The role of exosomes and microflora in establishing mucosal tolerance and the protection against allergic disease

Nina Almqvist

2008

UNIVERSITY OF GOTHENBURG

Till Izabella

We don't come alone, we are fire, we are stone

ABSTRACT

The breakdown of immune regulation to innocuous environmental antigens at mucosal sites can result in a number of different diseases such as, allergies and inflammatory bowel disease (IBD). Allergy is one of the most common diseases with a prevalence of up to 40% in children from developed countries. The healthy immune system prevents allergic sensitization by establishing immunological tolerance to innoccus antigens present at mucosal sites.

Oral administration of soluble protein antigens is a very effective way to establish antigen-specific tolerance to the ingested protein, a process known as oral or mucosal tolerance. This is an active process, which is maintained by the specific recognition of antigens by $CD4^+$ T-cells with a down regulatory function, and it is the default response to harmless antigens entering at mucosal sites. The process of oral tolerance starts with sampling of luminal antigens by the intestinal epithelial cells (IEC), processing and assembly with MHC II and subsequently a release of tolerogenic exosomes, small (40-90 nm) membrane bound vesicles of endocytic origin, produced by intestinal epithelial cells (IEC) and can be isolated from serum shortly after an antigen feed. We have previously shown that these exosomes potently transfer antigen-specific tolerance to naive recipients. Moreover, exosome-mediated tolerance is MHC class II dependent, which in turn requires an intact immune system in the fed donor. The hygiene hypothesis states that microbial exposure is required to properly educate the immune system. A full microbial flora in the gut generally provides the required stimuli for the maturation of the intestinal immune system and the intestinal epithelial cells to enable tolerogenic processing of orally administrated antigens. It is not known which individual bacterial species or what bacterial products that delivers the necessary signals.

The focus of this thesis was to further study the role of exosomes in oral tolerance and their capacity to protect against an allergic sensitization and whether microbial stimuli would effect the outcome of such response. We also wanted to examine the role of dendritic cells in exosome-induced tolerance, focusing on plasmacytoid dendritic cells (pDC).

We found that exosomes both isolated from serum and when isolated from intestinal epithelial cells in culture protect against an allergic sensitization in an antigen-specific manner. We could also show that the tolerant animals had higher levels of activated regulatory T cells in the draining lymph nodes indicating that exosome-induced tolerance is most likley mediated by regulatory T cells. Furthermore, we could also show that the tolerogenic effect of exosomes from serum could be enhanced when the gut epithelium was exposed to enterotoxin from *S. aureus* (SEA). When investingating the uptake of IEC derived exosomes by dendritic cells we could show that both conventional dendritic cells (cDC) and pDCs phagocytose exosomes. The capacity of pDCs to phagocytose have been questioned but our results indicate that they most readily ingest both exosomes and latex beads the size of exosomes. We also compared the capacity of the DCs to process and present the antigens carried by exosomes and found that pDCs induce higher antigen-specific T cell proliferation as compared to cDCs which suggest that pDCs in fact are better at both phagocytosis of IEC-derived exosomes as well as presenting the antigen they carry.

In conclusion, exosomes have the capacity to induce antigen-specific tolerance and protect against allergy. This exosome-induced tolerance could possibly be mediated by pDCs. Furthermore, in agreement with the hygiene hypothesis we could conclude that certain microbial stimuli, here SEA, does effect the tolerogenic processing, due to a more activated immune system in the gut.

ORIGINAL PAPERS

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

I. Lin XP, Almqvist N, Telemo E.
Human small intestinal epithelial cells constitutively express the key elements for antigen processing and the production of exosomes.
Blood Cells Mol Dis 2005;35(2):122-8.

II. Almqvist N, Lönnqvist A, Hultkrantz S, Rask C, Telemo E.
Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma.
Immunology 2008;125(1):21-7.

III. Almqvist N, Gerhmann U, Magnusson M, Telemo E.
Intestinal epithelial cell derived exosomes protect against an allergic sensitization and acts via pDCs in vitro.
In manuscript.

IV. Hultkrantz S, Almqvist N, Lönnqvist A, Östman S, Rask C, Telemo E, Wold A.
S. aureus enterotoxin facilitates tolerogenic processing of mucosally administered antigens.
In manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

APC	Antigen-presenting cell
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DC	Dendritic cell
cDC	Conventional dendritic cell
CLIP	Class II-associated invariant chain peptide
ESCRT	Endosomal sorting complex required for transport
FAE	Follicle-associated epithelium
FoxP3	Forkhead box P3
GALT	Gut associated lymphoid tissue
GITR	Glucocorticoid induced tumour necrosis factor related protein
HLA-DM	Human leukocyte antigen-DM
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
Ig	Immunoglobulin
IFN	Interferon
IEC	Intestinal epithelial cell
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy enteropathy, X-linked syndrome
iTreg	Induced regulatory T cell
LFA-1	Leukocyte function-associated antigen-1
LSEC	Liver sinusoidal endothelial cells
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MVB	Multivesicular body
nTreg	Natural regulatory T cell
NK cell	Natural killer cell
NKDC	Natural killer dendritic cell
NKT cell	Natural killer T cell
OVA	Ovalbumin
PBMC	Peripheral blood monocytes
pDC	Plasmacytoid dendritic cell
PP	Peyer's Patch
SCID	Severe combined immunodificient
SEA	*S.aureus* enterotoxin A
TGF-β	Transforming growth factor-beta
Th	T helper
TLR	Toll-like receptor
Tr1	Type 1 regulatory T cell
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

INTRODUCTION

The breakdown of immune regulation to innoccus environmental antigens at mucosal sites can result in a number of different diseases such as, allergies and inflammatory bowel disease (IBD). Allergy is one of the most common diseases with a prevalence of up to 40% in children from developed countries, and many of these individuals will continue to suffer allergic manifestation like asthma, rhinitis and food allergy in adulthood. The healthy immune system prevents allergic sensitization by establishing immunological tolerance to innoccus antigens present at mucosal sites. Oral administration of soluble protein antigens is a very effective way to establish antigen-specific tolerance to the ingested protein, a process known as oral tolerance. This is an active process that is maintained by the specific recognition of antigens by $CD4^+$ T-cells with a down regulatory function, and it is the default response to harmless antigens entering at mucosal sites [1-5].

We have previously proposed that oral tolerance is dependent on exosomes, which are nano-sized (40-90 nm) membrane bound vesicles of endocytic origin that are produced by intestinal epithelial cells (IEC) and can be isolated from serum shortly after an antigen feed. Transfer of these exosomes to naïve recipients induces antigen specific tolerance [6]. Moreover, we could show that these exosomes protect against an allergic sensitization [7]. Exosome-mediated tolerance is dependent on antigen-presenting molecules, such as MHC (major histocompatibility complex) class II, which in turn requires an intact immune system in the fed donor [8]. The hygiene hypothesis states that microbial exposure is required to properly educate the immune system [9]. A full microbial flora in the gut generally provides the required stimuli for the maturation of the intestinal immune system and the intestinal epithelial cells to enable tolerogenic processing of orally administrated antigens. It is not known which individual bacterial species or what bacterial products that delivers the necessary signals.

In this thesis I will discuss the biology of exosomes and the mechanisms of oral tolerance and the role of exosomes in this process. I will also briefly

discuss the effect of the microbiota on tolerogenic processing and finally, in an attempt to identify a possible target cell for intestinal-epithelial-cell derived exosomes, I will review plasmacytoid dendritic cells.

EXOSOMES

Exosomes are small, 40-90 nm membrane bound vesicles of endocytic origin that are secreted by a variety of cells in culture. They were described for the first time in 1981 as microvesicles containing 5´-nucleotidase activity secreted by neoplastic cell lines [10]. A few years later two independent groups reported secretion of small vesicles of endocytic origin by cultured reticulocytes. Using electron microscopy they observed these vesicles in the late endosomes, which by fusion with the cell membrane released the vesicles extracellularly. The supposed function of the exosome in this study was to remove the transferrin receptor from the cell surface [11, 12]. A decade later, in 1996, exosomes were for the first time shown to have an immunological function. Antigen pulsed B cells secreted exosomes originating from multivesicular bodies that activated antigen specific T cells [13]. Today we know that exosomes can be secreted by a variety of cells in culture and can be isolated in vivo from body fluids such as serum [14, 15], bronchoaveolar lavage [16] and urine [17]. So far intestinal epithelial cells [6, 18], T- and B-lymphocytes, dendritic cells, macrophages, placental trophoblasts, reticulocytes, mast cells, platelets and various neoplastic cells have been shown to produce exosomes [19, 20].

The formation process defines the exosome

The process of exosome formation starts with invagination of the cell membrane and formation of endosomes. Invagination and inward budding of the membrane of the late endosome then forms the exosome. Upon fusion with the cell membrane these multivesicular endosomes release exosomes extracellularly (Figure 1). So far two different mechanisms have been suggested which supports the idea that exosome formation and release is a highly regulated process. The first one is the identification of the endosomal sorting complex required for transport (ESCRT) in association with exosomes [21] and the second was just recently identified as ceramide-triggered budding [22]. The ESCRT sorts ubiquitinated proteins for transport in the endosomal network, however not all proteins found in exosomes are ubiquitinated. For review see [23].

There is as of yet no "exosome-specific" marker identified and hence they are characterised on morphological and biochemical criteria. Exosomes are commonly defined as small membrane bound vesicles originating from the cell surface and processed/modified intracellularly resulting in a multi-vesicular compartment that is emptied to the extra cellular space, thus releasing the exosomes. Due to their small size exosomes can only be visualized in electron microscope (Figure 2). As a result of the exosome formation pathway, some of the molecules found on the surface of exosomes are typically of endocytic/lysosomal origin e.g., CD9, CD63 and CD81 [24].

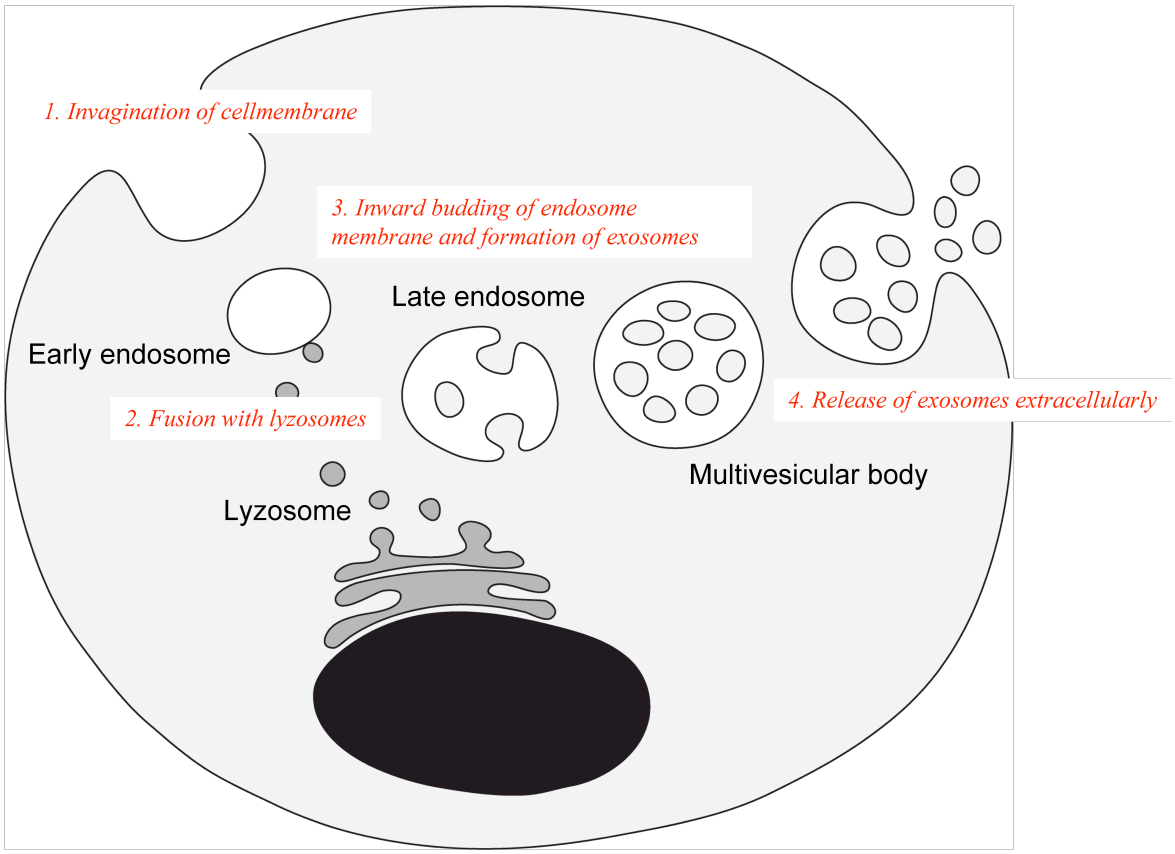

Figure 1. The process of exosome formation.

These molecules belong to a family of proteins called tetraspanins, which have been suggested to be involved in cell adhesion, activation, proliferation and antigen presentation. Exosomes from antigen presenting cells express MHC class I and II together with co-stimulatory molecules, like CD54 (ICAM-1), CD80 (B7.1) and CD86 (B7.2), which explains their capacity to activate T cells

[24, 25]. The enrichment of co-stimulatory molecules on the exosome seems to depend on the maturation and activation state of the producing antigen-presenting cell (APC). This indicates that they would also stimulate the immune system in different ways, e.g., exosomes from mature DCs are 50-100 fold more potent inducers of antigen-specific T cell activation in vitro as compared to exosomes from immature DCs. This effect was suggested to depend on the enrichment of MHC class II and the co-stimulatory molecules, ICAM-1 and CD86, on exosomes from mature DCs relative to exosomes from immature DCs [26]. Exosomes from intestinal epithelial cells express MHC class II along with e.g., CD63, CD81 and A33, which is a marker specific for IECs [6, 18]. We have shown that IEC-derived exosomes can induce antigen-specific tolerance [6], (III. Almqvist et al., in manuscript) but a different group have shown contradicting results, that is activation of the immune system with transfer of IEC-derived exosomes [27]. This suggests that IEC-derived exosomes can act as both suppressors and activators if the immune system. What effects the outcome of the immune response upon transfer of IEC-derived exosomes is unknown. Mast cells secrete exosomes expressing MHC class II, CD86, LFA-1 and ICAM-1, which mediates Mast cell-dependent B and T cell activation [28]. Recently it has also been reported that mast cell derived exosomes contain mRNA that can be transferred between cells [29] and may thus transfer a functional message. In general, exosomes express different surface markers and have different lipid composition [30] depending on what cell type they originate from and hence, they are likely to have different functions.

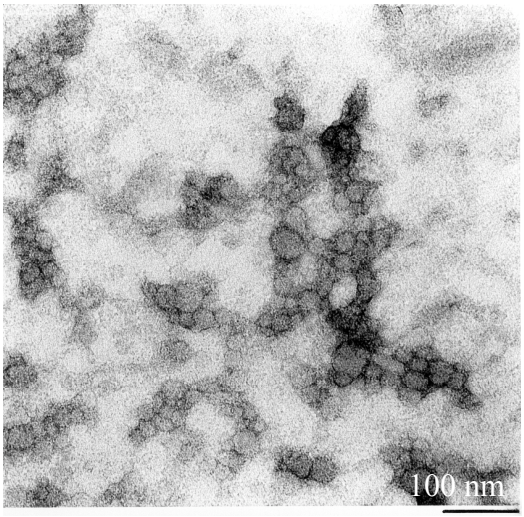

Figure 2. Exosomes isolated from intestinal epithelial cells in culture, viewed in Electron Microscope.

ANATOMICAL BASIS OF MUCOSAL TOLERANCE

The intestinal immune system is the largest and most complex part of the immune system. It encounters more antigen then any other part of the body and it also has to discriminate between harmful pathogens and beneficial antigens such as food proteins and normal gut flora. If the immune system fails to become tolerant to food antigens or components from the microflora the result might be hypersensitivity reactions such as allergies or chronic inflammatory conditions like IBD. A major tasks for the immune system of the gut is to prevent such reactions at the same time as being able to eliminate hazardous antigens. The usual response to harmless gut antigens is the induction of local and systemic tolerance, known as oral tolerance or mucosal tolerance [5]. It has been proposed that specific features of the mucosal tissue favours tolerance, e.g. the production of immunoglobulin A (IgA) antibodies and the relative abundance of the anti-inflammatory cytokines TGF-β and IL-10. Moreover, the mucosal tissue has a unique ontogeny and anatomical patterning, specialized cells and organs that are involved in the uptake of antigen, distinctive subsets of antigen-presenting cells and several unusual populations of B and T cells. In addition, the migration of lymphocytes to the intestine is controlled by a series of adhesion molecules and chemokine receptors specific for mucosal tissue [5]. A part from what is regarded as gut lymphoid tissue, the liver also seem to have a key role in the induction of oral tolerance and will in this context be discussed as a part of the anatomical basis for mucosal tolerance [31-33].

Gut-associated lymphoid tissue

The gut-associated lymphoid tissue (GALT) can be divided into effector sites and organized tissues. Effector sites consist of immune cells scattered throughout the epithelium and lamina propria of the mucosa and the organized tissue includes Peyer's patches (PP), mesenteric lymph nodes (MLN) and smaller lymphoid follicles (cryptopatches) distributed throughout the wall of the small and large intestines (Figure 3).

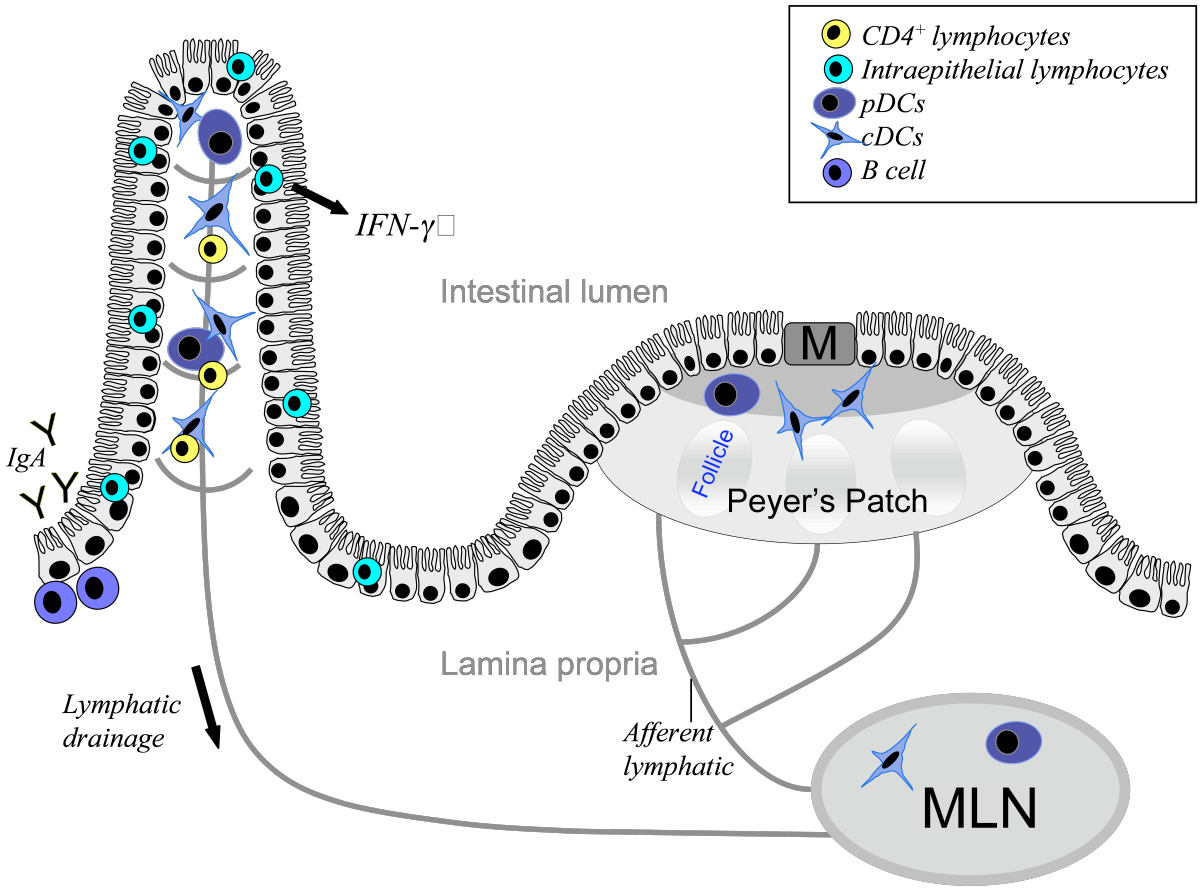

Figure 3. Schematic presentation of the gut-associated lymphoid tissue (GALT).

The lymphocytes of the mucosa are distributed in the lamina propria and in the epithelium. The B cells that enter the mucosa are redistributed to the lamina propria were they mature into IgA producing plasma cells. The $CD4^+$ T cells reside in the lamina propria and these are of particular significance to local immune regulation as this population contain regulatory T cells responsible for maintaining local tolerance to environmental antigens. The $CD8^+$ T cells preferentially migrate to the epithelium. Although, about 40% of the T cells in lamina propria are also $CD8^+$ [5]. The $CD8^+$ T cells in the epithelium, the intraepithelial lymphocytes, are of great importance for the induction of the MHC class II expression in the epithelial cells, which in turn enables antigen-presentation by the the latter (see below). The up-regulation of

MHC class II is dependent on the production of IFN-γ by these intraepithelial $CD8^+$ lymphocytes, which occurs in response to microbes present in the gut [34, 35]. This would explain why no MHC class II expression in the intestinal epithelium can be observed in neonate mice until 1 week post weaning [36] and also the total lack of MHC II expression in the small intestinal epithelium of SCID [8] and germ-free mice [37, 38].

The Peyer´s Patches are macroscopic lymphoid aggregates found in the submucosa of the small intestine and consist of large B cell follicles and T cell rich areas. This lymphoid tissue is separated from the lumen by a single layer of columnar epithelial cells called FAE (follicle-associated epithelium) and differs from the normal gut epithelium in that it has less digestive enzymes and less pronounced microvilli. It is also infiltrated by a large number of B cells, T cells, macrophages and dendritic cells. The FAE also contains M cells, which are specialized enterocytes that lack surface microvilli, but instead display microfolds in the membrane facing the lumen. M cells bind invasive pathogens and other particulate antigens and transport the material to underlying APCs, which then enter the T cell areas, and/or B cell follicles were they interact with naive lymphocytes. The B cells expand and undergo immunoglobulin class switch changing from IgM to IgA production under the influence of TGF-β and IL-10 either locally or after migrating to the mesenteric lymph nodes (MLN). The primed T cells exit the PPs through the draining lymphatic entering the MLNs where they proliferate. The MLNs are a set of large lymph nodes associated with, and responsible for the lymphatic drainage of the intestine where antigens and/or APCs transferred from PPs and mucosal lamina propria will interact with naïve B-and T cells. After residing in the MLNs for an undefined period of time, these lymphocytes enter the circulation through the thoric duct and eventually accumulate in the intestinal mucosa as effector cells. The homing of lymphocytes primed in the GALT to the gut mucosa depend on the down regulation of L-selectin (CD62 L) and the up-regulation of $\alpha_4\beta_7$ integrin. The $\alpha_4\beta_7$ integrin interacts with mucosal addressing cell-adhesion molecule 1 (MADCAM-1), highly expressed by the vasculature of the mucosal surfaces. Gut-derived T cells also express CCR9, a chemokine receptor allowing them to respond to ligand CCL25,

which is selectively expressed by epithelial cells in the small intestine. The homing imprint of the gut derived lymphocytes is generated at the very first contact with the mucosal professional APCs due to their unique production of retinoic acid [39, 40]. Lymphocytes, which are primed in the peripheral lymph nodes, display other homing markers and thus cannot migrate to the mucosal surfaces [41].

The epithelial cells of the small intestine provide a selective barrier by its tight junctions and the epithelial lining has long been considered to be impermeable to larger molecule such as protein antigens. Small intestinal epithelial cells constitutively express MHC class II both in humans and mice [36, 42-44] and for this reason the IECs have been suggested to be non-professional antigen-presenting cells and may be capable of presenting antigens directly to $CD4^+$ T cells [45-47]. However, these conclusions are some what in conflict with the anatomical features of the intestine; first the MHC class II expression of the epithelial cell is intracellular and second the $CD4^+$ cells are separated from the epithelial cells by a basement membrane and are rarley found in contact with the epithelial cell. We and others have previously shown that IECs in culture produced exosomes [6, 18]. These exosomes could be the actual transporters of antigens from the lumen through the IECs into the lamina propria and also further cross the endothelial barrier into the circulation. In order to examine the capacity of IECs to process and present antigens on exosomes in vivo we performed immunohistochemical analyses of healthy human biopsies from the small intestine (duodenum). We found that IECs express MHC class II, HLA-DM, Invariant chain (Ii) and cathepsin S at steady state. Invariant chain is a chaperone molecule which occupies the peptide groove upon the production of the MHC molecule in order to stabilize the latter. Invariant chain is degraded by, amongst others, Cathepsin S and left in the peptide groove is a small part of the Ii called CLIP. When the MHC is assemblied with antigens, CLIP is removed and replaced by a peptide, aided by HLA-DM (Figure 4). From these results we could conclude that the IECs have all the necessary components required to process and assemble antigens with MHC class II molecules, which also would enable them to produce exosomes bearing MHC class II-peptide complexes [42] as suggested in figure 4. More

extensive studies confirming our results have recently been published and it is now established that IECs can take up soluble protein antigens from the lumen through pinocytosis. This material is partly processed and assembled with MHC class II resulting in the formation of multivesicular bodies (MVB). Finally, exosomes from MVBs are released basolaterally (Figure 4) [6, 18, 48-50].

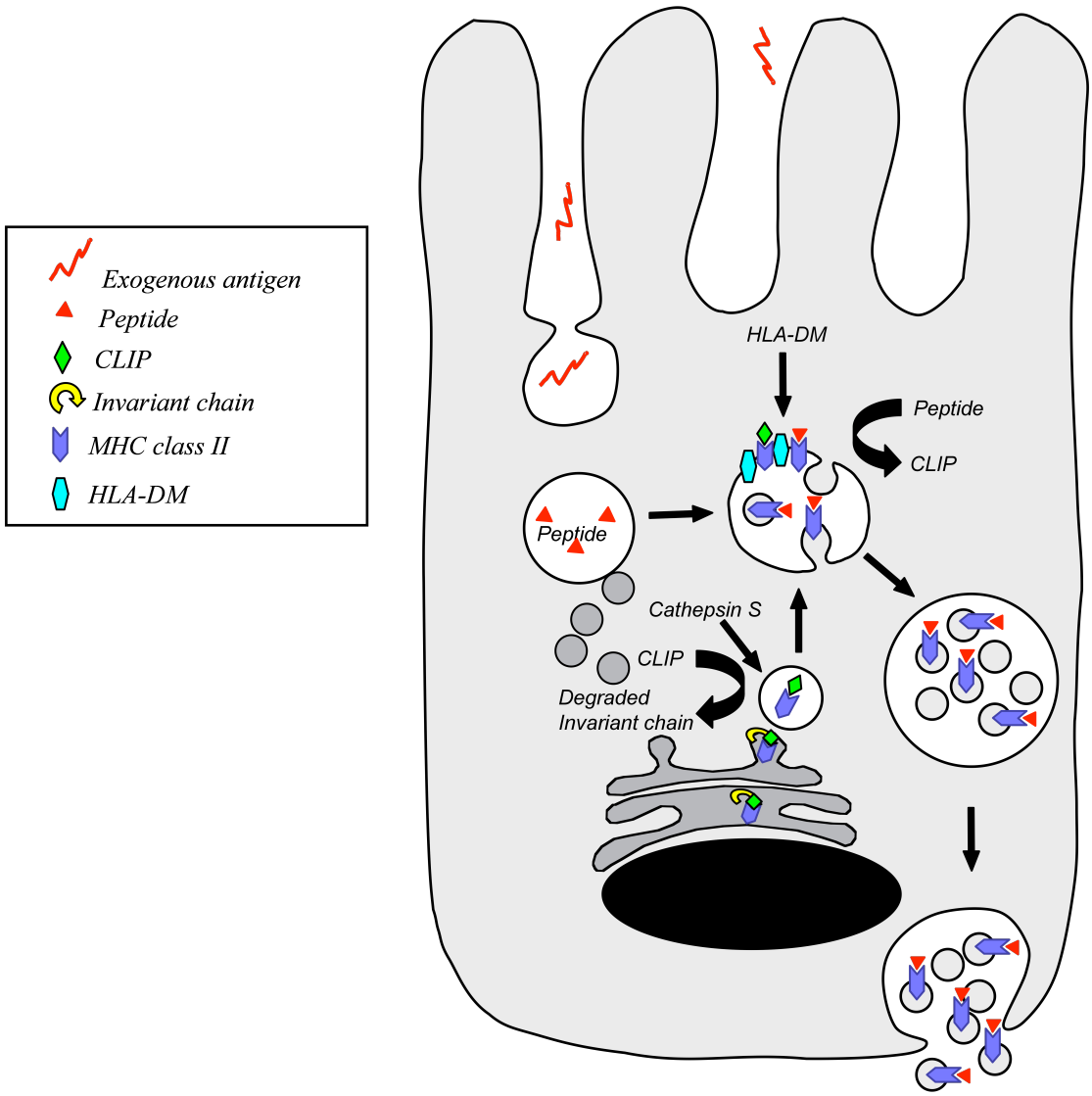

Figure 4. Antigen processing and loading on MHC class II molecules in the intestinal epithelial cell followed by exosome formation and release on the basolateral side. The exosomes carry MHC class II-peptide complexes on their surface.

The role of the liver in oral tolerance

The liver stands between the gastrointestinal tract and the systemic circulation and have been shown to play a key role in oral tolerance. The blood from the intestine, carrying harmless dietary and commensal antigens, enters the liver via the portal vein. Particulate matter the size of exosomes (<100nm) are filtered through the fenestration of the sinusoids into the space of Disse (the lymphoid tissue of the liver), where it interacts with hepatic APCs (Figure 5)[51, 52]. Under normal conditions the levels of TGF-β and IL-10 are high in the liver, making its environment very tolerogenic [53]. One of the best examples of immune tolerance in the liver is the phenomenal acceptance rate of allogenic liver transplant [54]. Consequently, when the resident APCs process and present the gut derived antigens to T cells, either locally or in the draining lymph nodes, a possible scenario would be an induction of antigen-specific regulatory T cells followed by systemic tolerance. In fact, studies have shown that portal drainage through the liver is a prerequisite for establishing tolerance to orally administered antigens [31, 32], confirming the key role of the liver in oral tolerance.

The organization of the liver gives easy access for circulating antigens and as a consequence enabling tolerance induction to the very same. The liver is composed of parenchymal hepatocytes with a network of narrow (5-7 mm) fenestrated blood vessels, the sinusoids. The sinusoids have no discrete membrane and it permits a slow passage of blood through the liver (25-250 mm/min), which together with its fenestration distinguishes the liver sinusoids from other vascular beds. Lining the sinusoids and bordering the space of Disse, are liver sinusoidal endothelial cells (LSEC), which allow a selective passage of small particles (Figure 5). A part from lymphocytes, the liver also contains resident macrophages called Kupffer cells and adjacent DCs patrolling the portal vein area. Dendritic cells, hepatocytes and lipocytes all produce significant amounts of TGF-β and IL-10 at steady state due to continous stimulation by bacterial products present in the portal blood [53]. The hepatic DCs are mostly localized around the portal triads and the central veins but they readily migrate through the fenestration into the space of Disse. Within the hepatic DC population four subsets have been identified so far:

myeloid CD8a$^-$CD11b$^+$ or lymphoid CD8a$^+$CD11b$^-$ DCs, pDCs and NKDC [53]. All of these subsets are generally immunosuppressive and have a fairly immature phenotype expressing MHC class II and only a few co-stimulatory molecules [53]. Hepatic DCs are difficult to activate and evidence suggests that they maintain their immature phenotype even as the leave the liver. It has been shown that hepatic DCs, loaded with particles, can migrate to the liver draining lymph nodes and still maintain their immature phenotype [51].

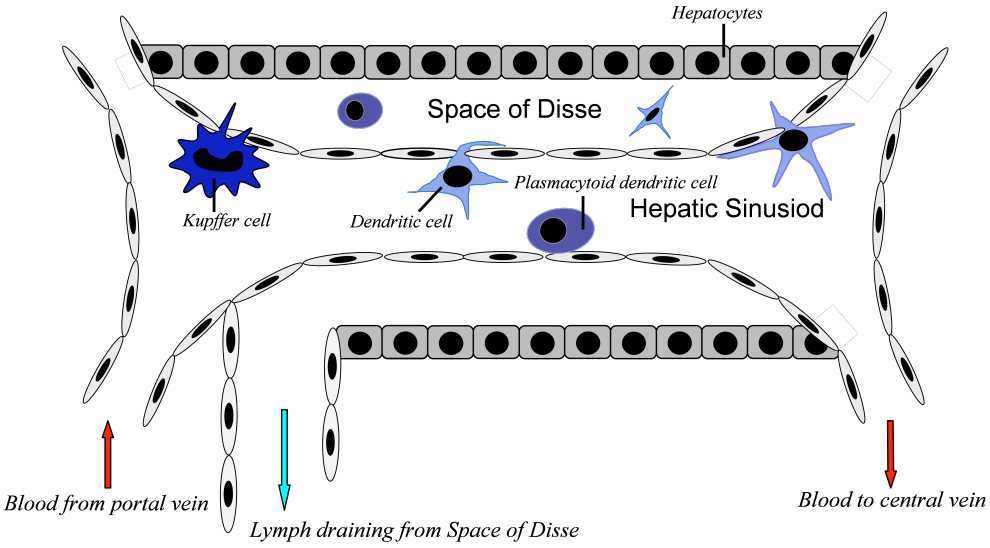

Figure 5. Schmatic presentation of the liver sinusoids.

The liver also contains an unusual population of resident lymphocytes amongst which CD8$^+$ T cells usually outnumber CD4$^+$ T cells. Both NK- and NKT cells are enriched relative to their proportions in lymphoid tissues. Several studies have shown that NKT cells also produce IL-10 in response to ceramides (glyko-lipid molecules) and in the liver a constant exposure of ceramides from the portal blood would perhaps lead to production of IL-10 by NKT cells. Consequently, NKT cells would thus contribute to the tolerogenic environment in the liver [55, 56]. Moreover, defects in NKT cell populations have been observed in diabetes [55-58] and experimental autoimmune encephalomyelitis [59], indicating a role for NKT cells in T cell homeostasis.

Regulatory T cells

Regulatory T cells are responsible for maintaining oral tolerance. They are educated to suppress the immune system in an antigen-specific manner upon oral administration of an antigen. There are two main types of $CD4^+CD25^+$ regulatory T cells, naturally occurring regulatory T cells (nTregs) and induced regulatory T cells (iTregs). The difference between the two is based on the antigen they respond to. Naturally occurring regulatory T cells are educated in the thymus and hence respond to self-antigens and induced regulatory T cells are educated in the periphery and can be induced to respond to any antigen, self or foreign. Another way to define the two types are intra- and extrathymic generated Tregs [60] which also reflects back on the antigen they recognize. Naturally occurring regulatory T cells are, as previously mentioned developed in the thymus as $CD4^+CD25^+$ and express high levels of the transcription factor forkhead box P3 (Foxp3). Foxp3 have recently been assigned the function as regulator of regulatory T cells development and function [61]. So far the tasks assigned to Foxp3 in regulatory T cells are repression of IL-2, activation of CTLA-4, CD25 and GITR. The Foxp3 gene was first identified as the defective gene in the mouse strain Scurfy, which is an X-linked recessive mutant that is lethal in hemizygous males within month after birth. The animals are exhibiting hyperactivation of $CD4^+$ T cells and overproduction of pro-inflammatory cytokines [62]. In humans, mutations in the Foxp3 gene are the cause of the genetic lethal disease IPEX (immune dysregulation, polyendocrinopathy, entheropathy, X-linked syndrom) [61]. Moreover, recent studies show that Foxp3 positive cells can be found shortly after birth and that autoimmune/inflammatory disease is a consequence of their depletion [63, 64].

The CD25 molecule is a component of the IL-2 receptor and is also functionally essential for Tregs. It has been shown that mice lacking IL-2 spontaneously develop T cell-mediated fatal lymphoproliferative/inflammatory disease with autoimmune components and hyperreactivity to commensal microbes [65]. Deficiencies in CD25 also results in similar symptoms and in humans the lack of CD25 is indistinguishable from IPEX [61, 66]. It is suggested that this is due to the deficiency or dysfunction of $Foxp3^+$ Tregs, which seems to depend on IL-2 signaling for their development, survival and function [67-69]. Natural Tregs

mediate suppression via cell-to-cell contact but the exact molecular interactions are not known. A recent studie suggests that this might occur through the inhibitory molecule CTLA-4 [70]. In vivo, the suppression is most likely also mediated via immunoregulatory cytokines such as TGF-β, IL-10 or IL-35 [61, 71]. Natural Foxp3$^+$ regulatory T cells specific for self-antigens will be enriched in the corresponding regional lymph node [72].

The intestinal immune system is dependent on regulatory T cells to prevent reactions against food proteins or the normal microbiota. It has been shown e.g. that depletion of Tregs or interfering with their function invariably induce inflammatory bowel disease [73]. The regulatory T cells residing in the gut, lamina propria, MLN and PPs are both natural Tregs and induced regulatory T cells. The induced regulatory T cells in the gut can be further divided into to main subsets Th3 and Tr1 regulatory T cells [5, 61]. Th3 cells are antigen-specific CD4$^+$CD25$^+$ and can also express Foxp3 and CTLA-4. They mediate supression by producing TGF-β. Tr1 cells are CD4 positive cells with suppressive functions attributed mainly to secretion of high levels of IL-10 secretion but also TGF-β. Their phenotype vary as they can be both CD25$^+$ and CD25$^-$ as well as both Foxp3$^+$ or Foxp3$^-$. The Foxp3$^+$ Tr1 cells have been shown to prevent colitis in an IL-10 dependent matter [74]. The role of IL-10 on Tr1 cell function and development needs to be further elucidated [75]. The Foxp3$^+$ Treg population in the gut are probably both natural Tregs and induced Tregs. The regulatory T cells involved in the mechanisms behind oral tolerance induction are most likely induced antigen-specific regulatory T cells, the Th3 type iTregs, first described by Weiner et al [76]. The norm recent years has been that induced Tregs mediate active suppression by the production TGF-β and IL-10 [77, 78] but a possible cell-to-cell mediated suppression by this subset should perhaps not be completely ruled out.

TGF-β is abundantly expressed in the gut and it is produced by both T cells and stromal cells. In addition to its immunosuppressive properties TGF-β is a critical factor for IgA class switching. It has recently been shown that TGF-β signals induce Foxp3 expression in CD4$^+$CD25$^+$ T cells and cause them to become regulatory in nature, both in vitro and in vivo [79-82]. Interestingly, a

recent study have shown that TGF-β induced Foxp3$^+$ regulatory cells transferred to newborn Scurfy mice can prevent disease. The authors therefore concluded that TGF-β-differentiated Foxp3$^+$ regulatory T cells possess all of the functional properties of thymic-derived nTregs [83]. Several studies have also shown that the development of Foxp3$^+$ Tregs can be further enhanced by retinoic acid, produced by GALT derived dendritic cells, together with TGF-β [84-86]. Hence, IEC-derived exosomes with MHC class II-peptide complexes or free antigen presented by retinoic acid producing DCs in the TGF-β rich milieu of the gut could induce Foxp3$^+$ Tregs and consequently induce tolerance to the ingested antigen.

MECHANISMS OF ORAL TOLERANCE

Oral administration of an antigen gives rise to a generation of $CD4^+$ T cells that down regulate the immune response and induce tolerance to the antigen [78, 87, 88]. The mechanisms behind oral tolerance and the induction of these regulatory CD4+ T cells remain largely unknown. It was believed for many years that the Peyer´s Patches were the main site for tolerance induction in the intestine and that the M cells were the only entrance for complex antigens. This commonly assumed pathway for antigen processing in the gut would come about as follows; M cells, which do not process antigen themselves, pass on the endocytosed material directly to B cells in the dome area and professional APC residing in either the epithelium or the underlying dome. The APCs, e.g. dendritic cells, move on to the T-cell areas or the B-cell follicles where they interact with naive lymphocytes. The lymphocytes that are primed in the PPs exit through the draining lymphatics to the MLNs, were they mature and migrate into the bloodstream through the thoracic duct and finally home to and accumulate in the mucosa. However, this scheme fails to address several alternative routes for antigen handling, for example;

- Transfer of free antigen and/or antigen loaded APCs from the PPs to the MLN, followed by local presentation to T cells.
- Transfer of antigens or IEC-derived antigen-loaded exosomes from the intestinal circulation to the liver through the portal vein.
- Local presentation of antigen by epithelial cells or antigen-loaded exosomes released from the latter to T cells in the lamina propria or local presentation via APCs.
- Uptake of antigens or IEC-derived exosomes by APCs migrating to MLN for presentation to T cells.

The lamina propria, PP, MLN and the liver are all sites which favour tolerance since the levels of IL-10 and TGF-β are high. Consequently, presentation at these sites would occur during tolerogenic conditions and the result is likely to be induced regulatory T cells which then, due to the unique set of homing molecules upregulated on gut derived lymphocytes, would home back to the mucosa.

In deed, several studies support the independence of PPs for oral tolerance induction e.g. tolerance to orally ingested antigens have been induced in animals lacking Peyer's Patches, both as a result of genetic modification [89, 90] and surgical removement [91]. Moreover, oral tolerance to protein antigens in B cells deficient mice seems to be normal despite that these animals do not have fully developed Peyer's Patches and almost entirely lacking M cells [92]. In contrast, there seems to be little doubt that the MLNs have a crucial role in oral tolerance induction. Studies using adoptive transfer of transgenic T cells show that antigen recognition occurs in the MLN within a few hours of feeding a protein antigen [5]. In addition it is impossible to induce oral tolerance in mice lacking MLNs [89, 90].

Exosome-induced tolerance

We have shown that one possible route for tolerance is via exosomes produced by intestinal epithelial cells (tolerosomes) [6, 93, 94], (III. Almqvist et al., in manuscript). The initial step in this process is active sampling by the small intestinal epithelial cells of the luminal content at the mucosal surface. The antigen is processed and peptides are loaded on MHC class II molecules, which are constitutively present in IECs [42]. Exosomes, carrying MHC class II-peptide complexes, are formed and released at the basolateral side of the IEC [6] (III. Almqvist et al., in manuscript). We believe that these exosomes are transported across the endothelium, which is supported by the fact that we can isolate exosomes from serum expressing the epithelial-specific A33 molecule [8] and that A33 stained structures are present in the endothelial cells of the capillaries adjacent to the IECs [42]. Moreover, we have shown that the exosomes isolated from serum 1h after an antigen feed, can transfer antigen specific tolerance when injected into naïve recipients [7, 93]. The recipient animals were protected against both Th1 and Th2 dominated responses. Already in 1983 Strobel et al showed that serum, obtained 1h after feeding an antigen and given intraperitoneally to recipient mice, had the capacity to induce suppression of delayed-type-hypersensivity (DTH) reactions [95]. A few years later the same group could show that parenteral administration of the antigen did not result in tolerance and therefore concluded that the "gut processing" of the antigen was a requirement to achieve tolerance via serum transfer [96]. We

believe that our research have contributed to identifying the tolerogenic serum-factor as exosomes secreted from the intestinal epithelial cells. The fate of the exosomes upon entering the circulation is not known, but a proportion most likely enters the liver where small particles (<100nm size) are filtered out from the blood into the space of Disse. Here the exosomes meet APCs that clears particulate matter of similar size as the exosomes (≈40nm) [51, 52]. Due to the tolerogenic environment in the liver [97], the message sent as a consequence of the processing and presentation of antigen-loaded exosomes by liver APCs is therefore likely to be one of tolerance induction. It has been shown in a previous study from our group that after oral administration of an antigen there is an induction of regulatory T cells in the liver draining lymph node, the celiac lymph node (CLN) [3]. The same study also shows that the CLN rapidly becomes engaged in the response to fed antigens and that the T-cells become activated within 6h and later develop into a distinct antigen specific T-cell population with a regulatory phenotype and a suppressive function [3]. These results have been confirmed in a recent study which also shows that regulatory T cells, expressing Foxp3, are induced in the liver draining lymph nodes after feeding an antigen orally [98]. Taken together these studies strengthens the idea of a central role for the celiac lymph node in tolerance induced by feeding an antigen, and suggests that CLN function as a "boot camp for regulatory T cells".

In summary, exosome-induced tolerance, summarized in figure 6, would contribute to local tolerance through direct presentation, in the lamina propria, to T cells, or via APC. Furthermore, the exosomes would be responsible for the systemic tolerance achieved upon oral administration of an antigen via two possible routes: exosomes transported by the circulation to the liver were they are taken up by APC which in turn mediate their message to T cells resulting in systemic tolerance due to induced regulatory T cells. Second, APC that have taken up exosomes in the lamina propria could migrate to MLN and present the exosome-derived antigen to T cells.

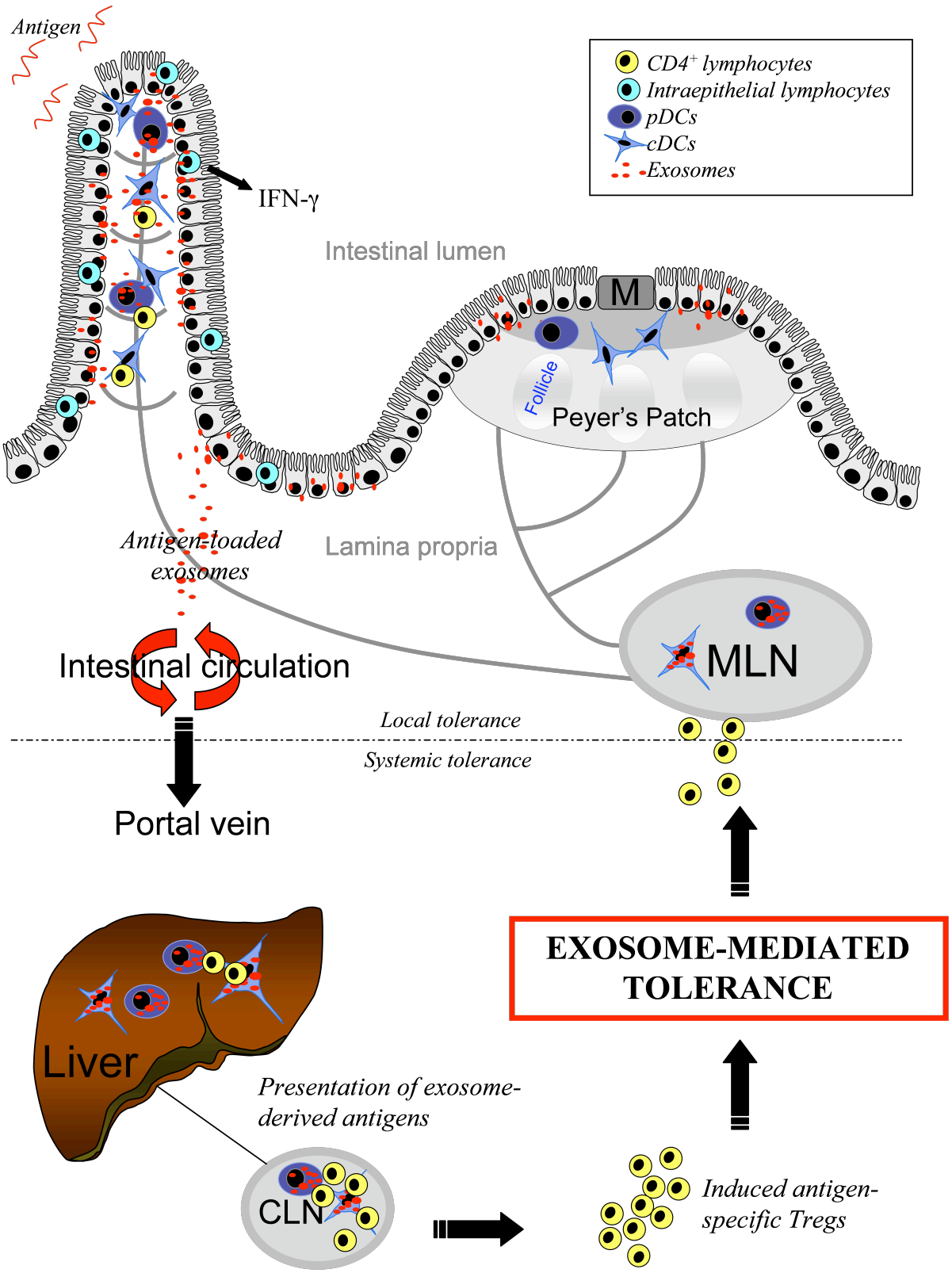

Figure 6. Overview of the fate of IEC-derived exosomes and their role in the induction of oral tolerance.

Effects of the microbiota on tolerogenic processing

In 1989 David Strachan formulated the hygiene hypothesis based on his studies of prevalence the of allergic disease in relation to socio-economic status and family size [9]. He proposed that microbial exposure was required in order to properly educate the immune system and that a decreased microbial exposure would lead to a failure of such stimulation and development of allergic disease. In recent years the Western countries have seen a substantial increase in certain diseases such as allergies, which have actually doubled in the last 20-40 years, [99, 100], inflammatory bowel disease [101-103] and organ-specific autoimmune disorders such as insulin-dependent type I diabetes and multiple sclerosis [104-106]. The reasons for an increase in prevalence among these diseases are not known but according to Strachans hygiene hypothesis it is related to the increased hygienic lifestyle in the modern Western society. A lifestyle associated with factors such as decreased bacterial load and reduced number of infections [9, 107]. The immune system of the gut is frequently exposed to different food antigens and commensal bacteria and must also discriminate these from pathogens. This complex interplay between immunity and tolerance to intestinal antigens makes the intestinal immune system extremely sensitive to lifestyle changes of the kind mentioned above. Changes of the commensal flora of the young child might alter the stimulation and maturation of the immune system and in fact, several longitudinal studies have demonstrated a disturbance in gut microbiota is preceded by the development of atopic disease (reviewed by [108]) and recently also an association between a microbiota of low diversity at 1 week of age and later allergy development [109]. The hygiene hypothesis and its relevance in gut immune system are also supported by experimental data e.g. germfree animals do not develop oral tolerance to the same extent as animals reared conventionally [110, 111]. Moreover, naturally induced regulatory T cells have reduced functional capacity in germ free animals [112].

As previously mentioned we have shown that exosomes can be isolated from serum 1h after an antigen feed and transfer antigen specific tolerance when injected into naïve recipients [7, 93]. We have also shown that exosome-mediated tolerance is MHC class II dependent and requires an intact immune system in

the fed donor [8]. Germ-free mice lack MHC II-expression in the small intestinal epithelium, which results in the formation of non-informative exosomes that without MHC class II lack antigen presenting capacity, and failure to induce regulatory T cells after oral antigen administration [111]. It is known that a full flora generally provides the required stimuli for the maturation of the intestinal immune system and the intestinal epithelial cells, but it is not known which individual bacteria or bacterial product that delivers the necessary signals. A collaborating group investigated whether neonatal mucosal exposure to *S.aureus* enterotoxin A (SEA) could influence the capacity to develop oral tolerance and reduce sensitization and allergy. *S. aureus* enterotoxins are amongst the strongest T-cell activators known and it has been shown that enterotoxins are taken up by small intestinal epithelial cells and strongly activate intraepithelial T cells [113]. The results from this group show that SEA pre-treated mice are more efficiently tolerised by OVA feeding. This suggests that strong T cell activation in infancy promotes the development of oral tolerance (Lönnqvist et al., unpublished data). In collaboration with this group we have examined the role of mucosal exposure to *S.aureus* enterotoxin A, regarding the capacity of tolerogenic processing by the intestinal epithelium in adult mice. Our results indicates that the *S.aureus* enterotoxin A potentiates the development of oral tolerance, and we show for the first time that this effect can be transferred to naive recipient mice by the adoptive transfer of serum. The results suggest that the exosome fraction produced by SEA-exposed epithelium more efficiently modulates the immune system into a tolerogenic response to a fed antigen (IV. Hultkrantz et al., in manuscript). We could show that the SEA-exposed animals had significantly higher numbers of intraepithelial lymphocytes. This indicates higher levels of IFN-γ and an up-regulation of MHC class II expression in the intestine. Antigenic processing by this 'highly activated' epithelium would consequently give more potent exosomes, that is exosomes with more MHC class II-peptide complexes on their surface and hence more efficient presentation of the antigen. In conclusion, bacterial stimuli are important both for the tolerogenic processing and the development of oral tolerance.

DENDRITIC CELLS INVOLVED IN ORAL TOLERANCE

Dendritic cell is a common name for multiple subtypes that vary in hematological origin, life cycle as well as functional properties but that share enough features to include them in a single family. Included in this family are pDCs, but these types of cells still differ enough from "conventional" dendritic cells (cDCs) to have its own subgroup. There are two main sites were DCs play a significant role in the induction of oral tolerance, in the gut-associated lymphoid tissue and in the liver. In both locations there are several different subtypes of DCs. The liver populations are described above and the DC subtypes found in GALT are both conventional $CD11c^{+}$ DCs and pDCs. In the Peyer's Patches there are $CD11c^{high}$ populations expressing CD11b but they are negative for CD8α, negative for CD11b but positive for CD8α, finally there are CD11c positive cells negative for both the other two markers. The same subsets are described for both lamina propria and MLN, the MLNs contain migratory DCs from lamina propria and PPs as well as resident DCs developed from blood-borne precursors. Plasmacytoid dendritic cells have been found in PPs and MLN and in the lamina propria [114, 115]. For review on different DC subtypes in GALT see [116]. As in the liver the DCs in the gut are highly tolerogenic at steady state producing high levels of IL-10. As a consequence, T cells activated by gut derived DCs produce higher levels of IL-10 and IL-4 than those activated by splenic DC. Furthermore, as mentioned previously DCs in the gut, both conventional (myeloid) DC and pDCs [117] induce Foxp3 expression and Tregs [116].

Plasmacytoid dendritic cells

Plasmacytoid dendritic cells was recognized as a DC family member quite recently [118, 119] but the cells have been known for several decades as "T-cell associated plasma cells", "plasmacytoid T cells" and "natural interferon-producing cells" [120, 121]. These aliases of pDCs refers to their unique capacity to quickly secrete large amount of type I interferons (IFN I) in response to viral infections together with their microscopic appearances of a plasmablast in an immature or non-activated state. In fact, they produce 10-100 times more IFN as compared to any other IFN producing cell. Plasmacytoid dendritic cells

need not to be infected to respond to a viral pathogen but can respond via toll-like receptors (TLR), which detect structural features of viral nucleic acids such as unmethylated CpG-rich DNA motifs or double-stranded RNA. The capacity to produce IFN-α in the absence of virus gene expression, i.e. without being infected, makes pDCs able to bypass these evasion strategies and produce a vigorous immune response [120]. In addition to secretion of IFN I, activated pDCs undergo phenotypic changes e.g. acquisition of dendritic morphology and upregulation of MHC and T cell stimulatory molecules enabling them to engage and activate naïve T cells [122-125]. Their antigen-presenting capacity is what justifies their inclusion in the DC family.

When comparing pDCs to cDCs they differ, first of all, in their migratory properties. Conventional DC percursors leave the bone marrow and via the blood enters lymphoid organs and peripheral tissue where they convert into resident or migratory cDCs [126]. These DCs have an immature phenotype, that is a low surface expression of MHC class II and co-stimulatory molecules and are dedicated to antigen sampling [127]. The resident DC will remain in this immature state unless they are activated, in which case they mature and up-regulate MHC class II and co-stimulatory molecules. The migratory DC on the other hand is constantly migrating from the tissue to the local lymph nodes and become mature upon reaching the latter [126]. Plasmacytoid dendritic cells develop fully in the bone marrow and then enter the bloodstream [126] and in steady state pDCs are present in the thymus and all secondary lymphoid tissue [128, 129]. Plasmacytoid dendritic cells are also abundant in both the liver and the intestine [116, 130, 131]. The migration and maturation of migratory cDCs occurs constitutively, suggesting a role for these cells in the transport of periheral self-antigens to induce T cell tolerance. Whether pDCs also contribute to this mechanism is still unknown and their migration pattern at steady state needs to be further examined. However, several studies show that pDCs are effectivly recruited to sites of infection while few of them appear to migrate to lymph nodes, a behavior more consistent with a role in antigen-presentation and/or immunomodulation at site of infection rather then a role in antigen transport to the local lymph nodes for presentation to T cells. A function, which appears to be carried out mainly by cDCs [127, 132].

Although pDCs have the capacity to present antigens they might not be as effective as cDCs, or at least not have the same role as cDCs. Both types can efficiently present endogenous antigens, that is peptides generated in the cytosol for presentation on MHC class I or if these peptides possibly accessed the endosomal route for presentiation on MHC class II. When it comes to exogenous antigens, which is antigens that have been captured from the extracellular environment, pDCs seem to perform poorly. Exogenous antigens are also antigen that cDCs can present with very high efficiency due to: high endocytic capacity, long-lived MHC class II complexes on their surface and the abilibty to cross-present. It has previously been suggested that pDCs does not have the capacity to phagocytose [118, 133] but recent studies have confirmed the opposite [33, 134, 135]. Moreover, it was recently shown that the particles phagocytosed by different DCs differ in size and the authors concluded that cDCs more readily phagocytose larger particles while pDCs seems to favour smaller particles for ingestion (20-500nm in size) [136]. We have performed studies where pDCs were incubated with either small (100nm) flourecsent latex beads or FITC stained IEC-derived exosomes and microscopically examined. We found that pDCs readily engulf both beads and exosomes. We have for the first time shown that pDCs not only phagocytose exosomes but also more efficiently then cDCs (III. Almqvist et al., in manuscript). Taken together this suggests that cDCs and pDCs might favour different exogenous antigens e.g. those carried by exosomes for uptake and presentation, perhaps even having non-overlapping roles as antigen presenting cells.

When cDCs encounter activation signals they increase their antigen uptake and upregulate the MHC class II synthesis, this is later down-regulated, which renders the DC with a cell surface displaying large amounts of stable long lived MHC class II complexes loaded with antigens captured at the time of activation. Consequently, mature cDCs lose their ability to present newly encountered antigens via MHC class II, including the antigens that they still endocytose. On the contrary, pDCs maintain their MHC class II synthesis and peptide loading upon activation. Moreover, the ubiquitination and turnover of MHC class II is not downregulated in activated pDCs and as a result they lack the ability to accumulate long lived MHC class II-peptide complexes on their

surface. Finally, cDCs have the ability to cross-present, which is the ability to present exogenous antigen on MHC class I. And how does pDCs compare? The data are controversial and both human and murine studies are in contradiction. For review on antigen presenting capacity of pDCs compared to cDCs, see [132].

A role for pDCs in oral tolerance?

In contrast to the description of pDCs as potent activators of an immune response, through the secretion of large amounts of IFN I, recent studies have pointed at pDCs as potent inducers of systemic tolerance both to self and non-self antigens[33, 134, 135]. One of these studies even appoints pDCs as the mediators of oral tolerance. In all of the above studies tolerance is mediated via induced Tregs, which have also been shown in several other studies [137-139]. The exact mechanism employed by pDCs when inducing regulatory T cells is still largely unknown. Recently, pDCs have been shown to produce indoleaminge 2,3-dioxygenase, this may contribute to their ability to induce tolerance [140, 141]. It has been suggested that IDO-expressing pDCs could have a role in regulating T cells homeostasis, and IDO may act on CTLA-4 expressing Tregs [142]. We have transferred exosome-pulsed pDCs, to recipient animals followed by allergic sensitization e.i. by sensitization and intranasal exposure to ovalbumin (OVA). The exosomes were derived from OVA-pulsed IECs and as a control IECs without OVA. The results showed no difference between the two groups and both groups were protected against an allergic response compared to control animals. These results suggest that the hyporesponsiveness to OVA challenge is induced by the administration of pDC to the mice in an antigen non-specific way. The mechanism behind this effect needs to be further examined (III. Almqvist et al., in manuscript). It has been shown that IFN-α enhances the maturation of human $CD11c^+$ cDCs, with IFN-α matured cDC leading to induction of IL-10 producing Tregs [143]. This could be a possible mechanism for pDCs through which they could influence the induction of tolerance.

Given that pDCs seems to be excellent mediator of antigen-specific tolerance taken together with their apparent fetish for ingesting smaller particles they

might be a good target-cell candidate for intestinal derived exosomes. There are two sites where phagocytosis of IEC-derived exosomes can effect induction of oral tolerance, most importantly the liver but also in the lamina propria. It has recently been shown that oral tolerance depend on pDCs in the liver [33]. We have shown that not only do pDCs internalize exosomes but also induce higher levels of antigen-specific T cell proliferation as compared to cDCs when given exosomes with antigen (III. Almqvist et al., in mauscript). This indicates that pDCs might be more efficient then cDCs at processing exosomes and "translating" the message they carry. If this is true for all types of exosomes remains to be investigated. We also found that IEC-derived exosomes express MFG-E8, a molecule known to facilite their uptake by dendritic cells [144], suggesting a mechanism by which IEC exosome uptake is acheived. This on the other hand would make them a target for cDCs as well, suggesting that exosomes targeting pDCs also involves other molecules. Our results are, to some extent, also supported by the previously mentioned study, which indicated that pDCs seems to favour smaller particles for ingestion (20-500nm) [136]. Perhaps pDCs are the actual mediators of oral tolerance.

EXOSOMES IN OTHER APPLICATIONS

Exosomes have been studied most extensivley in cancer therapy due to their T-cell stimulatory capacity. One of the pathogenic problems with cancer is the unresponsiveness of the immune system and a way to overcome this is to give the patient autologous APCs primed with tumour antigen. However, it has been shown that also the exosomes, derived from these primed DCs, most effectively trigger a tumour specific T cells response [145-147]. The advantage exosomes have over the whole cell in this case is their size; they spread through the system more efficiently then an activated dendritic cell, but still carry the same message. The tumour itself can also reduce antigen presenting functions of APCs through the release of soluble factors or by direct interaction with immune cells and exosomes are not affected by such factors. The tumour cells themselves have also taken advatage of the exosome pathway, they produce exosomes used as an immune evasion mechanism as tumour-derived exosomes have been shown to exert immune suppressing effects, e.g. via FasL:Fas interaction [148], TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) [149] or TGF-β [150]. A similar evasion mechanism as described for tumour, using the exosome pathway, has also been suggested as a mechanism for the semi-allogeneic featus to avoid being rejected by the maternal immune system. Exosomes are produced by placental trophoblasts as a way to induce 'tolerance' or immunological non-responsiveness to the featus during pregnacy [19, 151].

Depending on the maturation state of the secreting dendritic cell, the exosomes released from the latter appear to trigger the immune response in different ways. It has been shown that ICAM-1, which is more abundant on exosomes from mature DCs, is crucial for naïve T cell priming by exosomes [26]. Another study show that exosomes from IL-10 treated DCs are capable to suppress inflammation and collagen-induced arthritis and this occurs in a non-antigen specific manner [152]. This suggest that a tolerogenic DC secretes exosomes that have the same immunomodulatory effect. In conclusion, the current literature indicates that the outcome of an immune response to DC

derived exosomes depend on the activation/maturation state of the originating DC.

As mentioned previously, we have also shown that exosomes isolated from serum shortly after an antigen feed have the capacity to transfer tolerance to recipients and protect against allergic airway sensitization [7]. In addition, it has recently been shown that exosomes from B cells, isolated and cultured from human PBMC, can present allergen peptides and activate allergen specific T cells to proliferate and produce Th2 cytokines [153]. Taken together these findings suggests that exosomes from different sources may play a role in the development of asthma and allergy in at least two ways; either as a failure to induce effective tolerance or as enhancers of an already established allergic response. This makes exosomes highly interesting as therapeutic targets in anti-allergy treatment.

Exosomes are also believed to be utilised as a transport for retroviruses [154]. Gould et al suggests 'The Trojan exosome hypothesis' which states that retroviruses use the pre-existing nonviral exosome biogenesis pathway for the formation of infectious particles [155]. The exosome-like vesicles would contain virus particles undetectable to the host's own immune system. It has been shown that macrophages infected with HIV release HIV particles displaying, to a certain extent, similar molecules as exosomes [156]. In addition HIV virons assemble in the MVBs of macrophages, which is the site where exosomes are formed [157]. A different study has shown that HIV infected immature DC release exosomes that can transfer HIV to CD4+ T cells. They also show that the exosome-associated HIV was 10 fold more infectious then free virus particles [158]. Using exosomes as a transport is an excellent strategy to escape the host defences. The virus is protected inside a membrane bound vesicle that is readily taken up and processed by a number of different cells unaware of its infectious content, just like a true 'Trojan Horse'.

It has also been suggested that exosomes are contributing to the intracellular membrane exchange and the spread of prions [159, 160]. These studies show that infectious prion proteins, abnormally folded prion proteins (PrPs), scrapie

(PrPsc) are associated with exosomes. Furthermore the exosomes had the capacity to transfer the PrPsc to uninfected cells and transform normal PrPs into scrapie PrPsc. Exosomes enriched in PrP have also been isolated from sheep cerebral spinal fluid and is suggested by the authors to be a way of detecting abnormal forms of the prion [161].

CONCLUDING REMARKS

Exosomes are important players for passing on immunological information between cells and thus take active part in immune regulation. We propose that oral tolerance is dependent on exosomes released from the small intestinal epithelial cells upon ingestion of an antigen. We also belive that these exosomes could contribute to systemic tolerance as a result of oral administration of an antigen due to their uptake by APCs in the liver. The liver would act as a cross-over point, the place where orally administrated antigens could be presented to T cells without at the same time induce gut-homing receptors on the latter. This would enable the T cells to home to other tissue and reside there as memory cells, a scenario that is not possible when priming occurs in GALT since priming in GALT is most likley to result in a gut-imprint on the T cells. We also suggest that pDCs may play an important role in both the liver and the gut since they seem to readily ingest exosomes and efficiently translating their message. Recent evidence indicates that pDCs can effecively induce tolerance in different experimental settings, which would support the hypothesis that pDCs contribute to exosome-mediated tolerance. Moreover, the gut-processing of an antigen and the release of functional exosomes depend on MHC class II expression in the gut, which is in turn dependent on microbial stimuli. We have shown that the production of exosomes from the epithelium can be influenced by microbial stimuli and that the effect of the exosomes can actually be enhanced. In conclusion, we have show that exosomes secreted from intestinal epithelial cells can transfer antigen-specific tolerance and protect against an allergic response, a mechanism which can also be enhanced by using microbial stimuli as a way to improve the effects of the transferred exosomes. We have also shown that pDCs efficiently ingest IEC-derived exosomes and we suggest that they might be important mediators of antigens carried by IEC-derived exosomes. These findings are important for understanding the process of oral tolerance and the effects of exosomes and through more basic knowledge of the intestinal immune system one could easier find ways to prevent or cure disease were dysregulation of the intestinal immune system is the cause.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Förekomsten av allergier, inflammatoriska tarmsjukdomar och autoimmuna sjukdomar har ökat kraftigt i i-länder. Allergi är en av de vanligaste sjukdomarna och förekomsten har fördubblats de senaste 20-40 åren. Man räknar med att upp till 40 procent av alla barn i dessa länder har någon form av allergi. En förklaring till denna drastiska ökning ges av den så kallade hygienhypotesen. Den går ut på att en alltför hygienisk livsstil, det vill säga minskning av bakterier i omgivningen i kombination med minskat antal infektioner, medför bristande stimulering och följdaktligen ett mindre utvecklat immunförsvar.

Ett friskt immunförsvar förhindrar reaktioner mot ofarliga födoämen och bakterier genom att utveckla tolerans mot dem. De tillåts av immunförsvaret, medan skadliga bakterier och virus känns igen och elimineras. Det är helt avgörande att kroppens immunförsvar inte sätter igång liknande reaktioner mot ofarliga ämnen från den egna kroppen eller från maten vi äter. För att förhindra detta har immunsystemet utvecklat kontrollmekanismer för att tolerera ofarliga ämnen och förhindra till exempel födoämnesallergier.

Mag-tarmkanalen utsätts ständigt för födoämnen och bakterier från den normala tarmfloran. Den måste därför hålla immunsystemet i schack, för att undvika felaktiga reaktioner mot ofarligt material, och har utvecklat en kraftfull kontrollmekanism som kallas oral tolerans. Att inta ett ämne eller ett protein via munnen är ett effektivt sätt för att åstadkomma specifik tolerans mot detta ämne. Oral tolerans upprätthålls genom att kroppen utvecklar särskilda celler, regulatoriska T-celler, som gör immunsvaret mindre aktivt mot ämnet. Cellerna får också ett minne mot det specifika ämnet för att kunna känna igen det vid en senare tidpunkt. Ett första steg i processen är att tarmepitelcellerna, de celler som utgör barriären in till kroppen, samlar upp och bryter ner proteiner. På så vis gör epitelcellen om proteinet till en form som kan visas upp och kännas igen av kroppens andra celler. Det släpps ut på små (40-90 nanometer) vesiklar som kallas exosomer. Vi tror att exosomerna förs vidare med blodet och når levern, eftersom allt blod från mag-

tarmkanalen når levern för rening innan det når kroppens övriga delar. I levern finns celler som kan läsa exosomernas budskap och översätta det för de regulatoriska T-cellerna. De regulatoriska T-cellerna skulle i så fall utbildas i levern och sedan vandra ut i kroppen och förhindra att immunsvaret sätts in mot proteinet i fråga.

Vår forskningsgrupp har i en djurmodell tidigare visat att exosomer kan tas fram ur blodet från ett djur matat med ett visst protein. När exosomerna ges till en annan individ överförs toleransen mot ämnet som exosomen presenterar. I den här avhandligen har vi fokuserat på att förstå exosomers roll i oral tolerans. Vi ville undersöka om exosomerna kunde skydda mot allergi och om stimulering med bakteriella produkter skulle påverka utgången av toleransöverföring med hjälp av exosomer. Vi ville också ta ytterligare ett steg och försöka identifiera en cell som kan översätta exosomernas budskap och utbilda regulatoriska T-celler.

I den här avhandlingen kan vi i djurmodeller visa att exosomer kan skydda mot allergi. De djur som får exosomer med ett specifikt ämne blir inte lika allergiska när de senare utsätts för detta ämne. Vi kan också visa att stimulering med en bakteriell produkt gör immunsystemet i mag-tarmkanalen mer aktivt och förstärker effekten av exosomerna. Slutligen kan vi visa att en viss typ av cell, en så kallad plasmacytoid dendritisk cell, mycket effektivt kan plocka upp exosomer och förstå och presentera deras budskap.

Sammanfattningsvis drar vi slutsatsen att exosomer kan skydda mot allergi, möjligen via plasmacytoida dendritiska celler. Dessutom överensstämmer våra resultat med hygienhypotesen, vilket innebär att bakteriell stimulering effektiviserar oral tolerans tack vare ett mer aktiverat immunsystem i mag-tarmkanalen.

ACKNOWLEDGEMENTS

Jag är otroligt glad och tacksam för att jag kunnat genomföra arbetet som har lett till den här boken. Jag vill tacka alla som har varit med och bidragit, speciellt vill jag tacka:

Esbjörn, min handledare. Det har varit ett privilegium att ha obegränsad tillgång till dina oändliga kunskaper i immunologi. Ditt sätt att handleda har gjort mig självständig och gett mig enormt mycket självkänsla, både privat och i mitt arbete. Du har också varit en vän och ditt stöd i alla lägen har haft stor betydelse. Tack!

Mattias, min bi-handledare. Först och främst stort tack för att du kämpade så för att jag skulle kunna genomföra det här. Du har varit en fantastisk arbetskamrat och vän som sedan också blev handledare. Du har lärt mig allt jag kan om pedagogik och om att vara språkligt korrekt – och kanske en del om hur man är korrekt i andra sammanhang också. Tack vare dig är jag också mästare på att undvika satsradning, massage och hemligheter.

Stort tack till min andra bihandledare, **Hans**. Utan ditt stöd hade jag inte fått möjlighet att skriva den här avhandlingen.

Tack till **alla på avdelningen för Reumatologi och Inflammationsforskning** för en fantastisk stämning och gemenskap på arbetsplatsen! Extra stort tack till **Eva** och **Harriet**. Vad skulle jag ha gjort med alla papper utan er? **Kristina**, stort tack för ditt stöd.

Elin, min fantastiskt roliga vän. Tack för allt från toastmasteri till barnvakteri.

Susanne, du gav mig en väldigt välorganiserad start på min forskarutbildning. Var hade jag varit utan de kunskaperna…? Du är också en fantastisk vän med grym musiksmak.

Anna, tack för alla dagar i djurhuset med allt biktande. Du har inte bara varit en ovärderlig hjälp med arbetsbördan utan är också en mycket lojal vän.

Tack för allt stöd, nya och gamla rumskamrater. **Hanna**, jag har haft tur som haft dig som stöd de sista veckorna. **Sofi** och **Sylvie**, vi satt ihop tills rummet bokstavligen möglade.

Tack till vänner och kollegor. **Ulf** du slavade åt mig under min semester och jag fick till och med låna din bil till Roskilde. Jag ska bara ha doktorander som dig. Tack vare dig har jag också ytterligare ett manus i min avhandling. **Sofia**, för att du har lyssnat och gett råd. **Caroline**, för ditt stöd och dina positiva ord. **Anna-Karin**, för att du har lyssnat så ofta. **Mikael**, för ett eget fikarum och för att du ställer upp i semestertider. **Alexandra**, för vänskap. **Tove**, för din vänskap både till mig och Izabella.

Tack till gamla kollegor på Lungfarmakologiska gruppen; **Jan**, **Margareta**, **Carina**, **Madelaine**, **Karin**, **Apostolos** och **Hadi**.

Jenny, min bästa vän. Saknar ord! Det här känns oerhört lång borta från Lottas och förfest i lilla ettan på Drakblommegatan. Totalt tre barn, en familjemedlem mindre, en identitetskris, en ny pojkvän och en avhandling senare… Extra mycket kärlek till dig och dina barn, **Molly** och **Selma.**

Vänner ute i verkligheten, ni vet hur mycket ni har betytt. **Cilla**, **Camilla, Fredde** och **Jocke**. Nya vänner, utan att vara mindre viktiga, **Elenor** och **Marcus**.

Tack till familjen för all kärlek och omtanke; **Morfar** och **Mormor**, **Pappa** och **Gunilla**, **Mamma**, **Björn**, **Charlie** och **Emma**.

Gustaf, du är min klippa och jag hade inte velat göra det här utan dig. Ditt stöd har betytt allt den här sista tiden. Du vet och jag vill bara säga ”if the sun refused to shine…”

Izabella, du är mitt allt! Jag älskar dig som ingen annan och du gör mig till en bättre människa. Du är en fantastisk, speciell och extremt tålig liten tjej och utan dig hade jag inte klarat det här. Den här boken är till dig, min lilla skrutta.

REFERENCES

1. Challacombe, S.J. & Tomasi, T.B., Jr. Systemic tolerance and secretory immunity after oral immunization. *J Exp Med* **152**, 1459-1472 (1980).
2. Dahlman-Hoglund, A., Dahlgren, U., Ahlstedt, S., Hanson, L.A. & Telemo, E. Bystander suppression of the immune response to human serum albumin in rats fed ovalbumin. *Immunology* **86**, 128-133 (1995).
3. Hultkrantz, S., Ostman, S. & Telemo, E. Induction of antigen-specific regulatory T cells in the liver-draining celiac lymph node following oral antigen administration. *Immunology* **116**, 362-372 (2005).
4. Lundin, B.S. *et al.* Active suppression in orally tolerized rats coincides with in situ transforming growth factor-beta (TGF-beta) expression in the draining lymph nodes. *Clin Exp Immunol* **116**, 181-187 (1999).
5. Mowat, A.M. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* **3**, 331-341 (2003).
6. Karlsson, M. *et al.* "Tolerosomes" are produced by intestinal epithelial cells. *Eur J Immunol* **31**, 2892-2900 (2001).
7. Almqvist, N., Lonnqvist, A., Hultkrantz, S., Rask, C. & Telemo, E. Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. *Immunology* **125**, 21-27 (2008).
8. Ostman, S., Taube, M. & Telemo, E. Tolerosome-induced oral tolerance is MHC dependent. *Immunology* **116**, 464-476 (2005).
9. Strachan, D.P. Hay fever, hygiene, and household size. *BMJ (Clinical research ed* **299**, 1259-1260 (1989).
10. Trams, E.G., Lauter, C.J., Salem, N., Jr. & Heine, U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* **645**, 63-70 (1981).
11. Harding, C., Heuser, J. & Stahl, P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* **97**, 329-339 (1983).
12. Pan, B.T., Teng, K., Wu, C., Adam, M. & Johnstone, R.M. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol* **101**, 942-948 (1985).
13. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* **183**, 1161-1172 (1996).
14. Caby, M.P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G. & Bonnerot, C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol* **17**, 879-887 (2005).
15. Janiszewski, M. *et al.* Platelet-derived exosomes of septic individuals possess proapoptotic NAD(P)H oxidase activity: A novel vascular redox pathway. *Crit Care Med* **32**, 818-825 (2004).
16. Admyre, C. *et al.* Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *Eur Respir J* **22**, 578-583 (2003).

17. Pisitkun, T., Shen, R.F. & Knepper, M.A. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* **101**, 13368-13373 (2004).
18. van Niel, G. *et al.* Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* **121**, 337-349 (2001).
19. Mincheva-Nilsson, L. *et al.* Placenta-derived soluble MHC class I chain-related molecules down-regulate NKG2D receptor on peripheral blood mononuclear cells during human pregnancy: a possible novel immune escape mechanism for fetal survival. *J Immunol* **176**, 3585-3592 (2006).
20. Thery, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* **2**, 569-579 (2002).
21. Williams, R.L. & Urbe, S. The emerging shape of the ESCRT machinery. *Nature reviews* **8**, 355-368 (2007).
22. Trajkovic, K. *et al.* Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science (New York, N.Y* **319**, 1244-1247 (2008).
23. van Niel, G., Porto-Carreiro, I., Simoes, S. & Raposo, G. Exosomes: a common pathway for a specialized function. *Journal of biochemistry* **140**, 13-21 (2006).
24. Escola, J.M. *et al.* Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* **273**, 20121-20127 (1998).
25. Lamparski, H.G. *et al.* Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods* **270**, 211-226 (2002).
26. Segura, E. *et al.* ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* **106**, 216-223 (2005).
27. Van Niel, G. *et al.* Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* **52**, 1690-1697 (2003).
28. Skokos, D. *et al.* Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J Immunol* **166**, 868-876 (2001).
29. Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology* **9**, 654-659 (2007).
30. Laulagnier, K. *et al.* Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochem J* **380**, 161-171 (2004).
31. Callery, M.P., Kamei, T. & Flye, M.W. The effect of portacaval shunt on delayed-hypersensitivity responses following antigen feeding. *J Surg Res* **46**, 391-394 (1989).
32. Yang, R., Liu, Q., Grosfeld, J.L. & Pescovitz, M.D. Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. *J Pediatr Surg* **29**, 1145-1148 (1994).

33. Goubier, A. *et al.* Plasmacytoid dendritic cells mediate oral tolerance. *Immunity* **29**, 464-475 (2008).
34. Matsumoto, S. *et al.* Physiological roles of gammadelta T-cell receptor intraepithelial lymphocytes in cytoproliferation and differentiation of mouse intestinal epithelial cells. *Immunology* **97**, 18-25 (1999).
35. Taguchi, T. *et al.* Novel function for intestinal intraepithelial lymphocytes. Murine CD3+, gamma/delta TCR+ T cells produce IFN-gamma and IL-5. *J Immunol* **147**, 3736-3744 (1991).
36. Hughes, A. *et al.* Expression of MHC class II (Ia) antigen by the neonatal enterocyte: the effect of treatment with interferon-gamma. *Immunology* **72**, 491-496 (1991).
37. Matsumoto, S., Setoyama, H. & Umesaki, Y. Differential induction of major histocompatibility complex molecules on mouse intestine by bacterial colonization. *Gastroenterology* **103**, 1777-1782 (1992).
38. Okada, Y. *et al.* Effects of fecal microorganisms and their chloroform-resistant variants derived from mice, rats, and humans on immunological and physiological characteristics of the intestines of ex-germfree mice. *Infection and immunity* **62**, 5442-5446 (1994).
39. Bjersing, J.L., Telemo, E., Dahlgren, U. & Hanson, L.A. Loss of ileal IgA+ plasma cells and of CD4+ lymphocytes in ileal Peyer's patches of vitamin A deficient rats. *Clin Exp Immunol* **130**, 404-408 (2002).
40. Mora, J.R. *et al.* Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science (New York, N.Y* **314**, 1157-1160 (2006).
41. Campbell, D.J. & Butcher, E.C. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med* **195**, 135-141 (2002).
42. Lin, X.P., Almqvist, N. & Telemo, E. Human small intestinal epithelial cells constitutively express the key elements for antigen processing and the production of exosomes. *Blood Cells Mol Dis* **35**, 122-128 (2005).
43. Mayer, L. *et al.* Expression of class II molecules on intestinal epithelial cells in humans. Differences between normal and inflammatory bowel disease. *Gastroenterology* **100**, 3-12 (1991).
44. Mayrhofer, G. & Spargo, L.D. Subcellular distribution of class II major histocompatibility antigens in enterocytes of the human and rat small intestine. *Immunology and cell biology* **67 (Pt 4)**, 251-260 (1989).
45. Bland, P.W. & Warren, L.G. Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera. *Immunology* **58**, 1-7 (1986).
46. Blumberg, R.S. *et al.* Antigen presentation by intestinal epithelial cells. *Immunology letters* **69**, 7-11 (1999).
47. Kaiserlian, D., Vidal, K. & Revillard, J.P. Murine enterocytes can present soluble antigen to specific class II-restricted CD4+ T cells. *Eur J Immunol* **19**, 1513-1516 (1989).

48. Buning, J. *et al.* Multivesicular bodies in intestinal epithelial cells: responsible for MHC class II-restricted antigen processing and origin of exosomes. *Immunology* (2008).
49. Hundorfean, G. *et al.* Luminal Antigens Access Late Endosomes of Intestinal Epithelial Cells Enriched in MHC I and MHC II Molecules - In vivo Study in Crohn's Ileitis. *Am J Physiol Gastrointest Liver Physiol* (2007).
50. Zimmer, K.P., Buning, J., Weber, P., Kaiserlian, D. & Strobel, S. Modulation of antigen trafficking to MHC class II-positive late endosomes of enterocytes. *Gastroenterology* **118**, 128-137 (2000).
51. Matsuno, K., Ezaki, T., Kudo, S. & Uehara, Y. A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J Exp Med* **183**, 1865-1878 (1996).
52. Willekens, F.L. *et al.* Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. *Blood* **105**, 2141-2145 (2005).
53. Hsu, W., Shu, S.A., Gershwin, E. & Lian, Z.X. The current immune function of hepatic dendritic cells. *Cellular & molecular immunology* **4**, 321-328 (2007).
54. Mazariegos, G.V., Sindhi, R., Thomson, A.W. & Marcos, A. Clinical tolerance following liver transplantation: long term results and future prospects. *Transplant immunology* **17**, 114-119 (2007).
55. Baev, D.V. *et al.* Impaired SLAM-SLAM homotypic interaction between invariant NKT cells and dendritic cells affects differentiation of IL-4/IL-10-secreting NKT2 cells in nonobese diabetic mice. *J Immunol* **181**, 869-877 (2008).
56. Hong, S. *et al.* The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* **7**, 1052-1056 (2001).
57. Kanagawa, O., Militech, A. & Vaupel, B.A. Regulation of diabetes development by regulatory T cells in pancreatic islet antigen-specific TCR transgenic nonobese diabetic mice. *J Immunol* **168**, 6159-6164 (2002).
58. Sharif, S. *et al.* Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* **7**, 1057-1062 (2001).
59. Singh, A.K. *et al.* Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* **194**, 1801-1811 (2001).
60. Apostolou, I. *et al.* Peripherally Induced Treg: Mode, Stability, and Role in Specific Tolerance. *Journal of clinical immunology* (2008).
61. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. *Cell* **133**, 775-787 (2008).

62. Brunkow, M.E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature genetics* **27**, 68-73 (2001).
63. Kim, J.M., Rasmussen, J.P. & Rudensky, A.Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* **8**, 191-197 (2007).
64. Lahl, K. *et al.* Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* **204**, 57-63 (2007).
65. Malek, T.R. & Bayer, A.L. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* **4**, 665-674 (2004).
66. Caudy, A.A., Reddy, S.T., Chatila, T., Atkinson, J.P. & Verbsky, J.W. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J Allergy Clin Immunol* **119**, 482-487 (2007).
67. Antony, P.A. *et al.* Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol* **176**, 5255-5266 (2006).
68. Fontenot, J.D., Rasmussen, J.P., Gavin, M.A. & Rudensky, A.Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* **6**, 1142-1151 (2005).
69. Shevach, E.M. *et al.* The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* **212**, 60-73 (2006).
70. Wing, K. *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science (New York, N.Y* **322**, 271-275 (2008).
71. Sakaguchi, S. Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. *Annu Rev Immunol* **22**, 531-562 (2004).
72. Samy, E.T., Parker, L.A., Sharp, C.P. & Tung, K.S. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. *J Exp Med* **202**, 771-781 (2005).
73. Singh, B. *et al.* Control of intestinal inflammation by regulatory T cells. *Immunol Rev* **182**, 190-200 (2001).
74. Rubtsov, Y.P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546-558 (2008).
75. Roncarolo, M.G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* **212**, 28-50 (2006).
76. Faria, A.M. & Weiner, H.L. Oral tolerance and TGF-beta-producing cells. *Inflammation & allergy drug targets* **5**, 179-190 (2006).
77. Chen, Y. *et al.* Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature* **376**, 177-180 (1995).
78. Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science (New York, N.Y* **265**, 1237-1240 (1994).

79. Chen, W. *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* **198**, 1875-1886 (2003).
80. Pyzik, M. & Piccirillo, C.A. TGF-beta1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets. *J Leukoc Biol* **82**, 335-346 (2007).
81. Selvaraj, R.K. & Geiger, T.L. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. *J Immunol* **179**, 11 p following 1390 (2007).
82. Zheng, S.G., Wang, J.H., Gray, J.D., Soucier, H. & Horwitz, D.A. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* **172**, 5213-5221 (2004).
83. Huter, E.N. *et al.* TGF-beta-induced Foxp3+ regulatory T cells rescue scurfy mice. *Eur J Immunol* **38**, 1814-1821 (2008).
84. Benson, M.J., Pino-Lagos, K., Rosemblatt, M. & Noelle, R.J. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* **204**, 1765-1774 (2007).
85. Coombes, J.L. *et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**, 1757-1764 (2007).
86. Sun, C.M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**, 1775-1785 (2007).
87. Groux, H. *et al.* A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **389**, 737-742 (1997).
88. Karlsson, M.R., Kahu, H., Hanson, L.A., Telemo, E. & Dahlgren, U.I. Neonatal colonization of rats induces immunological tolerance to bacterial antigens. *Eur J Immunol* **29**, 109-118 (1999).
89. Spahn, T.W. *et al.* Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *Eur J Immunol* **31**, 1278-1287 (2001).
90. Spahn, T.W. *et al.* Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *Eur J Immunol* **32**, 1109-1113 (2002).
91. Enders, G., Gottwald, T. & Brendel, W. Induction of oral tolerance in rats without Peyer's patches. *Immunology* **58**, 311-314 (1986).
92. Alpan, O., Rudomen, G. & Matzinger, P. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J Immunol* **166**, 4843-4852 (2001).
93. Karlsson, M.R., Kahu, H., Hanson, L.A., Telemo, E. & Dahlgren, U.I. Tolerance and bystander suppression, with involvement of CD25-

positive cells, is induced in rats receiving serum from ovalbumin-fed donors. *Immunology* **100**, 326-333 (2000).
94. Karlsson, M.R., Kahu, H., Hanson, L.A., Telemo, E. & Dahlgren, U.I. An established immune response against ovalbumin is suppressed by a transferable serum factor produced after ovalbumin feeding: a role of CD25+ regulatory cells. *Scand J Immunol* **55**, 470-477 (2002).
95. Strobel, S., Mowat, A.M., Drummond, H.E., Pickering, M.G. & Ferguson, A. Immunological responses to fed protein antigens in mice. II. Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen. *Immunology* **49**, 451-456 (1983).
96. Bruce, M.G. & Ferguson, A. Oral tolerance to ovalbumin in mice: studies of chemically modified and 'biologically filtered' antigen. *Immunology* **57**, 627-630 (1986).
97. Knolle, P.A. & Gerken, G. Local control of the immune response in the liver. *Immunol Rev* **174**, 21-34 (2000).
98. Siewert, C. *et al.* Experience-driven development: effector/memory-like alphaE+Foxp3+ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *J Immunol* **180**, 146-155 (2008).
99. Verlato, G. *et al.* Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study. *J Allergy Clin Immunol* **111**, 1232-1238 (2003).
100. von Mutius, E., Weiland, S.K., Fritzsch, C., Duhme, H. & Keil, U. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. *Lancet* **351**, 862-866 (1998).
101. Farrokhyar, F., Swarbrick, E.T. & Irvine, E.J. A critical review of epidemiological studies in inflammatory bowel disease. *Scandinavian journal of gastroenterology* **36**, 2-15 (2001).
102. Langholz, E., Munkholm, P., Nielsen, O.H., Kreiner, S. & Binder, V. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962 to 1987. *Scandinavian journal of gastroenterology* **26**, 1247-1256 (1991).
103. Munkholm, P., Langholz, E., Nielsen, O.H., Kreiner, S. & Binder, V. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scandinavian journal of gastroenterology* **27**, 609-614 (1992).
104. Poser, S., Stickel, B., Krtsch, U., Burckhardt, D. & Nordman, B. Increasing incidence of multiple sclerosis in South Lower Saxony, Germany. *Neuroepidemiology* **8**, 207-213 (1989).
105. Pundziute-Lycka, A. *et al.* The incidence of Type I diabetes has not increased but shifted to a younger age at diagnosis in the 0-34 years group in Sweden 1983-1998. *Diabetologia* **45**, 783-791 (2002).

106. Rosati, G. *et al.* Incidence of multiple sclerosis in the town of Sassari, Sardinia, 1965 to 1985: evidence for increasing occurrence of the disease. *Neurology* **38**, 384-388 (1988).
107. Wold, A.E. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* **53**, 20-25 (1998).
108. Penders, J., Stobberingh, E.E., van den Brandt, P.A. & Thijs, C. The role of the intestinal microbiota in the development of atopic disorders. *Allergy* **62**, 1223-1236 (2007).
109. Wang, M. *et al.* Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol* **121**, 129-134 (2008).
110. Moreau, M.C. & Corthier, G. Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice. *Infection and immunity* **56**, 2766-2768 (1988).
111. Rask, C., Evertsson, S., Telemo, E. & Wold, A.E. A full flora, but not monocolonization by Escherichia coli or lactobacilli, supports tolerogenic processing of a fed antigen. *Scand J Immunol* **61**, 529-535 (2005).
112. Ostman, S., Rask, C., Wold, A.E., Hultkrantz, S. & Telemo, E. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol* **36**, 2336-2346 (2006).
113. Musch, M.W. *et al.* Bacterial superantigen-treated intestinal epithelial cells upregulate heat shock proteins 25 and 72 and are resistant to oxidant cytotoxicity. *Infection and immunity* **72**, 3187-3194 (2004).
114. Monteleone, I., Platt, A.M., Jaensson, E., Agace, W.W. & Mowat, A.M. IL-10-dependent partial refractoriness to Toll-like receptor stimulation modulates gut mucosal dendritic cell function. *Eur J Immunol* **38**, 1533-1547 (2008).
115. Rescigno, M. & Matteoli, G. Lamina propria dendritic cells: for whom the bell TOLLs? *Eur J Immunol* **38**, 1483-1486 (2008).
116. Coombes, J.L. & Powrie, F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* **8**, 435-446 (2008).
117. Bilsborough, J., George, T.C., Norment, A. & Viney, J.L. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology* **108**, 481-492 (2003).
118. Grouard, G. *et al.* The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* **185**, 1101-1111 (1997).
119. Siegal, F.P. *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science (New York, N.Y* **284**, 1835-1837 (1999).
120. Fitzgerald-Bocarsly, P., Dai, J. & Singh, S. Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine Growth Factor Rev* **19**, 3-19 (2008).

121. Ronnblom, L., Ramstedt, U. & Alm, G.V. Properties of human natural interferon-producing cells stimulated by tumor cell lines. *Eur J Immunol* **13**, 471-476 (1983).
122. Asselin-Paturel, C. *et al.* Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* **2**, 1144-1150 (2001).
123. Bjorck, P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* **98**, 3520-3526 (2001).
124. Kadowaki, N. *et al.* Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* **194**, 863-869 (2001).
125. O'Keeffe, M. *et al.* Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J Exp Med* **196**, 1307-1319 (2002).
126. Shortman, K. & Naik, S.H. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19-30 (2007).
127. Villadangos, J.A. & Schnorrer, P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* **7**, 543-555 (2007).
128. Asselin-Paturel, C., Brizard, G., Pin, J.J., Briere, F. & Trinchieri, G. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* **171**, 6466-6477 (2003).
129. Okada, T. *et al.* Murine thymic plasmacytoid dendritic cells. *Eur J Immunol* **33**, 1012-1019 (2003).
130. Lau, A.H. & Thomson, A.W. Dendritic cells and immune regulation in the liver. *Gut* **52**, 307-314 (2003).
131. Wendland, M. *et al.* CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc Natl Acad Sci U S A* **104**, 6347-6352 (2007).
132. Villadangos, J.A. & Young, L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* **29**, 352-361 (2008).
133. Robinson, S.P. *et al.* Human peripheral blood contains two distinct lineages of dendritic cells. *Eur J Immunol* **29**, 2769-2778 (1999).
134. de Heer, H.J. *et al.* Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* **200**, 89-98 (2004).
135. Ochando, J.C. *et al.* Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* **7**, 652-662 (2006).
136. Manolova, V. *et al.* Nanoparticles target distinct dendritic cell populations according to their size. *Eur J Immunol* **38**, 1404-1413 (2008).

137. Ito, T. *et al.* Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* **204**, 105-115 (2007).
138. Martin, P. *et al.* Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential. *Blood* **100**, 383-390 (2002).
139. Sharma, M.D. *et al.* Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J Clin Invest* **117**, 2570-2582 (2007).
140. Fallarino, F. *et al.* Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *J Immunol* **173**, 3748-3754 (2004).
141. Puccetti, P. & Fallarino, F. Generation of T cell regulatory activity by plasmacytoid dendritic cells and tryptophan catabolism. *Blood Cells Mol Dis* **40**, 101-105 (2008).
142. Mellor, A.L. & Munn, D.H. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* **4**, 762-774 (2004).
143. Ito, T. *et al.* Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* **166**, 2961-2969 (2001).
144. Veron, P., Segura, E., Sugano, G., Amigorena, S. & Thery, C. Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells. *Blood Cells Mol Dis* **35**, 81-88 (2005).
145. Amigorena, S. Anti-tumour immunotherapy using dendritic-cell-derived exosomes. *Research in immunology* **149**, 661-662 (1998).
146. Hsu, D.H. *et al.* Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides. *J Immunother* **26**, 440-450 (2003).
147. Zitvogel, L. *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **4**, 594-600 (1998).
148. Andreola, G. *et al.* Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med* **195**, 1303-1316 (2002).
149. Huber, V. *et al.* Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* **128**, 1796-1804 (2005).
150. Valenti, R. *et al.* Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer research* **66**, 9290-9298 (2006).
151. Taylor, D.D., Akyol, S. & Gercel-Taylor, C. Pregnancy-associated exosomes and their modulation of T cell signaling. *J Immunol* **176**, 1534-1542 (2006).

152. Kim, S.H. *et al.* Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* **174**, 6440-6448 (2005).
153. Admyre, C. *et al.* B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce T(H)2-like cytokines. *J Allergy Clin Immunol* (2007).
154. Pelchen-Matthews, A., Raposo, G. & Marsh, M. Endosomes, exosomes and Trojan viruses. *Trends Microbiol* **12**, 310-316 (2004).
155. Gould, S.J., Booth, A.M. & Hildreth, J.E. The Trojan exosome hypothesis. *Proc Natl Acad Sci U S A* **100**, 10592-10597 (2003).
156. Nguyen, D.G., Booth, A., Gould, S.J. & Hildreth, J.E. Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. *J Biol Chem* **278**, 52347-52354 (2003).
157. Kramer, B. *et al.* HIV interaction with endosomes in macrophages and dendritic cells. *Blood Cells Mol Dis* **35**, 136-142 (2005).
158. Wiley, R.D. & Gummuluru, S. Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection. *Proc Natl Acad Sci U S A* **103**, 738-743 (2006).
159. Fevrier, B. *et al.* Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* **101**, 9683-9688 (2004).
160. Vella, L.J. *et al.* Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *The Journal of pathology* **211**, 582-590 (2007).
161. Vella, L.J., Greenwood, D.L., Cappai, R., Scheerlinck, J.P. & Hill, A.F. Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid. *Veterinary immunology and immunopathology* (2008).