete exosomes in vitro but the secretion can vary with cell-cycle and can be increased by ligand interactions or stress conditions (255, 256).

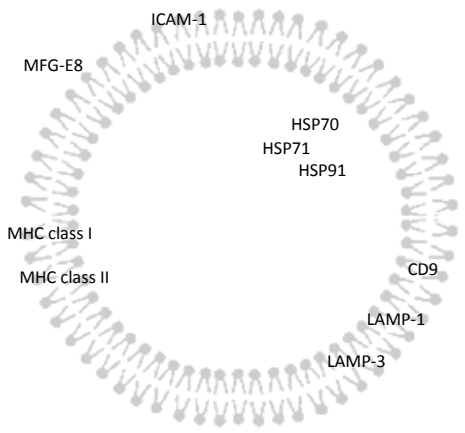

Figure 8. Exosomes are microvesicles released from different types of cells. They carry many different proteins, including the antigen presenting molecules MHC class I and II and proteins such as MFG-E8 and ICAM-1, that enable interactions with dendritic cells. Transmembrane proteins such as CD9, LAMP-1 and LAMP-3 indicate their endosomal origin. They also contain different heat shock proteins.

EXOSOMES AS MESSENGERS WITHIN THE IMMUNE SYSTEM

The "sms[4]" of the immune system. Exchange of membrane-derived proteins between cells of the immune system was discovered already in 1981, as it was observed that donor thymocytes of bone marrow chimeras acquired host-derived MHC molecules (257). Still, little is known about the fate of exosomes in vivo, but indirect evidence or in vitro studies have suggested different types of interactions between exosomes and recipient cells, including adhesion through lipids or ligand-receptor interactions, receptor-mediated internalisation of whole vesicles and fusion of exosomes with the plasma membrane (255). Electron microscopy has revealed that exosomes are present in tissue sections from tonsil germinal centers, where MHC-class II-bearing vesicles appear to be attached to follicular dendritic cells (258). As follicular DCs do not express MHC class II molecules, it is suggested that fDCs capture MHC II$^+$ exosomes produced by the surrounding B cells (255).

Local interactions. Exosomes derived from APCs can directly activate T-cell clones or pre-activated CD4$^+$ T cells, but they need to be captured by recipient DCs to activate naïve CD4$^+$ T cells (reviewed in (255)). Exosomes from IEC have been shown to interact with dendritic cells and strongly potentiate peptide presentation to CD4$^+$ T cells in vitro (259). CD11c$^+$ DC in the lamina propria interacts closely with the epithelium and appear to harbour intracellular fragments of IECs (24). A33, the specific marker for intestinal epithelium, as well as dietary antigens has been demonstrated in dendritic cells in the

[4] Short Message Service

mesenteric lymph nodes (24, 256). As mentioned earlier, a subset of dendritic cells migrate from the LP to MLN under steady state. Their collection of environmental antigens in the LP may be reinforced by the uptake of exosomes and part of the MHC II/peptide complexes they present to naïve $CD4^+$ T cells in the MLN may be derived from exosomes secreted by enterocytes. Exosomes may also travel in the lymph on their own, to interact with dendritic cells already present in the lymph nodes, which is supported by the presence of A33 staining in the MLN (256). As mentioned above, the exosomes are equipped with adhesion molecules that allow interaction with dendritic cells in target organs such as the lymph nodes and/or the liver. By the binding to ICAM-1 they may be captured by dendritic cells expressing LFA-1 and this interaction has been shown to be particularly important (260). MFG-E8 can work as a link between phosphatidylserine on the exosomes and integrins on the dendritic cells but do not appear to be essential for the interaction (261). Exosomes may also interact with other receptors for phosphatidylserine, such as the recently identified TIM-4 (262).

Reaching the systemic circulation. It seems that a substantial amount of intestinal derived exosomes reaches the peripheral circulation, as demonstrated by the fact that serum transfer from fed donors confers tolerance into naïve recipients, although not as potent as the one induced directly in a fed animal. The peripheral circulation could be reached either via the venous blood draining the gut, or via the lymphatic system. If the systemic circulation is reached via the blood vessels draining the gut, this certainly means that the concentration of exosomes is higher in the portal blood before the passage of the liver, as the liver is very efficient in clearing the blood of particulate material (263). If exosomes reach the peripheral circulation by the lymphatics, they are passing the MLN and reaches peripheral blood via truncus jugularis. This is supported by the presence of MHC class II in the exosome fraction of lymph fluid (unpublished observations) Thus, the liver and its draining lymph nodes along with the MLN may be a major final destination for intestinal derived exosomes reaching the circulation, as will be elaborated below.

Immune deviation by exosomes derived from enterocytes. As described above, exosomes produced *in vivo* have been shown to induce antigen-specific tolerance when isolated from the serum of fed donor mice and administered intraperitoneally to naïve syngenic recipient mice, subsequently challenged systemically by a Th1 or Th2-dominated reaction (191, 249). Results of the immunomodulatory property of exosomes obtained from IEC cultured *in vitro* are conflicting. While Karlsson et al found that exosomes isolated from antigen-pulsed intestinal epithelial cells (IEC-17) induced humoral as well as cell-mediated tolerance in naïve recipient rats, van Niel et al demonstrated that exosomes derived from a mouse epithelial cell line (MODE-K, derived from C3H/HeJ mice) instead primed recipient mice for a humoral response (256). In paper IV, we tried to explore the immunomodulatory capacity of a second murine cell line, IEC4.1 that is derived from BALB/c mice. By using exosomes derived from this cell line cultured with OVA peptide

and a low concentration of IFN-γ (100 U), we obtained a tolerising effect in recipient mice in a model of OVA induced allergic airway inflammation. However, exosomes from IEC cultured with a higher concentration of IFN-γ (250 U) had no such effect, no matter if given in the same or a higher dose. Thus, in our hands IFN-γ appeared to be a molecular switch for the resulting effect of IEC exosome on a subsequent systemic challenge. The data are too preliminary to draw firm conclusions from, but they raise some interesting questions.

IFN-γ as a molecular switch? Different experiments, ranging from the basal studies of oral tolerance induction (264), via serum transfer experiments of IFN-γ substituted SCID mice (248) and EM-studies of antigenic trafficking (246) and also our disparate results described above (paper IV), indicates that this is a fundamental factor. A basal level of IFN-γ stimulation is apparently necessary for antigen processing and the formation of a tolerising factor in serum. On the other hand, a too high level of IFN-γ stimulation seems to break tolerance and instead has the capacity to induce priming of the immune system.

The effect encircled – donor or recipient? Induction of oral tolerance can be blocked by previous IFN-γ treatment (264). In this case, the effect may have impact on either (1) the cells producing exosomes, leading to quantitative or qualitative differences of the exosomes produced or (2) on the immune cells that interact with the exosomes and subsequently mediate the information to naïve T cells. In contrast, by manipulation of donors (for collection of serum after feeding) or treatment of in vitro cultured cells (for isolation of their secreted exosomes), and the subsequent transfer to naïve recipients, the impact of the circumstances for exosome production can be studied separately. Based on that reasoning, it can from different studies be concluded that an irradiated (233) or defect (as in SCID mice) (248, 265) immune system of the serum donor interferes with tolerance induction in the recipient. Reconstitution of the irradiated donor mouse with normal spleen cells (233) or IFN-γ treatment of the SCID donor mouse (248) restores the capacity to generate a tolerogenic serum factor. This points to a role of IFN-γ-producing IEL as stimulators of the absorptive epithelium in order make them produce functional exosomes. However it has been shown that IFN-γ treatment directly prior to feeding (264), bacterial stimuli concomitant with an antigen feed (266, 267) or an ongoing active intestinal inflammation of the antigen fed mouse (268) can abrogate oral tolerance induction. It cannot be said for certain that this effect operates on the production of exosomes and not on the immune cells subsequently interacting with them.

Exosome-associated characteristics. With the factors that may have an impact on exosome secretion in focus, what would then be the qualitative or quantitative entities that may be affected by IFN-γ stimulation? The MHC class II/peptide density of the exosomes is one such factor, since a too low level or the complete lack of MHC class II

in the IEC would result in non-informative exosomes. Indeed, the MHC class II expression of enterocyte-derived exosomes is strongly upregulated by IFN-γ stimulation and exosomes obtained in the absence of IFN-γ have been found unable to induce an immune response (256). Unfortunately, we have not yet been able to reliably quantify the relative MHC class II levels on exosomes produced by enterocytes stimulated with different concentrations of IFN-γ and we have not studied the in vivo-effect of exosomes produced in the absence of IFN-γ stimulation in our system. IFN-γ also increases the expression of MHC class I as well as the total amount of exosome proteins produced by enterocytes ((192) and own observations). However, with the currently used strategy to quantify the total protein content of the exosomes (i.e the Bradford assay), it is not possible to tell if there is more exosomes produced or if there is a higher protein content of each exosome.

Exosomes derived from mature DCs commonly carry the co-stimulatory molecules CD80 and CD86, and have been shown to efficiently prime immune responses for rapid graft rejection (260). By contrast, exosomes from immature DC have been described to promote graft survival and reduce inflammation in a model of septic chock (269, 270). In the steady state, neither enterocytes nor their secreted exosomes express co-stimulatory molecules. Under inflammatory conditions, co-stimulatory molecules can be expressed on the enterocytes in parallel with upregulation of MHC class II (239, 240, 271) and it is possible that this changes the message of the exosomes.

It is also possible that endogenous ligands for pattern recognition receptors may convey signals of tissue distress from one cell to another. For example, exosomes from different cell types including enterocytes may express HSP70 (255), which is an endogenous ligand for TLR4 (272). However, the knowledge in this area is still limited. It has been suggested that heat shock proteins may exchange peptides between MHC class II molecules, from the ones brought by the exosomes, to the ones formed by the recipient dendritic cell (256).

In our model (paper IV), we pulsed the epithelial cells with the OVA peptide 323-339, which is the dominant T-cell-epitope in I-Ad expressing mouse strains . Exosomes from the pulsed epithelial cells and non-pulsed control cultures were administered to naïve mice, which were then immunized twice by peritoneal injections with native ovalbumin adhered to aluminiumhydroxide and challenged with ovalbumin intranasally. This means that about four additional, T-cell epitopes was present at the time for immunization and challenge, as well as three-dimensional B-cell epitopes. A tolerogenic effect induced by the initial antigen-encounter, in the form of OVA-peptide in complex with MHC class II on exosomes, was therefore later challenged by a more potent antigen in the airway-inflammation model. It is possible that a clearer effect would be seen with a different strategy. It has been demonstrated that exosomes must be loaded with a more complex antigen than a single peptide to evoke a potent in vivo T-cell response (273) and it would be of interest to repeat the experiment using an ovalbumin hydrolysate to load the exosomes.

Tolerance induction and anatomical aspects of the liver. As it is clear from serum transfer experiments that exosomes with the potential to confer tolerance reach the systemic circulation after feeding of an antigen, the positioning and physiology of the liver suggests a role in oral tolerance induction. The liver derives its portal venous inflow from the gastrointestinal tract, and has also been shown to play a critical role in tolerance induction. Long-lasting suppression of delayed-type hypersensitivity (DTH) was induced for cellular alloantigens given by portal venous injection, but not when administered by the intravenous route (274, 275). Further, it has been demonstrated that portal drainage through the liver is a prerequisite for establishing oral tolerance (276, 277). The venous outflow from the intestines is collected in the portal vein and passed through the liver before reaching other parts of the body. In the liver, the blood is slowly sieved through the liver sinusoids, which are lined by a fenestrated endothelium. The fenestrations, or pores, are letting particles as big as 100 nm through, so the blood is basically filtrated in the sinusoids. Blood cells are remaining in the lumen of the sinusoid while proteins and particles can pass through the fenestrations and into the space of Disse. This allows for interaction with hepatocytes and other cells in this area, such as DCs and Kupffer cells. In this way, the cells in the vicinity of the liver sinusoid can closely monitor the antigen load brought by the venous outflow from the intestines and thus also filter out particles with the size of exosomes. Further, the liver accumulates dendritic cells from the blood that effectively endocytose particulate matter of similar size as the exosomes (263). DCs can undergo blood-lymph translocation via the hepatic sinusoids, which are suggested to act as concentrators of blood DCs into regional hepatic lymph nodes (278). The liver draining celiac lymph node may therefore become an important site for immunomodulation in response to blood-borne antigens, in particular gut-derived antigens (Figure 9).

The unique microenvironment of the liver. DCs constantly transport apoptotic self-material from peripheral tissues to T cell areas of draining lymph nodes and they present peptide-MHC complexes to T cells. In the absence of danger signals, immature DCs may generate T cells with regulatory properties; this represents a physiological process in which a random selection of mainly self-antigens is used to tolerise T cells in the periphery. The liver constantly receives not only self-material but also exogenous antigens from the gut. Danger-signalling molecules such as LPS and other breakdown products of bacteria are present and accompanied by antigens from the diet. Despite this, liver-derived DCs have been shown to be poor inflammatory T cell stimulators. On the contrary, they produce the anti-inflammatory cytokine IL-10 (279, 280) and induce T cells, which in their turn produce IL-10 (280, 281). This might be explained by the specific microenvironment in the liver. The Kupffer cells, which reside in the liver

sinusoids, respond to physiological concentrations of LPS (ranging from 10 pg/ml to 1 ng/ ml) in the portal-venous blood by producing IL-10, TNF-α, TGB-β and prostanoids, all which have been shown to promote the development of tolerance inducing APCs (282-284). NKT cells, which are abundant in the liver, have been shown to produce large amounts of IL-10 and IL-4, presumably in response to certain bacterial products presented *via* the CD1 molecules on DCs (285). It has also been suggested that NKT cells may help in the induction of oral tolerance (286). In this manner, the unique microenvironment in the liver might cause "differential" activation/maturation of resident DCs, whereby favouring the induction of tolerance to self-antigens, as well as microbial and dietary antigens. The liver is under surveillance by a heavy traffic of DCs, which are capable of phagocytosis before they eventually migrate to the T cell area of the liver draining celiac lymph node (278). The sinusoidal endothelial cells that reside in the liver have also been shown to have antigen capturing and presentation capacities even for naïve T cells, at least *in vitro* (287).

Antigen-specific responses in the liver-draining lymph node. As the liver provides a unique microenvironment for the conditioning of tolerogenic DCs and has been shown to play a critical role in tolerance induction, we wanted to investigate antigen-specific T cell responses in the celiac lymph node (CLN) that drains the liver, pancreas and stomach (288). Studies of the induction of regulatory T cells after oral administration of protein antigens have focused on the mucosal immune system i.e. the Peyer's patches (PP) and the mesenteric lymph nodes (MLN) nodes (289-292). On this background, our aim in paper II was to elucidate the potential role of the liver in the priming of $CD4^+$ regulatory T cells in response to a fed protein antigen by evaluating the possible induction of regulatory T cells in the liver draining celiac lymph node (CLN) as well.

Using the adoptive transfer model and similar systems, several groups have investigated the activation of antigen-specific T cells during establishment of oral tolerance, but with conflicting results. Some groups suggests that responses to orally administered antigens are initiated locally in the gut (293-296) while other reports simultaneous activation of antigen-specific T cells throughout the animal after feeding (297, 298). In paper II, we found that differences in the kinetics of the response between different lymphoid compartments were readily detected. Antigen-specific cells in the PP were very rapidly activated, as determined by the upregulation of CD69 and CD25, while cells in the MLN and CLN were activated somewhat later, but still faster than cells in peripheral lymph nodes (PLN) and the spleen.

Peripheral induction of Treg – a special role of the CLN? Immunoregulatory cytokines are central in the T-cell mediated suppression involved in oral tolerance, especially TGB-β (208, 209, 299). However, several groups have also suggested a role for CTLA-4 (292, 300, 301), particularly in the induction phase (301). Zhang et al reported that oral administration of OVA to the DO11.10 mouse resulted in an increase of $CD25^+$

T cells, which had an elevated expression of CTLA-4 and produced increased amounts of TGB-β. Adoptive transfer of $CD4^+CD25^+$ T cells from fed DO11.10 mice suppressed DTH responses in naïve BALB/c mice (289). We also found a high proportion of bright $CD25^+$ T cells, particularly in the CLN and PLN, which also expressed a high level of GITR (paper II). In addition, the majority of the $CD25^{bright}$ cells in the CLN, but not in PLN, expressed CTLA-4 and CD103 (the integrin also known as α_E that forms a heterodimer with β7). Thus, it appears that cells of the putative regulatory phenotype were induced particularly in the CLN. Expression of CD103 may indicate preferential homing of these cells to the mucosal epithelium of the intestine and have been associated with an effector/memory phenotype of regulatory T cells (302). However, the *in vitro* suppressive properties of antigen-specific cells isolated from MLN, CLN and PLN were equal after oral OVA-administration of DO11.10 mice and we were not able to detect upregulation of FoxP3 of the antigen-specific T cells in any of the lymph nodes. Siewert et al later demonstrated induction of FoxP3 by antigen-specific T cells after oral antigen administration. Using a model very similar to ours, they found that the *de novo*-induced expression of FoxP3 was largely confined to the CLN, as considerably lower proportion of the antigen-specific cells in the MLN, PP and spleen were induced to express FoxP3 (303). The majority of the $FoxP3^+$ Tregs in the CLN also coexpressed CD103. Induction of $FoxP3^+CD103^+$ Treg in the CLN was not observed after intraperitoneal immunization of the antigen together with an adjuvant, suggesting that this response is restricted to antigen-specific activation under tolerogenic conditions (303).

Contribution of different suppressive mechanisms. Earlier studies have found a partial blockade of the suppression by orally tolerised T cells by the addition of antibodies specific for the IL-10 receptor as well as for the TGF-ß receptor (289). In paper II, we found no influence by neutralising IL-10 on the suppression mediated by antigen-specific cells isolated from OVA-fed DO11.10 mice. Anti-TGF-ß antibodies, on the other hand, almost completely reversed the suppression mediated by cells isolated from the PLN, and had also a great influence on the suppression mediated by cells from MLN. The role for TGF-ß in the PLN of orally tolerised animals has been shown previously (299). Interestingly, the neutralising anti-TGF-ß antibody did not have the same effect on suppressor cells isolated from the CLN. More than 50 % of the proliferation remained despite the addition of the blocking antibody. This implicates the influence of an additional mechanism of suppression by the transgenic cells isolated from the CLN. It would be interesting to evaluate the potential dependency of cell-cell contact in this system, and also the influence of anti-CTLA-4 antibodies.

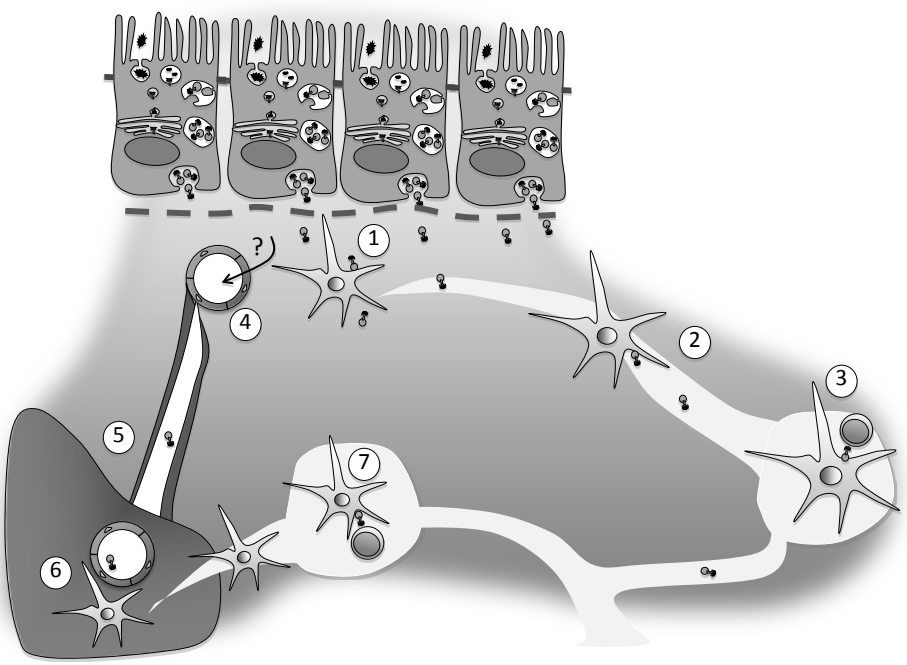

Figure 9. Hypothesis for the fate of the exosomes derived from intestinal epithelial cells. Exosomes released from the basolateral side of the enterocytes pass through the basolateral membrane. (1) In the lamina propria, they may then interact with dendritic cells. (2) The exosomes may be transported in the afferent lymph by the migrating dendritic cell or travel on they own. (3) MHC class II/peptide complexes from the exosomes can be presented to naïve $CD4^+$ T cells. Exosomes may also pass the lymph node and reach the systemic circulation by the efferent lymph. (4) Alternatively, they may reach the circulation by entering blood vessels in the lamina propria. (5) The exosomes reach the liver by the portal vein. (6) They may there be taken up by dendritic cells and transported to the liver draining celiac lymph node (7) so that their carried antigens can be presented to naïve T cells. The microenvironment in the lamina propria and the liver conditions the dendritic cells so that a tolerogenic rather than an aggressive immune response is generated. This involves the induction of regulatory T cells imprinted to home back to the lamina propria.

"We are an environment to an uncountable number of symbiotic, commensal and pathogenic organisms, each of which has had evolutionary time to learn how to use and misuse our immune system. As we expand our picture of the immune system from an army of lymphocytes patrolling the body for foreigners to an integrated group of communicating tissues, all working to maintain tissue integrity and health, we will necessarily need to include the signals from the non-self organisms that take advantage of that health or that help maintain it." Polly Matzinger, 2007

The hygiene hypothesis. Epidemiological investigations show a correlation between improved hygienic conditions and an increase in the incidence of inflammatory bowel disease, allergies and autoimmune disease (304-306). The observed increase of diseases associated with misdirected immune responses may be a consequence of the parallel decrease in infectious diseases i.e. decreased microbial load in developed countries (304) as well as the altered enteric flora that follows with reduced microbial exposure (307-309).

In parallel, NOD mice housed under specific pathogen-free conditions have an increased incidence of diabetes compared to those housed under conventional conditions and diabetes in NOD mice can be prevented by microbial stimulation of the young mice (reviewed in (304)). Even the laboratory mouse bred in a conventional environment is most likely less exposed to microbes compared to its feral relative and probably has a relatively deprived immune system as a result.

Alternative explanations for the observed increase in autoimmunity and atopic disease, such as differences in diagnosis, genetic factors and other environmental factors must of course be considered. However, the rapid increase as well as data from studies of populations migrating from one country to another, suggest that the contribution of genetic factors is small compared to the contribution of the environment. Neither underdiagnosis nor environmental factors such as air pollution seem to relate to the incidence of asthma. In epidemiological studies, the risk of allergy has been inversely linked to family size and birth order, factors associated with more frequent childhood infections and/or increased microbial exposure. Children raised on farms are also protected from the development of allergy.

Intestinal flora of the neonatal. In young mammals, the gut-associated lymphoid tissue is not yet fully developed and the maturation of the immune system is dependent on microbial stimuli (310). Increasing evidence demonstrate that the commensal gut flora can influence the function of epithelial cells, dendritic cells, B cells and T cells, although the precise mechanisms are still unknown (311). Particular microorganisms that provide a better protection for atopic disease and sensitisation to food allergens have not yet been identified in European neonates (312). Possibly the reduced diversity and descreased turnover rate of the enteric flora are more relevant than any individual bacterial strains.

High microbial exposure, measured as the level of endotoxin in the environment, is associated with a lower risk for asthma and atopic sensitization (313) and it has been shown that endotoxin facilitates the development of oral tolerance in various experimental systems (314-316). Among $CD4^+$ T cells, $CD45RB^{low}CD25^+$ T cells have been found to selectively express some toll-like receptors, e.g. TLR-4 (317) and signalling through these may be important to expand subsets of regulatory T cells in the periphery. As mentioned above, the commensal flora could also be important in providing a normal endotoxin load to the liver. Under non-inflammatory conditions, i.e. low endotoxin levels, antigen-processing cells in the liver are conditioned by the hepatic microenvironment to produce IL-10 and express low levels of co-stimulatory molecules (318).

Staphylococcus aureus (S. aureus) is a gram-positive bacterium that colonizes foremost the anterior nares, but also the throat, intestines and perineum. Probably as a consequence of reduced pressure from conventional gut bacteria, it is also increasing as a resident colonizer in the gut flora of infants in Western societies (319). Almost half of *S. aureus* strains isolated from the enteric flora of Swedish infants produce one or more toxins, where SEC is the most common (23%) followed by SEA (13%) (319).

S. aureus *enterotoxins*. SEA is constitutively expressed by the strains that carry the gene, in contrast to most other enterotoxins whose expression is tightly regulated by environmental conditions (320, 321). Ingestion of enterotoxins causes food poisoning with vomiting and diarrhea also in low amounts (<1μg). However, infants colonized with enterotoxin-producing strains of *S. aureus* do not suffer from increased gastrointestinal symptoms (319). Instead, a correlation was found between colonization with *S. aureus* in the first weeks of life and a decreased risk of developing food allergy (322). Children harbouring enterotoxin-producing *S. aureus* strains were further found to have higher numbers putative regulatory T cells in peripheral blood by 4 months of age, as well as a lower production of Th2-cytokines in response to birch allergen (Karlsson et al, in manuscript). No other bacterial group was associated with such putative Treg expansion.

Superantigens connects MHC class II and the TCR. The enterotoxins produced by *S. aureus* belong to a group of toxins known as superantigens, as they are the most potent stimulators of T cells known. Without the requirement of antigen processing, they bind to MHC class II molecules outside the peptide-binding groove and are then able to bind specific Vβ-domains of the TCR and activate a large proportion of the T cells, regardless of their antigen specificity. *S. aureus* strains can secrete an array of superantigens including enterotoxins (SE) A-E, G-J and the toxic shock syndrome toxin-1 (TSST-1), as well as the enterotoxin-like toxins K-R and U. A range of superantigens is also produced by *Streptococcus pyogenes*, including the pyrogenic exotoxins responsible for scarlet fever and streptococcal toxic shock syndrome. All the superantigens are potent T cell mitogens but they have different affinity for MHC class II alleles and stimulate distinct TCR Vβ-subsets. The affinity to MHC class II is 10-100 times stronger than for the TCR, and the

binding to MHC class II is very stable., which is important for the potency of the superantigens. The superantigens have a slightly higher affinity for human compared to murine MHC class II molecules and as a result, 4-5 times lower concentrations are required to stimulate human peripheral blood lymphocytes than murine peripheral blood lymphocytes (reviewed in (321)). Mice transgenic for human MHC class II are more sensitive to superantigens than their wild type littermate (323, 324). SEA is the most potent superantigen and can use all the three human class II isotypes (HLA-DR, DP, DQ) as well as the murine I-A^d and I-E^d to stimulate T cell responses (325).

Effects of enterotoxins on the immune system. Intragastrically administered *S. aureus* enterotoxins rapidly cross the intestinal epithelium in an intact and fully functional form, reaching maximum serum concentration after 2 h (326). Superantigens stimulate a defined T cell oligoclonal repertoire, potentially more than 20% of the total T cell repertoire, by binding to the variable part of the β-chain. For example, SEA stimulates murine T cells bearing Vβ 1, 3, 10, 11, 12 and 17 and preferentially the human Vβ 6.3, 7.3, and 7.4, while SEB binds preferentially to members of the murine Vβ7 and Vβ8 families and to Vβ 3.2 in humans. The toxic shock associated with these toxins is attributed to the overflow of cytokines (e.g. TNF, IL-2, IFN-γ) that are released during the massive T cell activation. Nude mice and mice lacking T cells with the relevant Vβ elements are resistant to the toxic effects of SEB (327). Local and systemic activation of the immune system probably also accounts for some of the symptoms associated with food poisoning, although the underlying mechanisms are not very well understood. A role for histamine release has been proposed and receptors on vagal afferent neurons are important in SEA-triggered emesis.

In addition to the interaction with Vβ-chains and in contrast to SEB, SEC1 and TSST, SEA can also bind specifically to γδ TCR T cells expressing the Vγ9 region, that constitute the large majority of peripheral γδ T cells (328). The Vγ9-chain is only expressed by a minor fraction of the γδ T cells in the thymus, which indicates an extrathymic expansion of these cells, possibly driven by SEA specifically (328).

The enteric flora is the major stimulus for the development of the mucosal immune system, since the lymphoid tissue of germfree animals is poorly developed. Several studies have found that the ability to induce oral tolerance is reduced in the absence of an enteric flora (316, 329-332). The observation that children colonized with *S. aureus* in their first weeks in life seemed to be protected from food allergy later in life, raised the hypothesis that stimulation with *S. aureus* enterotoxins could have an impact on oral tolerance induction. Indeed, Lönnqvist et al showed that treatment of mice pups with SEA during their first two weeks of life increased their tolerance to a fed protein antigen later in life (333). The fed antigen-dose was chosen to induce partial tolerization in untreated control mice and tolerance was tested in a model of allergic airway inflammation.

Microbial influence on antigen processing. There are several ways in which bacterial stimuli could influence the development of oral tolerance. The expression of MHC II in IECs is dependent on IFN-γ production from intraepithelial lymphocytes in response to bacterial colonisation (334). Lack of MHC II in the IEC due to immaturity (335, 336) or knockout (337), results in a failure to produce regulatory T cells after oral administration of antigen. Intestinal epithelial cells from SCID mice do not express MHC II and can therefore not produce a tolerogenic serum factor, however, this can be reversed by the treatment with IFN-γ (248). It is also shown *in vitro* that the release of exosomes from IEC is significantly increased by the presence of IFN-γ (191, 192). Thus, by induction of IFN-γ production, the commensal bacterial flora could facilitate sampling of luminal antigens (338) and induce MHC II-expression and functional exosome formation in the IEC (193, 246).

Effect of SEA on antigen processing. To test whether this effect could be achieved by the treatment with SEA, we designed an experimental set-up based on serum transfer from fed donor mice (paper III). First, we confirmed that tolerance was accomplished in the Th2-driven model of allergic airway inflammation after serum transfer from fed donor mice (249). We then treated adult donor mice with SEA in the drinking water for 5 days. After a washout period of three days one dose of ovalbumin was given orally and serum was collected one hour later. We found that recipients of serum from donors treated with SEA prior to antigen feeding became more tolerant by the OVA-feeding. This was demonstrated as a lower degree of allergic airway inflammation, as measured by infiltration of eosinophils in bronchoalveolar lavage fluid, compared to recipients of serum from donors fed the antigen without prior SEA-treatment. Improved tolerance was further demonstrated by lower production of Th2-cytokines by antigen-stimulated cultures of lung tissue cells from the recipients of serum from SEA-pretreated, OVA-fed mice. The effect was dependent on the tolerizing antigen feeding, as recipients of serum from non-fed SEA-treated control mice were no more tolerant than controls receiving serum from untreated donors. Thus, the treatment of SEA increased the ability to convert the fed antigen into a tolerogen. It could be argued that this effect is simply due to higher serum-concentration of the fed antigen as a result of increased permeability after SEA-treatment, but is unlikely since it has been shown that the serum-concentration of unprocessed antigen is unrelated to tolerance induction by serum-transfer (231, 232).

We also observed that the SEA-treated donor mice had higher numbers of $CD8\alpha^+$ intestinal intraepithelial lymphocytes, in line with previous studies of the effect of SEA-treatment in the rat (339). Superantigens induce substantial production of IFN-γ (340-342) by the intestinal intraepithelial lymphocytes are the in the intestinal mucosa (247). In response to the locally produced IFN-γ the intestinal epithelium upregulates MHC class II (247, 343, 344). In our SEA treated mice we observed a clear tendency towards a higher expression of MHC class II in the IECs after SEA-treatment, but the effect was not statistically significant.

Taken together, these observations indicate that the treatment of SEA, by stimulation of the intestinal epithelium via the IEL, increases tolerogenic processing. One may speculate that the formation of the formation exosomes is increased in the epithelial cells activated directly by SEA, or indirectly via IFN-γ. Further studies are necessary to characterise the mechanism for the effect of SEA on the epithelium.

Influence on the induction of regulatory T cells as the final outcome? It would also be of interest to characterize the subsets of T cells in the lamina propria following SEA-treatment, with regard to phenotype and production of IL-10 and TGB-β. Lönnqvist et al found that SEA-treatment of mice early in life resulted in an increased frequency of gut-homing ($CCR9^+$ $\alpha4\beta7^+CD103^+$) $FoxP3^+$ $CD4^+$ T cells in the mesenteric lymph nodes ((333) and Lönnqvist et al, unpublished data). In this model, neonatal treatment with SEA via the i.n. or i.p. route also enhanced the capacity to develop oral tolerance, although slightly less efficiently than the p.o. exposure (333). Other experimental animal studies have indicated that exposure of *S. aureus* superantigens favours the development of regulatory T cells, as repeated i.v. injection of SEA results in the induction of IL-10-producing $CD4^+$ T cells (345) and induction of $CD4^+$ T cells with a potent contact-dependent suppressive function (346). Further, repeated i.p. injections of SEB induced suppressive $CD4^+CD25^-$ T cells (347) and repeated i.n. administration has been shown to protect against lethal systemic SEA challenge by a mechanism dependent on IL-10 (348). As mentioned above, children that were colonized with enterotoxin-producing *Staphylococcus aureus* early in life were found to have an expansion of putative regulatory T cells ($CD4^+CD25^+CTLA\text{-}4^+$) (Karlsson et al, in manuscript). Thus, repeated stimulation with super antigen can induce subsets of regulatory T cells, similar to the induction of regulatory T cells seen in chronic infections.

This implicates that repeated stimulation of superantigens can induce regulatory T cell subsets, by a mechanisms that appears to involve production of IL-10. For the enhancement of oral tolerance in neonatal mice, the p.o. route appears to be most efficient, involving induction of gut-homing $CD4^+$ T cells. The individual steps in this chain of events are mainly unknown. A clue might come from the unpublished data by Lönnqvist et al showing that dendritic cells in the mesenteric lymph nodes of SEB-treated mice prime T cells by mechanism dependent on retinoic acid. Repetitive stimulation with low concentrations of superantigens may activate only local T cells to produce cytokines (IFN-γ, IL-2, TNF-α) that stimulate the epithelium to efficient tolerogenic processing of soluble antigens and favour a tolerizing conditioning of local antigen-presenting cells. Another interesting factor in this context may be the "T-cell immunoglobulin-domain and mucin-domain" (TIM)-4, expressed by dendritic cells in lymphoid tissue. Being a receptor for phosfatidylserine, TIM-4 has been shown to mediate the engulfment of apoptotic cells and has been suggested to be involved in the intercellular signalling mediated by exosomes (262). TIM-4 is expressed by dendritic cells in the jejunal mucosa and is further upregulated by the exposure of SEB (349). In models of concurrent exposure of SEB and food antigen, this modulation of intestinal dendritic cells has been suggested to drive Th2

polarized immune responses via interaction by TIM-4 on the dendritic cells and TIM-1 expressed on T cells (349). Current knowledge indicate that TIM-4 can act as a regulator of immune responses through several different pathways, with different outcomes (350), and TIM-4 may possibly aid in the uptake of exosomes derived from intestinal epithelial cells as a link in the natural handling of soluble luminal antigens.

The importance of timing. The timing of superantigen-exposure in relation to the introduction of novel antigens is probably a critical factor. Repetitive oral administration of SEB *together* with a novel food antigen was shown to induce immune responses that evoked anaphylaxis upon oral challenge (267). This effect was associated with enhanced production of Th2-type cytokines and reduced expression of FoxP3 and TGB-β, suggesting a Th2-biased response with a parallel loss of Treg cell function. The reaction was most severe in the LPS insensitive C3H/HeJ mouse strain (267), that is more prone to develop food allergy than other mouse strains (351). The adverse effect could be overcome by the administration of a higher dose of food antigen (267). SEB has also been shown to induce upregulation of GITR-L on monocytes, resulting in a reversible inhibition of the contact-dependent suppression exerted by natural regulatory T cells and their concomitant proliferation (352). This could represent a mechanism to expand the Treg population while temporarily inhibit Treg suppression. This may also explain the observed abrogation of suppression by Treg from patients with atopic dermatitis after SEB stimulation (353).

The significance of maturation. The age and immunologic development of the experimental animals also needs to be considered for the outcome of an experiment In mice, oral tolerance can not be induced during the first week of life and the mechanism is not fully developed until 5-6 weeks of age. In paper III we found, contrary to the results in a previous study from our lab, that SEA administered to adult mice were able to improve the induction of oral tolerance. An explanation for this disparity may be found in the nature of the different experimental models. In the Lönnqvist et al study, tolerance was induced by direct feeding of antigen to the mice. The dose regimen for induction of oral tolerance was chosen to achieve an intermediate tolerance in mice 6 weeks of age, in order to be able to detect an improvement by the SEA-treatment in neonatals. In the older mice in this study, the induction of oral tolerance by feeding of an antigen alone was more effective and any further effect of SEA might have been difficult to detect. Compared to our findings in paper III, the degree of tolerance that is transferred by serum from an antigen-fed donor to a naïve recipient is not nearly that complete, which makes an improved effect more easily detected.

In summary, the effect of *S. aureus*-derived superantigens in the modulation of the immune system is still not fully comprehended. Although the sudden exposure of higher systemic concentration is highly toxic and results in potentially lethal immune responses

by the host – especially if human - via the simultaneous activation of high T-cell numbers, the advantage for the microorganism is likely obtained by the action of lower concentrations locally. Modulation of the immune system in favour of the development of regulatory T cells may benefit the colonizing bacteria. In turn, stimuli from the microbiota are instrumental to the development of the intestinal as well as the systemic immune system.

The interactions between the intestinal microbiota and the immune system is probably most dynamic in the young individual, where the microbiota has not yet established a stable community and the immune system is under development. In the first years of life, regulatory T cells express homing receptors that guide their migration to intestinal secondary lymphoid tissue (146), which points out the significance of the gut as the major site of antigen-exposure in early life. A delayed or altered colonization of the neonate may therefore have a significant impact.

To sum up. There is an inverse correlation between the increase in autoimmune and allergic diseases and the reduced microbial exposure, accompanied by a decreased frequency of childhood infections and an altered composition of the intestinal flora in infancy. *S. aureus*, accompanied by different clostridias, belong to the strains that have increased on the expense of gram-negative strains such as *E. coli* as a gut colonizer of neonates and may be considered a sign of a too hygienic life style. Indeed, studies have shown that babies that develop allergies are more often colonized with *S. aureus* and have higher counts of clostridia (354). However, the action of superantigens produced by certain strains of *S. aureus* might in part compensate for the reduced microbial stimuli in Western societies.

In the steady state, dendritic cells in an immature state continuously circulate from tissues, where they have captured self-antigens, as well as innocuous environmental proteins. Despite the epithelial barrier and other mechanisms of exclusion, the mucosal immune system interacts with luminal antigens and is developed and activated in response to the commensal flora. Production of anti-inflammatory cytokines such as IL-10 and TGB-β during immune responses at mucosal surfaces is important to prevent chronic inflammation. There are accumulating evidence that bacterial antigens are critical for the generation and/or the expansion of regulatory T cells. The commensal flora is therefore probably important to promote the maintenance of an adequate population of regulatory T cells. Lowered levels of natural adjuvants may result in an impaired development of regulatory T cells. When the natural diversity of the gut flora is becoming more limited, certain potent microbial agents such as SEA may partly compensate.

Concluding remarks

Regulatory T cells derived from the thymus are now well established in their role in the regulation of immune responses. Their significance is unquestioned as depletion of this subset causes an uncontrolled inflammation with the characteristics of autoimmunity as well as inflammatory bowel disease and allergy. Several molecules critically involved in their generation, maintenance and function have been identified from mutations found in humans and by experimental animal models. In paper I, we found that CD25+ regulatory T cells were efficiently in the control of auto-aggressive T cells in adults, but that the corresponding population from cord blood were less prone to suppress responses to self antigens. This might reflect the need for activation and expansion in the periphery, as a part of the maturation process of the regulatory T cell population.

It is now acknowledged that additional subsets of regulatory T cells can be induced from naïve T cells in the periphery under the right influences. The intestine is the organ with the most urgent need of regulation because of the high load of foreign antigens of dietary and microbial origins. It appears that particularly efficient mechanisms for the induction of regulatory T cells have evolved in this region, both in lymphoid tissue directly adjacent to the gut and in the lymph node draining the liver that in turns receives the venous outflow from the intestines. In paper II we found a high proportion of antigen-specific T cells with the phenotype and function of regulatory T cells in the liver-draining celiac lymph node after feeding.

The chain of events that leads to the generation of regulatory T cells and oral tolerance is not fully understood but it has been shown that gut-processing of a fed antigen is required to generate a tolerogenic serum factor, that upon transfer confers oral tolerance to naïve recipients. Exosomes derived from intestinal epithelial cells (IEC) are found in the fraction of serum that possesses the tolerogenic qualities. The exosomes carry MHC class II/peptide-complexes and can be taken up by antigen-presenting cells and influence immune responses. Stimuli from the intestinal flora are important for the induction of oral tolerance and the generation of a tolerogenic serum factor, but their exact nature is not identified. Disorders caused by defects of immune regulation are geographically and in time associated with decreased microbial exposure and changes in the establishment of the intestinal microbiota. While *Staphylococcus aureus* is gut-colonizer associated with a more hygienic life-stile, children colonized with *S. aureus* at a very early age appear to be protected from the development of food allergy. Many of the gut-colonizing *S.aureus* strains produce enterotoxins. In paper III, we found an increase in the tolerogenic capacity of serum from fed animals that were pre-treated with *S. aureus* enterotoxin A (SEA). The mechanism behind this finding is not evident but the increased effect was associated with an elevated number of intraepithelial lymphocytes (IEL) in SEA-treated donor mice. We hypothise that SEA activates the IEL to produce IFN-γ, which leads to

changes in the IEC that may include upregulation of MHC class II. These changes increase the capacity of the IEC to process a fed antigen into a tolerogenic form.

Exosomes derived from antigen-pulsed IEC cultured *in vitro* have been shown to be able to be able to inform the immune system *in vivo*, but the outcome has differed in two previous studies. We investigated this further and were able to show that exosomes produced by antigen pulsed IEC showed a partial protection against allergic airway sensitisation, however this response seemed to be dependent on the level of IFN-γ used to stimulate the IECs during the exosome production.

The regulation of the immune system is increasingly appreciated to be finely tuned and dynamic. Function is the combined effects of genes and environment and immune homeostasis is the integrated result of many mechanisms in balance. Understanding the normal mechanisms of the regulation of immune responses in health is fundamental for successful treatment of diseases caused by a break in the tolerance. Identifying relevant factors needed for the accurate development of the immune system in the young individual is perhaps even more relevant, as chances are better to prevent than to cure.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Under de senaste decennierna har man kunnat se en kraftig ökning av sjukdomar som orsakas av att immunsystemet reagerar fel. Immunsystemet är kroppens försvar och ska skydda mot infektioner som orsakas av t ex bakterier och virus och samtidigt skilja dessa inkräktare från kroppens egna strukturer, eller för den delen alla ämnen som finns i vår omgivning men som inte utgör något hot för kroppen. Immunsystemet är kraftfullt och felaktiga reaktioner kan göra stor skada. Angrepp på egna strukturer orsakar autoimmuna sjukdomar och allergier uppstår när försvaret riktas mot exempelvis pollen eller vanliga födoämnen.

Det finns olika teorier för varför sjukdomar av det här slaget har ökat så mycket. En tänkbar förklaring ges av den s.k. hygienhypotesen, vilken förklarar ökningen av autoimmuna sjukdomar och allergier med att vi i västvärlden numera är mindre utsatta för infektioner och överlag har mindre bakterier i vår omgivning. Enligt hygienhypotesen behöver immunsystemet en viss grad av mikrobiell stimulering att det ska fungera korrekt.

I immunsystemet finns både aggressiva celler som förstärker immunreaktionerna och reglerande celler som dämpar dem och det gäller att balansen mellan de olika krafterna är rätt. Aggressiva reaktioner måste snabbt kunna sättas igång när kroppen invaderas av bakterier eller virus men däremellan måste de aggressiva cellerna hållas i schack av de reglerande cellerna. Vi har studerat balansen mellan de aggressiva cellerna och de reglerande när vi stimulerat dem med olika kroppsegna strukturer. Vi fann att dessa reglerande celler mycket effektivt dämpar farliga immunsvar hos friska vuxna. Vi har även undersökt hur cellerna fungerar i navelsträngsblod från nyfödda och fann att de reglerande cellerna inte är lika effektiva så tidigt i livet. Troligen behöver de aktiveras och mogna innan de fungerar lika bra som hos vuxna.

När immunsystemet utvecklas lär det sig att skilja på det som är kroppseget och det som är främmande. Men för att balansen mellan de aggressiva och de reglerande cellerna ska bibehållas måste immunsystemet fortsätta att lära sig att skilja på vän och fiende i takt med att vi kommer i kontakt med nya ämnen. Det finns en särskild mekanism som gör att de ämnen vi äter accepteras av kroppen och att det bildas reglerande celler som skyddar oss från att reagera aggressivt på ämnen i maten. Detta sker genom att proteiner i födan bryts ner i tarmen och förpackas på ett sätt så att immunsystemet förstår att de är ofarliga. Tarmepitelcellerna, de celler som utgör tarmens väggar, bildar små blåsor (exosomer) som är ca en miljon gånger mindre än en cell och som innehåller bitar från proteinerna i födan. Eftersom de är så små kan dessa blåsor spridas i kroppen med lymfan och blodet för att sedan fångas upp av immunsystemets celler och lära dem att födoämnena är ofarliga. Många av dem hamnar troligtvis i levern eftersom den är bra på att fånga upp små partiklar från blodet och vi har dessutom kunnat visa att det bildas särskilt många reglerande celler i anslutning till levern.

Denna mekanism, att förpacka födoämnen i små blåsor som kan tala om för immunsystemet att dess innehåll är ofarligt, är avancerad. Många olika steg är involverade och det finns mycket som tyder på att bakterierna i tarmen är viktiga för att processen ska fungera. Vår hygieniska livsstil har gjort att bakteriefloran är annorlunda nu jämfört med för några decennier sedan, speciellt hos de riktigt små barnen. Detta kan vara särskilt betydelsefullt eftersom immunsystemet hos dem fortfarande är under stark utveckling. Det verkar som att om immunsystemet inte stimuleras tillräckligt mycket av bakterier fungerar förpackningen av födoämnen sämre och immunsystemet lär sig inte att födoämnena är ofarliga. Detta skulle till exempel kunna förklara varför allergier blivit vanligare. Vi har undersökt hur förpackningen av ett födoämne i små blåsor påverkas av ett ämne som bildas av en bakterie, enterotoxin A från stafylokocker. Enterotoxin A är mycket kraftfullt och 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. Vi fann att bakterieämnet gör att det bildas fler eller bättre små blåsor i blodet efter att man ätit vilket leder till att födan accepteras bättre. Denna studie gjordes på möss och det går inte att veta säkert om det fungerar likadant hos människor. Skulle det fungera på liknande sätt hos människa finns det en möjlighet att under kontrollerade former stimulera immunsystemet och kompensera för den minskade mängden bakterier i vår omgivning och följaktligen minska förekomsten av allergier.

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