Mucosal immune regulation in intestinal disease

The role of bacterial products, food components and drugs

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Mucosal Immune Regulation in Intestinal Disease

The role of bacterial products, food components and drugs

Regulering van de mucosale afweer bij darmziekten

De rol van bacteriële producten, voedselbestanddelen en medicijnen

(met een samenvatting in het Nederlands)

Proefschrift

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Nothing is a waste of time
if you use the experience wisely.

Auguste Rodin
French Sculptor
(1840-1917)

In herinnering aan mijn vader

Contents

Chapter 1

General Introduction

The intestine represents the largest contact surface between the body and the external environment and is fundamental for the uptake of nutrients and fluids. In addition, the adult intestine is the home to an estimated 10^{14} commensal microbiota that constitute one of the most dense bacterial habitats known in biology (1), which exceeds the quantity of cells (approximately 10^{13} cells) of the human body (2). Furthermore, the intestine is subject to invasion by pathogens, to which the gut-associated lymphoid tissue (GALT) has to mount an appropriate immune response whereas the innocuous food antigens and the commensal flora need to be tolerated. Maintaining intestinal integrity therefore requires a tightly regulated immune homeostasis to keep the balance between tolerance and immunity (3, 4). An imbalance in immune homeostasis contributes to the induction of intestinal diseases such as inflammatory bowel disease (IBD), food allergies and cancer (2, 5, 6).

1. The intestinal immune environment

The gastrointestinal system has several layers of defense against pathogens, consisting of non-specific protective mechanisms (for example gastric acid, antimicrobial peptides, secretory IgA), a physical barrier provided by intestinal epithelial cells and the GALT (7).

A single cell layer of polarized intestinal epithelial cells (IEC) constitute a barrier between the lumen on the apical side and the host connective tissue (lamina propria) at the basolateral side. The IEC are joined firmly together by tight junctions to prevent penetration of luminal content (8, 9). The apical side of the IEC consists of a brush border that hinders the attachment of microorganisms, but contributes to the uptake of nutrients (10). This physical barrier is reinforced by the presence of a continuous layer, composed of heavily glycosylated mucin, secreted by goblet cells (11). In addition to this physical barrier, IEC (and especially paneth cells) secrete antimicrobial peptides such as defensins and calprotectins that directly kill bacteria (12). IEC also contribute to the secretion of IgA from lamina propria B cells into the gut lumen (13). Dimeric complexes of IgA (secretory IgA or sIgA) cross the epithelium and bind to bacteria and antigens hereby removing microorganisms from the gut lumen (14).

The GALT consists of the organized lymphoid organs in the intestine such as Peyer's patches (PP), mesenteric lymph nodes (MLN) and isolated lymphoid follicles (ILF) that are distributed along the whole intestinal tract. Furthermore, immune cells such as dendritic cells (DCs) that samples antigen directly from the lumen (15), and T and B cells can be found throughout the lamina propria. Intestinal epithelial lymphocytes

(IEL) are located within the epithelial cell layer, and comprise mostly T cells (figure 1). The organized lymphoid organs within the intestine form the induction site of the immune response and the immune cells in the lamina propria and the epithelium are predominantly responsible for effector responses within the intestine (16).

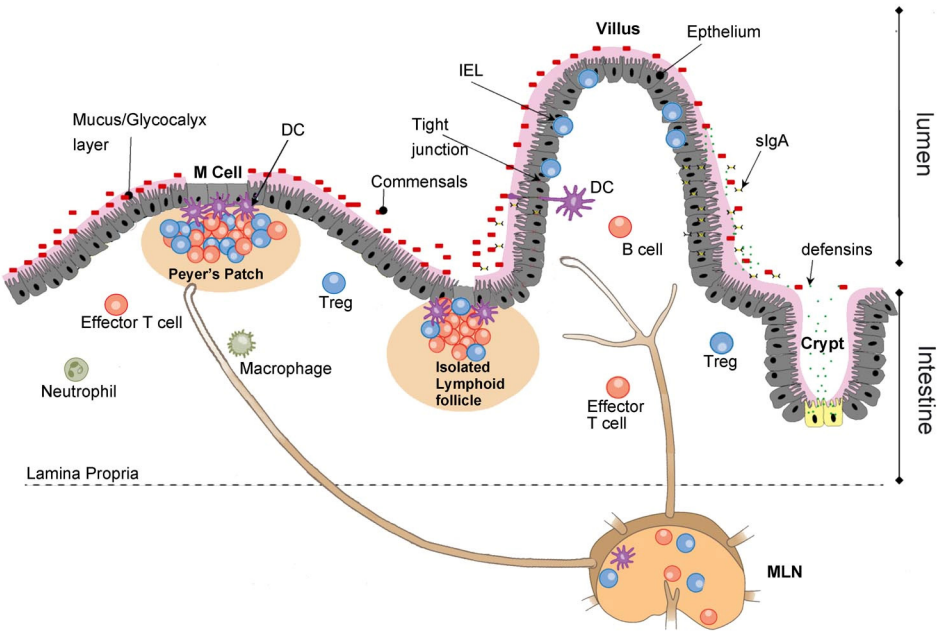

Fig. 1: Intestinal immune environment
The gut luminal content is separated from the lamina propria by a single layer of epithelial cells (IEC) that are joined firmly together by tight junctions. IEC are protected by a mucus layer, antimicrobial peptides (including defensins) and antigen-specific secretory IgA (sIgA). Inductive sites of the intestinal immune system include Peyer's patches (PP), isolated lymphoid follicles (ILF) and mesenteric lymph nodes (MLN). Luminal antigens are transported into PP or ILF via microfold (M) cells for sampling by dendritic cells (DCs). In turn, DCs can initiate immune responses in PP, ILF or MLN. Effector sites of intestinal immune system include the lamina propria and the epithelium. Scattered intestinal lymphocytes in the epithelium (IEL) monitor epithelial damage, while the lamina propria contains large numbers of (effector and regulatory) T cells, IgA-producing plasma B cells, macrophages and DCs. These DCs sample luminal content via cellular extensions, migrate to MLN where they present the antigens to lymphocytes. Activated lymphocytes migrate back to lamina propria as a result of expression of gut-homing receptors. Adapted from Magalhaes (18) and Izcue (5).

The organization of the PP is comparable to that of lymph nodes, with distinct B cell follicles and T cell areas. The lymphoid areas of the PP are separated from the intestinal lumen by the follicle-associated epithelium (FAE). This FAE differs from IEC, and contains specialized cells, called microfold (M) cells. M cells have the capacity to capture soluble antigens, apoptotic epithelial cells or bacteria from the gut lumen, and transport them to the sub-epithelial dome (SED) of PP for sampling by DCs (16-18). From there the DCs can move to the T cell areas and/or B cell follicles, where they prime naive lymphocytes. The lymphocytes which are primed in the PP migrate to the MLN and undergo further differentiation. As a result of the expression of gut homing receptors, they migrate back to the lamina propria. B cell blasts mature in IgA-producing plasma cells, whereas T cells in the lamina propria are of particular importance to local immune regulation. Besides PP, immune responses in the small and large intestine can be initiated in ILF. The formation, structure and function of ILF seem to be comparable to those of PPs, but their appearance and size highly depends on the presence of commensal bacteria (19).
In addition to uptake of luminal content via M cells as described above, lamina propria DCs can directly sample luminal contents by extensions that reach between epithelial cells into the intestinal lumen (15). Soluble antigen may also enter the lamina propria via trans- or para-epithelial transport, followed by antigen uptake by lamina propria DCs. Subsequently, these DCs migrate to the MLN to present antigens to naive lymphocytes (17). The constant interaction between luminal content and the GALT contribute to the maintenance of intestinal homeostasis (20, 21).

2. Immune regulation in the intestine

2.1 IEC in intestinal immune activation and regulation

Intestinal DCs are involved both in the maintenance of tolerance towards commensal flora, and in the generation of protective immune responses towards pathogens (22). The usual response to harmless gut antigens is the induction of local and systemic immunological tolerance, known as oral tolerance. Intestinal $CD103^+$ (integrin αE) DCs, constantly sampling luminal contents under steady state conditions, can imprint naïve T cells in MLN to become forkhead box P3 (FoxP3) expressing regulatory T cells (Tregs). These antigen-specific Tregs contribute to inhibition of immune responses to commensal bacteria and dietary antigens (22). $CD103^+$ DCs in the MLN also drive the expression of gut homing receptors on recently activated T and B cells,

thereby limiting their potential to cause systemic inflammation (23, 24). Furthermore, CD103$^+$ DCs in PP and lamina propria drive IgA B cell differentiation in the absence of T cell signaling. The regulatory imprinting capacity of CD103$^+$ DC appears to be highly dependent on their ability to convert vitamin A through aldehyde dehydrogenase family 1 (ALDH1) in its metabolite retinoic acid (RA) (fig. 2).
The local intestinal micro-environment contributes to formation of "tolerogenic" CD103$^+$ DCs. IEC are producers of transforming growth factor β (TGF-β) and interleukin-10 (IL-10) (1, 20). In addition, IEC are also able of converting vitamin A through ALDH1 in RA (25). Conditioning of mucosal DCs in the presence of these IEC-derived factors results in upregulation of CD103 and expression of ALDH1 in the DCs, thus formation of "tolerogenic" CD103$^+$ DCs (22) (fig. 2).

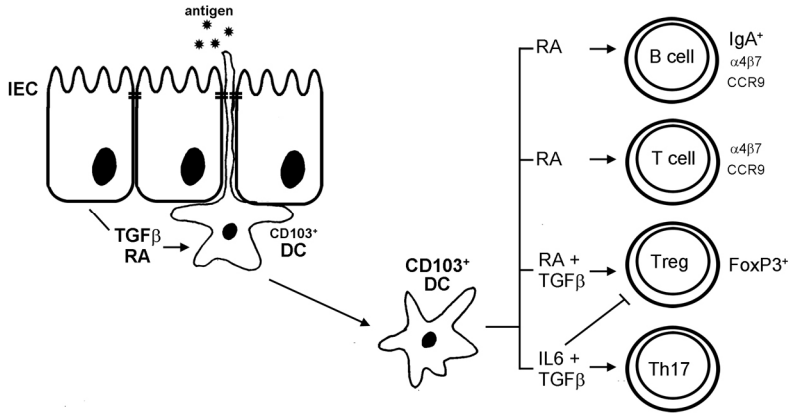

Figure 2. Intestinal DC function in relation to RA, TGF-β and IL-6
In response to commensal bacteria, IEC will produce TGF-β and RA, hereby conditioning CD103$^+$ mucosal DCs to become "tolerogenic" DCs. These CD103$^+$ DCs migrate to MLN and promote the expression of gut homing receptors (α4β7 and CCR9) by lymphocytes, the generation of FoxP3$^+$ Tregs, and class switching to IgA in the presence of RA and TGF-β. In the presence of the inflammatory cytokine IL-6 (and absence of RA) CD103$^+$ DCs will promote the formation of Th17 cells instead of Tregs.

In contrast to signals leading to tolerogenic CD103$^+$ DCs, IEC are able to condition CD11b$^+$ DCs in PP through thymic stromal lymphopoietin (TSLP). In the presence of TSLP, CD11b$^+$ DCs will preferentially promote T helper 2 (Th2)-type responses (26).

The IEC-mediated conditioning of DCs appears highly dependent on their basal activation in response to commensal flora (20). IECs show qualitatively distinct responsiveness to commensal and pathogenic bacteria, and therefore the IEC may act as a sensor for current environmental conditions, and instruct nearby DCs accordingly (26). Similar to pathogenic bacteria, commensal organisms will be detected by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and C-type lectins (27, 28). In contrast, these bacteria do normally not initiate the pathogenic inflammatory response (29). The recognition of commensal bacterial components by PRRs has been shown to be of highly importance for protection against gut injury (27, 28). The nuclear factor-κB (NF-κB) activation as a result of PRR-signaling is, however, dampened by a variety of protective mechanisms, amongst others the induction of peroxisome proliferator-activated receptor-γ (PPARγ) (30) and blocking of polyubiquitination of phosphorylated IκBα (31). PRR-mediated recognition of the normal flora also induces cytoprotective heat shock proteins (HSP)25 and HSP72 in colonic epithelial cells protecting the gut epithelium from nonspecific damage (6). The fermentation of starch by commensal flora into short chain fatty acids such as butyrate also add to protection from detrimental immune activation. It has been shown that butyrate strongly inhibits the production of pro-inflammatory cytokines IL-12 and tumor necrosis factor α (TNFα), and augments the production of regulatory cytokines such as IL-10 (32). Together these processes illustrate the contribution of commensal bacteria to intestinal immune hyporesponsiveness. In addition to commensal bacteria, it has been shown that dietary antigens (proteins) also contribute to maintenance of intestinal homeostasis (33).

In contrast to commensal bacteria, pathogens that are recognized by host PRRs do not dampen the NF-κB-mediated response. Furthermore, pathogens exhibit virulence factors to infect the host (18). As a result of NF-κB activation, IEC will produce IL-8 to attract neutrophils that engulf intruders and produce pro-inflammatory cytokines including TNFα and IL-6. The presence of IL-6 together with TGF-β will promote the $CD103^+$ DC-mediated induction of Th17 cells instead of Tregs (fig. 2), resulting in a protective immune response and pathogen clearance (34). The close relationship between the pathways of Th17 and Treg induction may account for a balanced immune regulation upon infection, thereby limiting the risk for derailed responses.

2.2 Regulatory T cell responses and regulatory cytokines

Next to conditioning of DCs, tolerance to commensal bacteria and harmless food is mediated by various types of regulatory lymphocytes. Effector mechanisms of regulatory lymphocytes encompass the production of suppressive cytokines such as IL-10 and TGF-β (5). Both cytokines can be produced by a variety of immune cells, including DCs, T cells but also IEC. The anti-inflammatory effect of IL-10 is partly based on its ability to reduce the antigen-presenting capacity of DCs (35). In addition, IL-10 stimulates the induction of non-inflammatory Th2 responses and of regulatory T cells (22). TGF-β is a pleiotropic cytokine that can suppress DC- and T cell responses (36, 37), but also contributes to the maintenance of epithelial barrier integrity (38, 39). Furthermore, TGF-β together with IL-6 is involved in the induction of proinflammatory Th17 cells (34). Next to secretion of regulatory cytokines, T-cell mediated immune suppression also includes the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) (40, 41).

The most investigated regulatory T cell subset in the body comprises $CD4^+CD25^+FoxP3^+$ Tregs. These Tregs can arise in the thymus (naturally occurring Tregs or nTregs) and are able to suppress immune responses via direct cell-cell contact mediated through signaling of CTLA-4 (40, 41). However, production of IL-10 and TGF-β also contribute to their regulatory capacity (5). In addition, the $CD103^+$ DC subset can imprint naive T cells to become antigen-specific Tregs. These inducible Tregs mainly reside in the intestine and their regulatory function depends on the production of TGF-β and IL-10. Feeding oral antigens can also induce $CD4^+$ ($FoxP3^+$) Tregs (Th3 cells) that mainly produce TGF-β.

Although the expression of FoxP3 is associated with regulatory functions, other cell types that lack FoxP3 expression can dampen immune responses as well, and act as regulatory cells. This includes Tr1 cells that produce IL-10 and probably also IFN-γ and may control inflammatory responses to dietary antigens (42). Other cells that have shown to possess regulatory capacities includes CD8 T cells (43), NK(T) cells (44) and γδ T cells (44-47).

γδ T cells are an important source of TGF-β (47), they regulate the restitution of IEC in response to epithelial damage (48) and contribute to the induction of IgA (49).

Together, these various cell subsets contribute to maintenance of intestinal homeostasis, and prevent excessive immune activation in response to pathogens.

2.3 Eicosanoids and immune regulation

A more direct mechanism that contributes to the maintenance of immunological homeostasis in response to innocuous luminal antigens includes cyclooxygenase-2 (COX-2)-dependent polyunsaturated fatty acid (PUFA) metabolites (50). PUFAs in cell membranes act as a substrate for COX enzymes and result in the production of various eicosanoids, including prostaglandins (PG), thromboxanes and leukotrienes (LT). Prostaglandin 2 (PGE_2) is the most prominent metabolite, and possesses both pro-inflammatory and anti-inflammatory capacities (51, 52). However, the panel of eicosanoids that is produced by COX-2 depends on the type of PUFA incorporated in cell membranes. There are two principal classes of PUFAs: the *n-6* and the *n-3* family (fig. 3) and these are not interconvertible in animals (53). The difference between *n-3* and *n-6* PUFA-derived eicosanoids is that most of the mediators formed from n-3 PUFAs are anti-inflammatory, whereas those formed from n-6 PUFAs (including PGE_2) are predominantly pro-inflammatory or show other disease-propagating effects (51) (fig. 3). Modulation of eicosanoid production can therefore contribute to maintenance of intestinal homeostasis.

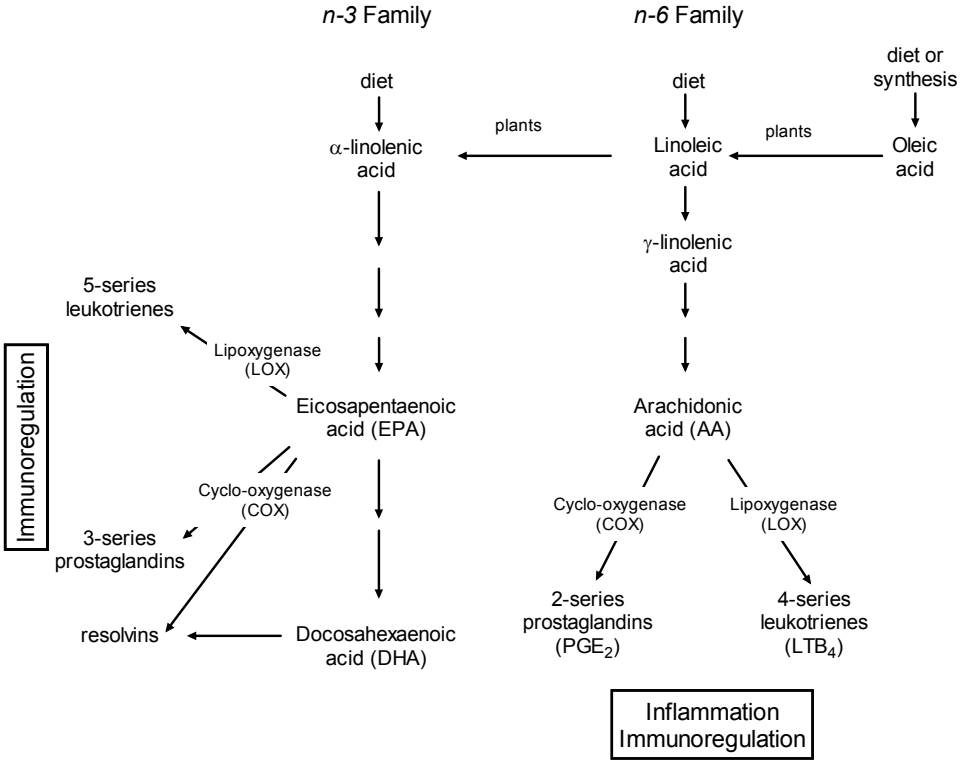

Fig.3: Schematic overview of polyunsaturated fatty acid (PUFA) metabolism
The essential *n-6* polyunsaturated fatty acid (PUFA) linoleic acid is the precursor of the membrane phospholipid arachidonic acid (AA). Conversion of AA by cyclo-oxygenase (COX) and lipoxygenase (LOX) enzymes will result in prostaglandins and leukotrienes that contribute to inflammation or immunoregulation. Metabolism of the essential *n-3* PUFA α-linolenic acid will result in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in cell membranes. Conversion by COX- en LOX-enzymes will result in the production of immunoregulatory mediators. Adapted from Calder (53).

2.4 Nonspecific regulation

In addition to restricting innate immune responses to commensal bacteria, host cells can actively modulate the inflammatory response by modifying the proinflammatory microbial molecules themselves. For example, the enzyme alkaline phosphatase (AP) can detoxify lipopolysaccharide (LPS) by dephosphorylation of the lipid A moiety being a 100-fold less toxic than the unmodified LPS (54). Intestinal AP (iAP) is expressed on apical surfaces of microvillus membranes of IEC and is secreted at low

levels into intestinal lumen (55). The expression of iAP responds to dietary intake and also coincides with the establishment of the normal flora (56). A study in zebrafish demonstrated that iAP is an essential factor for maintaining proper gut homeostasis (57). Administration of iAP has been shown to possess a therapeutic effect in several LPS-mediated diseases, including sepsis and lung toxicity (58-60).

3 Intestinal inflammation

Disturbance in intestinal homeostasis may result in host inflammatory responses to the commensal bacteria and lead to chronic intestinal inflammation seen in inflammatory bowel disease (IBD) (61). Two main types of IBD exist: Crohn's disease (CD) and ulcerative colitis (UC) (62) in which patients suffer from chronic diarrhea and weight loss, abdominal pain, fever, and fatigue. Both CD and UC are characterized by mucosal ulcerations, which is patchy in CD but continuous in UC. In CD, ulcers penetrate into the gut wall, and fistulous tracts may develop between loops of bowel or to the skin (6). IBD is a chronic condition without a medical cure, which commonly requires a lifetime of care. It can cause significant morbidity, but most patients are able to live normal productive lives. Over a longer time period 75% of patient with CD and 25 to 30% of those with UC will require surgery (63).

The disturbance in intestinal homeostasis which is underlying IBD can be the result of disruption of the intestinal epithelial barrier. This exposes the intestinal immune system to a increased amount of proinflammatory stimuli that overrule intestinal regulation. Another reason for disturbed homeostasis can be impaired regulation, in which the normal immune response to commensal bacteria ends in a chronic inflammation. Other factors that can contribute to loss in intestinal homeostasis comprise genetic defects and immunodeficiencies (5).

Irrespective of the trigger, neutrophils are early responders to injury (63). TLR-signaling on the basolateral side of IEC resulting in the production of IL-8 contributes to this neutrophil influx. Neutrophils release antimicrobial peptides and reactive oxygen intermediates that may cause further tissue damage. Neutrophils also produce high amounts of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 resulting in macrophage and DC recruitment. In addition to this innate response, increased microbial encounter also activate intestinal DCs (via PRRs) to produce high amounts of IL-23 (and/or IL-12). In addition, IL-23 stimulates IEC and DCs to produce pro-inflammatory cytokines and stimulates the induction of Th1 and Th17 T cells. The presence of IL-6 together with TGF-β also induces Th17 cells. Th17-derived cytokines

including IL-17 will further enhance production of pro-inflammatory cytokines, whereas Th1-derived IFN-γ will enhance intestinal permeability. Together, this leads to a vicious circle of intestinal inflammation (64) (fig. 4).

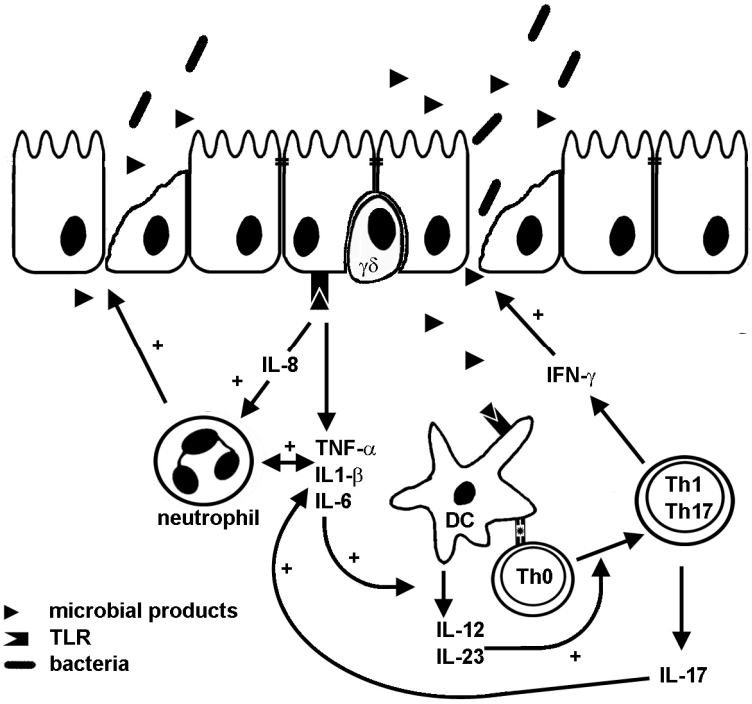

Figure 4: Induction of intestinal inflammation
Disturbance of intestinal homeostasis will result in epithelial injury and activation of pattern recognition receptors including Toll-like receptors (TLR) in intestinal tissue. As a result, IEC-derived IL-8 will attract neutrophils to the intestine which in turn will produce high amounts of the proinflammatory cytokines TNF-α, IL-1β and IL-6. In response to TLR-signaling and in the presence of pro-inflammatory cytokines, intestinal DCs start to produce high amounts of IL-12 and/or IL-23. Naive T cells will subsequently differentiate in Th1 / Th17 cells, respectively producing high amounts of IFN-γ and IL-17. IFN-γ further enhances intestinal permeability, whereas IL-17 further enhances pro-inflammatory cytokine production, together leading to a vicious circle of intestinal inflammation.

4 Food hypersensitivity responses

Responses to ingested food proteins normally result in the induction of oral tolerance. Conceivably, a breakdown in oral tolerance mechanisms or a failure to induce oral tolerance induction will result in an adverse reaction to food. Adverse reactions to food

can be divided in toxic and non-toxic reactions. Toxic reactions are inherent to the food and occur in every individual when eating an appropriate amount. Non-toxic reactions only occur in susceptible individuals and include non-immune mediated reactions (food intolerance) and immune mediated reactions (food allergy/hypersensitivity). The majority of food hypersensitivity responses are IgE-mediated reactions, also referred to as type I food allergy (65).

In type I food allergy, antigen (food protein) uptake across the epithelial barrier is followed by an allergic cascade. Antigen will be processed by DCs, and presented to naive T cells (fig. 5). In combination with costimulatory molecules such as Jagged-2 and OX40-L and the presence of IL-4, IL-10 and/or TSLP, naive T cells differentiate into Th2 cells. As a result, Th2 cytokines including IL-4, IL-5 and IL-13 will be produced by these cells, which results in activation of B cells that preferentially produce antigen-specific IgE. Secreted IgE is distributed systemically and binds to mast cells and basophils. Upon re-exposure to the antigen, cross-linking of the IgE on the cell surface of mast cells induces degranulation and release of mast cell mediators. Mast cell mediators include histamine, cytokines and proteases, which are able to elicit an anaphylactic reaction (66).

In the Western world, food allergy is reported in 1-4% of the whole population for any food, of which 0-3% is allergic to milk and 0.2-1.6 % to peanut. The prevalence of food allergy is highest in the first years of life, and up to 8% of children younger than 4 years of age experience food allergic reactions. The incidence of food allergies have increased during the second half of the twentieth century, but a plateau may now have been reached (67, 68).

Remarkably, a small number of food products account for 90% of all food allergic reactions, and include cow's milk, eggs, peanuts, tree nuts, soy, wheat and (shell)fish (69). Food allergies to peanut, tree nuts and (shell)fish are not easily outgrown. In case of peanut allergy, accidental ingestion of even minute quantities of peanut can immediately provoke life-threatening symptoms, so patients must maintain strict avoidance. However, owing to the frequent use of peanut-based products in common foods, complete avoidance is difficult and accidental ingestions are frequent. The risk of accidental ingestion leads to significant psychological distress in individuals diagnosed for food allergy (70).

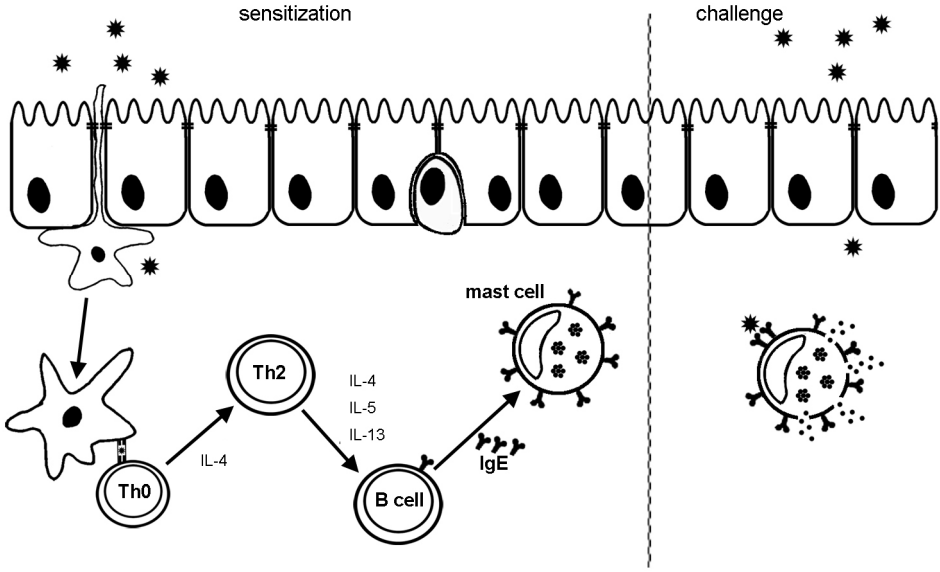

Figure 5: Induction of type I food allergic responses
During sensitization allergens cross the epithelial barrier and are processed by DCs. The cytokine milieu together with costimulatory molecules on DCs will result in differentiation of naive T cells into Th2 cells. Th2 derived IL-4, IL-5 and IL-13 contribute to the activation of B cells that preferentially produce antigen-specific IgE which in turn will bind to mast cells. Upon challenge with allergens, the membrane-bound IgE are cross-linked by interaction with the antigen. Cross-linking results in degranulation and release of mast cell mediators and in clinical symptoms.

5 Experimental models for intestinal inflammation and food allergy

Animal models are widely used to gain more insight in the complex mechanisms underlying the disturbance of intestinal homeostasis resulting in IBD and food allergy, and for identifying therapeutic strategies. In the present thesis, two different mouse models to mimic intestinal inflammation are used: dextran sulfate sodium (DSS)-induced colitis and trinitrobenzene sulfonic acid (TNBS)-induced colitis. Both models are based on disruption of the intestinal barrier, resulting in increased exposure to commensal bacteria, and are characterized by influx of neutrophils in the intestine, accompanied by oedema formation, crypt loss and loss in goblet cells. The inflammation in TNBS colitis depends on the presence of effector (CD4) T cells in contrast to DSS colitis which is T cell independent. Both models induce an acute intestinal inflammation that resolves within days after last exposure to the inducing

compound (e.g. DSS or TNBS), and are especially suitable to investigate early immune responses to intestinal damage.
To study IgE-mediated food allergy a mouse model for hypersensitivity responses to peanut was used (71). Mice are exposed to repeated doses of peanut extract (PE) in the presence of the mucosal adjuvant cholera toxin (CT) which results in the induction of PE-specific IgE in serum and mast cell degranulation upon oral challenge. This model facilitates to investigate factors that modulate allergic sensitization. In addition, in some studies described in this thesis an oral tolerance peanut model is used that enables investigation of factors that contribute to abrogation of oral tolerance to food products.

6 Thesis outline

All studies described in this thesis are performed to gain more insight in the role of environmental factors (bacterial products, food components, drugs) in intestinal immune regulatory processes contributing to intestinal inflammation and food allergy.

The first part of the thesis describes the effects of bacterial products on intestinal inflammation. Bacteria and bacterial products like LPS may contribute to the induction and/or maintenance of intestinal inflammation. **Chapter 2** investigates whether treatment with intestinal alkaline phosphatase (iAP), a natural occurring enzyme that is able to detoxify LPS, has therapeutic value to overcome epithelial damage of the intestine as a result of colonic inflammation. This study was performed in a mouse model using DSS to induce intestinal epithelial damage.
In contrast to LPS, bacterial (and endogenous) heat-shock proteins (HSP) are thought to improve regulatory function of immune cells. The studies described in **chapter 3** investigate whether administration of bacterial HSP70 protects against the induction of experimental DSS or TNBS colitis.
The next part of this thesis focuses on sensitization to food proteins. Animal models designed to study the sensitization to food proteins need a mucosal adjuvant to induce specific responses. **Chapter 4** describes the changes in DC and γδ T cell populations during establishment of allergic sensitization. The role of γδ T cells in sensitization to PE is investigated in **chapter 5**, by means of blocking the γδ-TCR using a specific antibody, and the use of transgenic mice, devoid of functional γδ T cells.
The last part of the thesis focuses on environmental factors that can modulate sensitization to food proteins. The effect of the non-steroidal anti-inflammatory drug (NSAID) diclofenac on sensitization to PE is studied in **chapter 6. Chapter 7**

describes the modulation of sensitization to PE via intervention with polyunsaturated fatty acid-enriched diets.
Finally the studies described in this thesis are summarized and discussed in **chapter 8.**

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Chapter 2

Intestinal alkaline phosphatase contributes to the reduction of severe intestinal epithelial damage

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Abstract

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the intestine and is accompanied by damage of the epithelial lining and by undesired immune responses towards enteric bacteria. It has been demonstrated that intestinal alkaline phosphatase (iAP) protects against the induction of inflammation, possibly due to dephosphorylation of LPS. The present study investigated the therapeutic potential of iAP in intestinal inflammation and epithelial damage.

Intestinal epithelial damage was induced in C57BL/6 mice using DSS and iAP was administered 4 days after initial DSS exposure. Loss in body weight was significantly less in iAP-treated mice and accompanied with reduced colon damage (determined by combination of crypt loss, loss of goblet cells, oedema and infiltrations of neutrophils). Treatment with iAP was more effective in case of severe inflammation compared to situations of mild to moderate inflammation. Rectal administration of LPS into a moderate inflamed colon did not aggravate inflammation. Furthermore, soluble iAP did not lower LPS-induced NF-κB activation in epithelial cells *in vitro* but induction of cellular AP expression by butyrate resulted in decreased LPS response.

In conclusion, the present study shows that oral iAP administration has beneficial effects in situations of severe intestinal epithelial damage, whereas in moderate inflammation endogenous iAP is sufficient to counteract disease-aggravating effects of LPS. An approach including iAP treatment holds a therapeutic promise in case of severe IBD.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial diseases characterized by an excessive intestinal inflammation and disruption of the intestinal epithelial lining. Evidence is growing that activation of mucosal immune responses by commensal enteric bacteria is an important factor in the etiology of IBD (1-3). In line with this, certain genetic polymorphisms related to microbial responses, including those of TLR4, have been associated to susceptibility to IBD (4). Conceivably, lipopolysaccharide (LPS) which binds to TLR4 may be involved in aggravating IBD. Methods to detoxify LPS may therefore be an interesting therapeutic venue in IBD. Interestingly, it has been shown that the enzyme alkaline phosphatase (AP) can detoxify LPS by dephosphorylation of the lipid A moiety (5, 6).

AP is a naturally occurring enzyme with ubiquitous distribution among cell types and tissues. The intestinal isoform of AP, intestinal AP (iAP) (7), is expressed in high amounts in the intestinal lining (8, 9) and is considered a differentiation marker of intestinal epithelial cells (IEC)(10-12). iAP expression by differentiated IEC is inhibited in UC patients possibly by pro-inflammatory cytokines like TNF-α which are abundantly present in inflamed tissue (9, 13). iAP appears essential in controlling responses towards intestinal flora and maintaining epithelial integrity, and loss of iAP expression increases susceptibility to inflammation and sepsis (8, 14, 15).

Previous studies have demonstrated that in addition to homeostatic and disease-protective effects iAP administration may also have therapeutic effects in several LPS-mediated diseases, including sepsis and lung toxicity (16-19). Recently, it has been demonstrated that administration of AP protects against the induction of DSS-mediated intestinal epithelial damage and inflammation in rats, when given prior to the DSS insult (9). In the present study, we investigated the effect of administration of iAP in mice after initiation of disruption of intestinal epithelial lining by DSS. Results show beneficial effects of iAP administration, but only in cases of severe intestinal injury and inflammation. Additional *in vitro* data suggest that in mild cases of epithelial damage, endogenous AP may suffice to protect against further disease aggravation by LPS.

Materials and Methods

Mice and reagents

Twelve-week-old female specific pathogen-free C57BL/6J mice purchased from Charles River (France) were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum.* The experiments were approved by the Animal Experiments Committee of the Utrecht University.

Clinical grade bovine intestinal AP (iAP) from Biozyme (Blaenavon, United Kingdom) was obtained through AM-Pharma (Bunnik, The Netherlands). Other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Induction of intestinal epithelial damage and treatment with iAP and LPS

Mice were divided in treatment groups of 5 to 10 animals and body weight, stool-consistency and behaviour were recorded daily during the experiment. To induce acute intestinal epithelial damage, C57BL/6 mice were provided with drinking water containing 2% (w/v) DSS (MP Biomedicals, Aurora, OH; MW 35-50 kD) for 5 consecutive days, followed by normal drinking water until they were sacrificed (day 12). No differences in water consumption between treatment groups were observed.

Oral treatment with iAP started 4 days after start of DSS exposure and was continued until mice were sacrificed. Treatment was performed by daily intra-gastric gavage of 100 U of iAP in Tris-HCl buffer, pH 7.2 in a 100 µl bolus. Vehicle-treated animals received 100 µl of Tris-HCl buffer alone.

In case of LPS exposure mice received 100µl LPS (O111:B4, Sigma; 100 µg as low dose or 500 µg as high dose) dissolved in PBS via rectal enema under isofluran/O_2 anaesthesia four days after initial DSS exposure and dosing was repeated every other day (4 times in total).

Evaluation of intestinal inflammation

After mice were sacrificed the colon was dissected from anus to caecum. The colon length was recorded and subsequently the faeces were carefully removed. Colon was weighed and then cut longitudinally, and the full length of half of the colon was fixed in phosphate-buffered formalin and embedded in paraffin. Routine 5 µm sections were

prepared and stained with haematoxylin-eosin (HE) and evaluated in a blind coded fashion by two independent investigators (MBS and RB).
Histological structural alterations were assessed by considering following parameters scored on a 0-3 scale (0=no alterations; 1= mild mucosal alterations in less than 1/3 of the colon; 2= moderate mucosal and submucosal alterations involving up to half of the colon; 3 = severe alterations in more than half of the colon): oedema; crypt loss; loss of goblet cells and leukocyte infiltrations. The histological score was calculated as the sum of the scores for each category and resulted in a maximum score of 12 in the most severe situation. Evaluation of independent investigators were averaged and resulted in a histological score for each mouse.

Determination of disease grade

Disease severity in individual animals was ranked from 0 to 4 based on maximum body weight loss, effect on colon length and colon weight, histological score, myeloperoxidase (MPO) activity and TNFα release from distal colon samples (Table 1). Scores per parameter were averaged resulting in a disease grade for every individual mouse.

Table 1: Ranking of disease parameters

grade	**maximum body weight loss** (%)	**colon length** (cm)	**relative colon weight** (mg/g BW)	**histological score** (hist. score)	**TNF-α release** (ng /g colon)	**MPO activity** (*10^3 U / g colon)
0	< 2	> 8	< 8.5	< 1	< 1.5	< 1.5
1	2 - 5	7 - 8	8.5 - 10	1 - 3	1.5 - 3	1.5 - 3
2	5 - 10	6.5 - 7	10 - 12	3 - 6	3 - 6	3 - 6
3	10 -15	6 – 6.5	12 – 14	6 - 9	6- 10	6- 10
4	> 15	< 6	> 14	> 9	> 10	> 10

Myeloperoxidase (MPO) activity

A part of the dissected colon of 20 to 40 mg (exact weight was recorded) was snap-frozen in liquid nitrogen and stored at -20 °C. Tissue was thawed and homogenized by means of a Braun Potter in 500 µl of ice-cold Tris-HCl lysis buffer (15 mM), pH 7.4, containing 300 mM NaCl, 2 mM $MgCl_2$, 1% triton X100 and 1 µg/ml of the protease inhibitors leupeptin A and aprotinine. Homogenate was centrifuged for 7 min at 700xg, supernatant was taken and centrifuged for another 10 min. at 12000xg after which 10 µl of the supernatant was diluted 5 times in 0.5% hexadecyltrimethylammonium bromide (HETAB) in 50 mM potassium phosphate buffer pH 6.0. MPO activity was determined by adding 150 µl ready to use 3,3',5,5'-tetramethylbenzidine substrate (TMB) and increase in absorbance at 630 nm was recorded for at least 15 minutes. One unit of MPO activity was defined as the amount of enzyme causing an increase in absorbance of 0.001 per 5 min. The result was normalized for colon tissue weight.

Colon culture and cytokine analysis

A distal sample of the dissected colon of 30 to 50 mg (exact weight was recorded), was washed three times in ice-cold PBS, containing 40 mg/ml glucose, 100 IU/ml penicillin-streptomycin and 250 µg/ml fungizone and incubated in 0.5 ml complete RPMI1640 (Invitrogen Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Greiner Bio-one, Germany) for 24h at 37 °C, 5% CO_2. Supernatant was collected and stored at -20 °C until analysis was performed.
IL-12 and IL-1β were determined by sandwich ELISA. IL-12 capture and biotin antibodies were obtained from BD Pharmingen (Erembodegem, Belgium), IL-1β antibodies were from R&D (Minneapolis, MN). Highbond plates (Costar 3590) were coated overnight at 4 °C with 2 µg/ml rat anti-mouse IL-1β or rat anti-mouse IL-12 p40/p70 in PBS, washed with PBS-Tween 20 and blocked with PBS-Tween 20/1% BSA for 4h at room temperature. Samples, IL-1β and IL-12 standards were added in several dilutions and incubated overnight at 4 °C. After washing, plates were incubated with 0.25 µg/ml goat anti-mouse IL-1β conjugate or 0.5 µg/ml rat anti-mouse IL-12 conjugate diluted in PBS-Tween 20/BSA for 1h at room temperature. Plates were washed and incubated with streptavidin-HRP (0.1 µg/ml, Sanquin, Amsterdam, The Netherlands) diluted in PBS-Tween 20/BSA for 45 min at room temperature. After the final washes, TMB substrate (0.1 mg/ml) was added and the color reaction was stopped after 15 minutes with 2M H_2SO_4. Absorbance was measured at 450 nm. IL-10 (BD Pharmingen) and TNF-α ELISA (Biosource, Nivelles, Belgium) were performed

according to the instructions of the manufacturer. Cytokine levels were normalized for weight of colon tissue used in culture.

Cell culture

The murine small intestinal cell line m-ICc12, stably transfected with a NF-κB luciferase reporter construct, was a kind gift from Dr. A. Vandewalle (INSERM, Paris, France (20, 21)). m-ICc12 cells were cultured in DMEM/F12 (1:1 v/v), supplemented with 100 IU/ml penicillin/streptomycin, 60 nM selenium, 5 g/ml bovine transferrine, 2 mM glutamine, 5 g/ml bovine insulin, 2 g/l glucose, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 2% FCS, 50 µg/ml hygromycin and 15 mM HEPES. Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

AP activity

m-ICc12 cells were grown to confluence in 75 cm^2 flask and incubated with sodium butyrate for 48h. To quantify cell numbers, images were taken at 6 different spots by means of an inverted microscope (Olympus CKX41 with a DP-20 camera) and cells were counted using imaging software (Olympus). Cells were rinsed tree times with 0.9% NaCl and harvested in 2.5 mM glycine buffer (pH=9.6) with a rubber policeman. Cell lysates were mixed with 2.4 mg/ml p-nitrophenylphosphate in glycine buffer, supplemented with 8.5 mM MgCl2, and increase in absorbance at 405 nm was recorded for 1h. One unit of AP activity was defined as the amount of enzyme causing the hydrolysis of 1 µM of p-nitrophenylphosphate per min (pH=9.6; 25 °C). The result was normalized for the amount of cells.

NF-κB activation and MIP-2 release

m-ICc12 cells were grown to confluence in 96-wells plates (Greiner Bio-one, Germany) and subsequently incubated with different amounts of iAP for 30 min, or with sodium butyrate for 48h. LPS (0.1 – 10 ng/ml; O111:B4, Sigma) or mouse IL-1β (0.1 – 1 ng/ml; e-Bioscience, San Diego, USA) was added, and cells were cultured for another 4h. Culture supernatant was harvested and stored at -20 ° until determination of MIP-2. Cells were washed once with PBS, and lysed with 20 µl lysis buffer (Promega, Madison WI, USA). Luciferase activity was recorded by means of a luminometer (LUMIstar OPTIMA, BMG Labtech) using a luciferase assay kit (BioThema, Sweden). MIP-2 ELISA (KOMA Biotech., Seoul, Korea) was performed according the instructions of the manufacturer.

Statistics

Data from animal studies are presented as the group mean ± standard error of the mean (SEM) and analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). A Kruskal-Wallis test was performed for all groups together. When appropriate, Mann-Whitney tests were performed to determine differences between two groups. Differences were considered significant when p values were < 0.05. Data from in vitro tests are presented as mean ± standard deviation (SD) and analysed using GraphPad Prism software. Two-way ANOVA was performed followed by Bonferroni post-hoc test. Differences were considered significant when p values were < 0.05.

Results

iAP treatment ameliorated DSS-induced intestinal epithelial damage.

Previously, it has been demonstrated that iAP can reduce the severity of DSS-induced inflammation in rats. In that particular study iAP was administered during induction of intestinal epithelial damage (9). To investigate the therapeutic effect of iAP in intestinal inflammation, C57BL/6 mice were first exposed to DSS for 5 days, and treated with iAP from day 4 onward. Body weight loss was observed from day 4 and reached maximum at day 8 (fig. 1a). Observed changes in stool consistency (loose stool or even absence of stool from day 5 to 12; data not shown) confirmed the induction of colon inflammation. Significant reduction of colon length (fig 1b) accompanied with markedly increased colon weight (fig 1c) was observed in DSS-exposed mice in comparison to mice that received normal drinking water (healthy subjects: colon length 8.2 ± 0.4 cm; relative colon weight 8.6 ± 0.7 mg/g BW, data not shown). These data were indicative of colon inflammation.

Oral administration of iAP was found to diminish DSS-induced body weight loss and effects on colon length and weight (fig 1 b-c). In addition, histological scoring (fig. 1d-h) confirmed the beneficial effect of iAP administration although MPO activity in colon homogenates (fig 1i), serving as a marker for activity of neutrophils, was not reduced. Of the cytokines evaluated only TNF-α was significantly reduced (fig 1k). Importantly, water intake was not different between vehicle-treated and iAP-treated groups (data not shown), indicating that the exposure to DSS was comparable between groups.

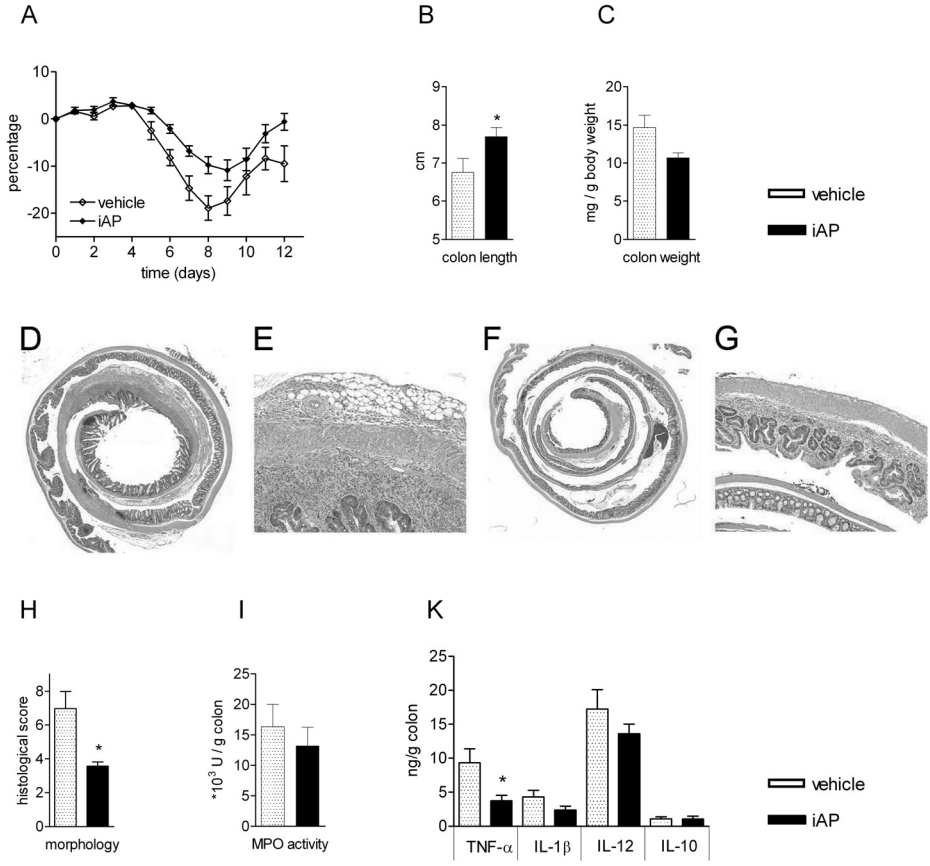

Figure 1: Treatment with iAP after DSS-induced intestinal damage.
DSS was administered via drinking water during 5 days, followed by normal drinking water. iAP was administered daily from day 4 and continued until mice were sacrificed. **B-K:** data obtained on day 12; group size: n=6 (vehicle) and n=10 (iAP) **A.** body weight changes during experimental procedure. **B.** colon length. **C.** relative colon weight. **D-G:** H-E stained paraffin sections from vehicle-treated (**D-E**) or iAP-treated (**F-G**) colon. Magnitude x40 (**D; F**) and x200 (**E;G***)*. Color images on page 166. **H.** changes in colon morphology, scored as described in materials and methods **I.** MPO activity in colon homogenate. **K.** cytokine levels in supernatant of cultured distal colon sample. Data are represented as mean ± SEM. * $p<0.05$ significantly different from vehicle-treated group.

Beneficial effects of iAP were more pronounced in situations of severe inflammation.
In the described experiment DSS exposure resulted in severe intestinal epithelial damage and a clear effect of iAP treatment was found on colon inflammation. The experiment was repeated several times and overall results indicated that the beneficial effects of iAP were most obvious in cases with severe epithelial damage. To assess the influence of disease severity we calculated a disease grade for every individual animal (95 in total used in 6 experiments and of which 44 were vehicle-treated and 51 were iAP-treated) based on maximum body weight loss, colon length and weight, histological changes, MPO activity and TNFα release in the colon. By ranking disease severity in this way effects on both clinical appearance and colon inflammation are taken into account. In the different experiments performed individual mice clearly displayed a wide range of disease severity after DSS exposure (fig 2), but more importantly, it is apparent that mice treated with iAP do not reach disease scores as high as vehicle-treated mice, confirming that iAP is most potent in case of severe inflammation. Also when disease scores were evaluated per experiment the beneficial effects of iAP were most distinct in those experiments in which intestinal epithelial damage was most severe (fig 2b).

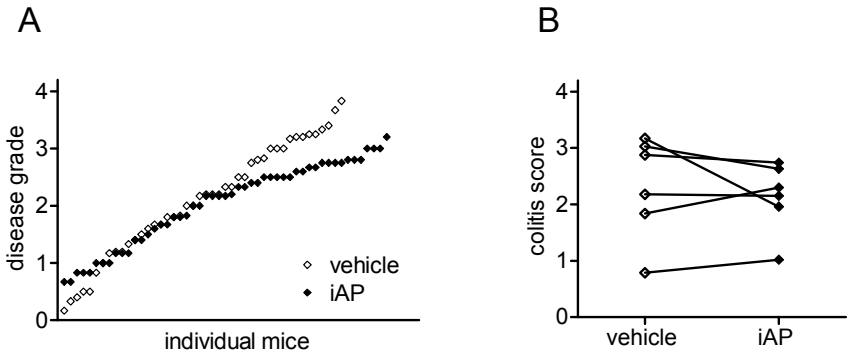

Figure 2: iAP effect in relation to severity of intestinal inflammation.
Disease grading is defined as described in materials and methods. **A.** each dot represents one single animal, open symbols: vehicle treatment, closed symbols: iAP treatment.
B. each line represents one separate study. Symbols represent average disease grade per treatment group (n=6 to 10) within one study.

Rectal LPS administration did not aggravate DSS-induced intestinal epithelial inflammation.

In systemic diseases such as sepsis the beneficial effect of iAP was attributed to its capacity to detoxify LPS (6). To investigate the direct influence of LPS on DSS-induced intestinal epithelial damage, LPS was administered rectally to mice which were already exposed to DSS for four days. DSS exposure was continued for 1 day, after which animals received normal drinking water. Mice received 100 µg or 500 µg LPS via a rectal enema 4 times every other day starting on day 4 of DSS exposure. These doses are in the range of the total amount of LPS present within the lumen in case of gram-negative bacterial infection (22). The severity of disease induced in this experiment was moderate (disease score of 1.90 ± 0.12). As shown in fig 3a, LPS administration did not significantly alter DSS-induced body weight loss. In addition, no effects were found on colon length, weight or on morphology of the colon after LPS administration (fig 3b-d). Furthermore, LPS treatment did not significantly alter TNF-α and IL-1β release from distal colon samples (fig 3e). Data shows that local LPS administration does not substantially modulate a moderate colonic inflammation.

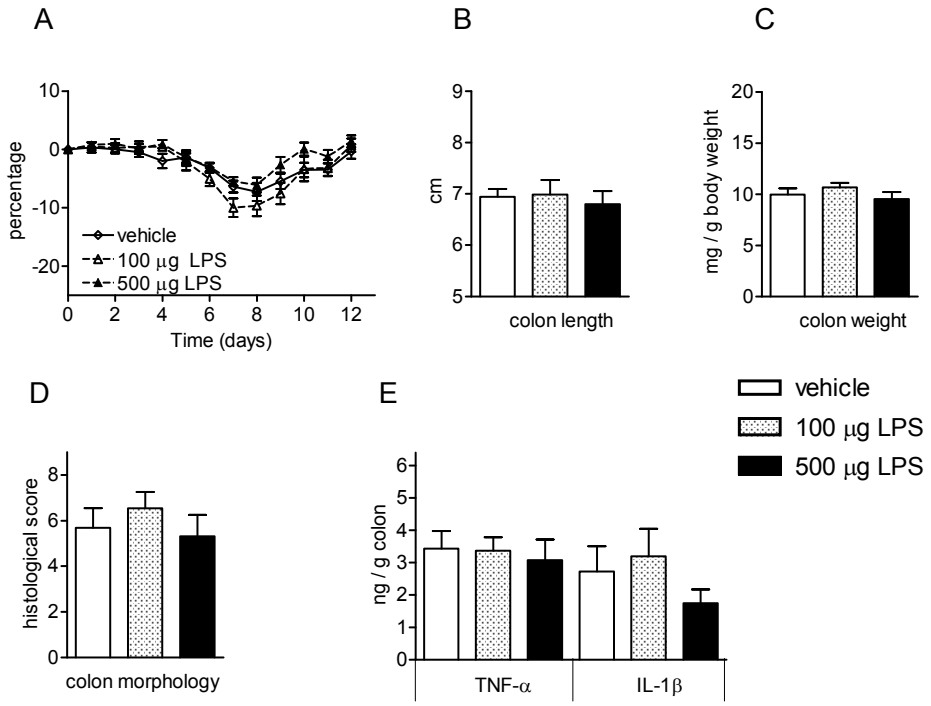

Figure 3: Effect of rectal LPS administration on intestinal inflammation
DSS was administered via drinking water during 5 days, followed by normal drinking water. iAP was administered daily from day 4 and continued until mice were sacrificed. **B-E:** data obtained on day 12, group size n=9 (vehicle and 100 µg/ml LPS), n=7 (500 µg/ml LPS). **A.** body weight changes during experimental procedure. **B.** colon length. **C.** relative colon weight. **D.** changes in colon morphology, scored as described in materials and methods. **E.** cytokine in supernatant of cultured distal colon sample. Data are represented as mean ± SEM.

iAP had no effect on LPS-induced NF-κB activation and MIP-2 release in intestinal epithelial cells.

Next, we investigated whether iAP could directly modulate LPS-induced epithelial cell responses. Mouse intestinal epithelial cells (m-ICc12), stably transfected with a NF-κB-luciferase reporter construct, were incubated with iAP for 30 min, after which various amounts of LPS and IL-1β were added. NF-κB activation and MIP-2 release were analyzed after 4h of stimulation. Both LPS and IL-1β were able to stimulate m-ICc12 cells to activate NF-κB and to release MIP-2. iAP was not able to decrease LPS- or IL-1β-induced NF-κB activation (fig. 4a-b) or MIP-2 release (fig. 4c).

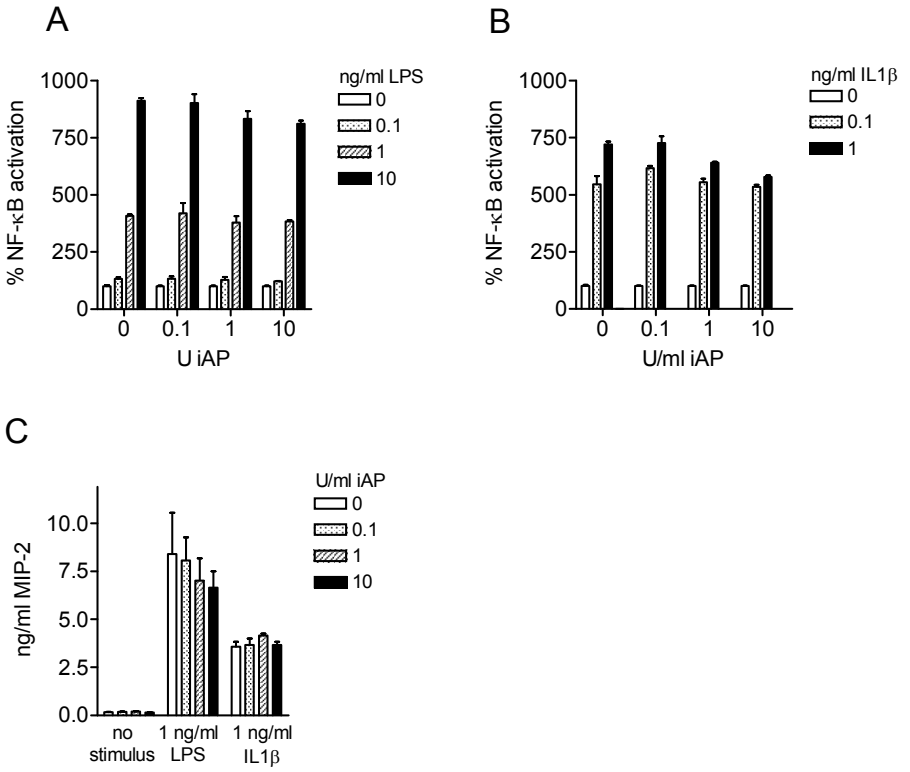

Figure 4: Effect of iAP addition on NF-κB activation and MIP-2 release in m-ICc12 cells *in vitro*. m-ICc12 cells were cultured to confluence, incubated with indicated doses of iAP for 30 min, and subsequently stimulated with LPS or IL-1β for 4h. **A-B:** NF-κB activation is measured using a luciferase reporter construct and expressed as percentage of control (no stimulus). **C.** MIP-2 is measured in culture supernatant using ELISA. Data are presented as mean ± SD from a triplicate from one representative experiment out of three.

Culturing intestinal epithelial cells in the presence of butyrate resulted in inhibition of LPS-induced NF-κB activation.

Addition of sodium butyrate to intestinal epithelial cells has been shown to induce cell differentiation accompanied by up-regulation of intracellular AP activity in several intestinal epithelial cell-lines (10) Therefore, we studied the effect of sodium butyrate on LPS-induced NF-κB activation by m-ICc12 cells. Addition of sodium butyrate to m-ICc12 cells resulted in immediate inhibition of cell proliferation and induced

morphological changes in the cultured cells (fig 5a) and an increase in AP activity (fig 5b). In the presence of sodium butyrate less NF-κB activation was induced by LPS (fig. 5c). In contrast, sodium butyrate did not affect the response of m-ICc12 cells to IL-1β thus excluding a direct effect on NF-κB activation (fig.5d). The results indicate that butyrate specifically inhibits LPS-mediated NF-κB activation in m-ICc12 cells, accompanied by up-regulation of AP activity and cell differentiation.

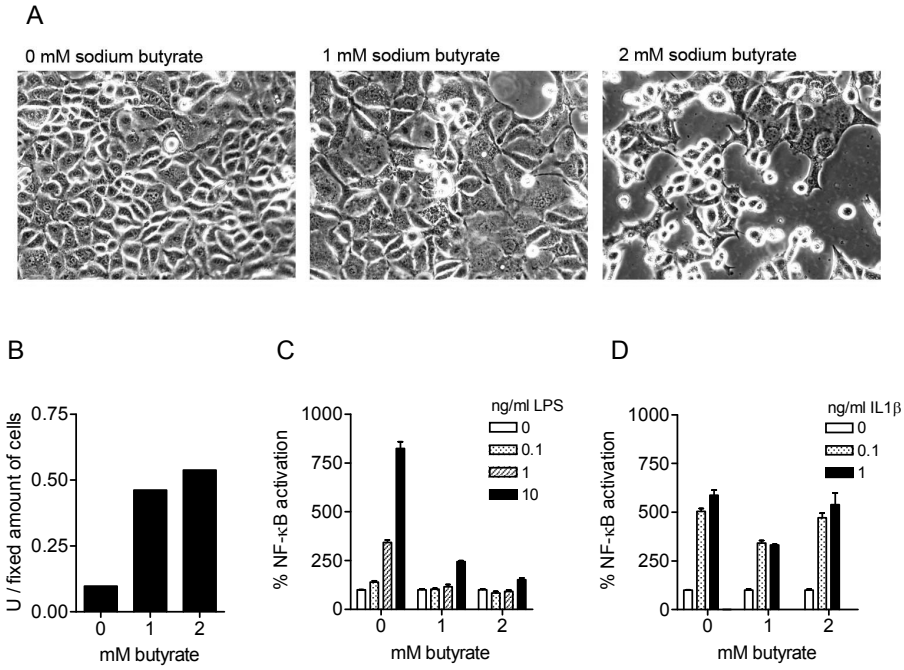

Figure 5: Effect of butyrate on LPS-induced NF-κB activation of m-ICc12 cells.
m-ICc12 cells were cultured to confluence and incubated with indicated amounts of sodium butyrate for 48h. **A.** phase images of cells after 48h culture with indicated concentrations of sodium butyrate, magnitude x200 **B.** AP activity is expressed as arbitrary units per fixed amount of cells. **C-D.** cells were subsequently stimulated with LPS or IL-1β for 4h. NF-κB activation is measured using a luciferase reporter construct and expressed as percentage of control (no stimulus). Data are presented as mean ± SD from a triplicate from one representative experiment out of three

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Discussion

Growing evidence exist that host inflammatory responses to commensal bacteria and their products contribute to IBD (3). Therefore, strategies to modify proinflammatory microbial molecules for example detoxification of LPS by AP, may add to treatment of IBD.

In the present study, we show that oral treatment with iAP is of benefit to mice with existing (DSS-mediated) intestinal inflammation and damage of the intestinal epithelial lining. This extends knowledge on the therapeutic potential of iAP obtained in a recent study that reported that administration of iAP is able to prevent DSS-mediated intestinal inflammation in rats (9). Intriguingly, our data shows that iAP is only therapeutically effective when the inflammation exceeded a certain disease grade. In those cases, colon inflammation was less pronounced, body weight loss less severe and body weight recovery more quickly.

One mode of action by which iAP may have its therapeutic effect is via the dephosphorylation and hence detoxification of luminal LPS (6, 23). However, addition of high amounts of LPS directly into the lumen of injured colon did not aggravate a moderate DSS-induced colonic injury. These findings, together with the presented *in vitro* data may explain why administration of iAP is only beneficial in cases of severe intestinal damage. Notably, our *in vitro* data demonstrate that addition of iAP to intestinal epithelial m-ICc12 cells did not inhibit LPS-induced NF-κB activation, but that culturing m-ICc12 epithelial cells in the presence of butyrate, known to upregulate iAP (10-12), inhibited the NF-κB -response to LPS but not to IL-1β. So, endogenous production of iAP protects against LPS-induced cellular responses. These findings are in line with a recent other study showing that LPS-induced phosphorylation of IκB-α and RelA/p65 and activation of NF-κB is reduced in AP-transfected intestinal HT29 cells that produce the enzyme endogenously (8). In addition, a recent study in zebrafish demonstrated a protective effect of iAP expression against LPS-mediated inflammation (14).

In general, differentiated epithelial cells produce high amounts of iAP. Our data suggest that in cases of moderate intestinal epithelial injury, cell-bound iAP may exert sufficient modulating capacity, administration of iAP is not of additional benefit and addition of LPS is unable to increase disease severity. However, in case of severe inflammation with profound epithelial damage, loss of differentiated cells results in reduced iAP expression, and addition of exogenous iAP will have a therapeutic effect. This hypothesis fits with findings by Tuin et al (9) demonstrating a reduced AP

expression in colon biopsies of patients with UC and CD and in rats with DSS-induced intestinal epithelial damage, although in that study a comparison between the efficacy of iAP and the severity of the disease was not made.
The anti-inflammatory capacity of butyrate is well-documented both *in vitro* and *in vivo* (13, 24, 25), and several clinical studies have demonstrated beneficial effects of butyrate in IBD (26, 27). Therefore, the data shown here indicate that the relieve of colitis by butyrate may be at least partly attributable to the induction of cellular expression of iAP resulting in interference with LPS-mediated effects.
Together, the findings in the present study add to the growing evidence that iAP is of significance in maintaining gut homeostasis (15), and importantly demonstrates that iAP not only prevents intestinal inflammation but may indeed also have therapeutic effects, in particular in cases of severe colon inflammation. AP may thus be considered in treatment strategies of patients with severe colitis.

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Chapter 3

Mucosal administration of HSP70 leads to partial disease suppression in mouse models for intestinal inflammation

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Abstract

Inflammatory bowel disease (IBD) is characterized by a chronic inflammation of the intestine. It appeared that insufficient regulation of the immune response against commensal bacteria contributes to disease etiology. Recently, the anti-inflammatory capacity of heat shock proteins (HSP) has been demonstrated in animal models of inflammatory diseases. For instance, administration of mycobacterial HSP70 results in the induction of IL-10-producing regulatory T cells. It was therefore of interest to investigate if HSP70 was able to diminish intestinal inflammation. Oral HSP70 treatment of mice prior to induction of intestinal inflammation with dextran sulfate sodium (DSS) could not ameliorate loss in body weight, and altered stool consistency. Furthermore, colon inflammation (determined microscopically by combination of crypt loss, loss of goblet cells, oedema and infiltrations of neutrophils) was not altered upon HSP70 treatment. Oral or intranasal treatment with HSP70 was able to diminish body weight loss in a model using tri-nitrobenzene sulfonic acid (TNBS) to induce intestinal inflammation. Oral treatment with HSP70 resulted in increased IFN-γ and IL-10 production in draining lymph nodes, but HSP70 treatment was not able to decrease colon inflammation. In conclusion, HSP70 had only marginal effects in diminishing intestinal inflammation in both DSS and TNBS mouse models for colitis, possibly due to the acute character of both models leaving no time to activate sufficient regulatory capacity of HSP70 immunization.

Introduction

Inflammatory bowel disease (IBD), like Crohn's disease (CD) and Ulcerative Colitis (UC), is characterized by extensive inflammation of the gastrointestinal tract. Genetic predisposition, lifestyle (socio-economic status) and environmental factors contribute to the etiology of these diseases (1). The commensal flora has an important contribution to disease, as abnormal immune responses towards microbiota occur abundantly in IBD patients (1-3).

In healthy individuals the responses to the luminal content are extensively regulated to prevent immune activation against harmless exogenous antigens, including commensal bacteria (4, 5). In IBD, however, the mucosal immune system remains continuously activated, resulting in a chronically inflamed intestine (6). This state of chronic activation is the consequence of a disturbance in the balance between immune activation and regulation, and can result from several factors, including impaired barrier function, enhanced immune activation or impaired regulation (4, 7).

Increasing evidence suggests that T cell responses to heat shock proteins (HSP) may have disease-regulatory activities in experimental models of inflammatory diseases such as arthritis, diabetes and atherosclerosis (8). Altered HSP function has been associated with immune dysfunction in several diseases including diabetes and arthritis (9).

HSP are intracellular proteins that possess chaperone capacities, assist in refolding of damaged proteins and contribute to maintenance of cellular integrity during stress. (10). They are divided in subclasses based on their molecular weight, and consist of constitutive and stress-inducible forms. In general, HSP expression enhances the activation of antigen presenting cells (APC) and contributes to appropriate immune responses to viral and bacterial infections. In addition, it is becoming clear that increased expression of inducible HSP family members in inflamed tissues results in the induction of anti-inflammatory regulatory T cell responses (8, 11). It was found that these regulatory T cells (Tregs) recognize highly conserved sequences of the HSP proteins, and are cross-responsive to mycobacterial homologous proteins (12). In experimental models of arthritis, diabetes and atherosclerosis, administration of exogenous (mycobacterial) HSP60 or HSP70 has been shown to suppress disease via the induction of T cells that produced high amounts of the suppressing cytokine IL-10 (12).

It was therefore hypothesized that the administration of bacterial HSP would result in the induction of HSP-specific IL-10-producing Tregs. Endogenous HSP would be

upregulated upon inflammation (cell stress), and further stimulate HSP-specific Tregs to suppress the inflammation via the production of IL-10 (13).
In the present study, the protective capacity of HSP70 immunization was assessed in two mouse models for acute intestinal inflammation, dextran sulfate sodium (DSS)-induced colitis and trinitrobenzene sulfonic acid (TNBS)-induced colitis. DSS exposure is known to result in intestinal epithelial damage accompanied by stress and upregulation of HSP (14). Rectal TNBS exposure of previously sensitized mice results in a T cell-mediated inflammation in response to the induced intestinal tissue damage. We found that administration of HSP70 via the tolerance-inducing oral and intranasal routes only partially altered the outcome of intestinal inflammation in these models. We propose that the acute character of both models leaves no time to activate sufficient regulatory capacity of HSP70 immunization.

Materials and methods

Mice and reagents

Twelve-week-old female specific pathogen-free BALB/cAnNCrl mice purchased from Charles River (Germany) were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum.* The experiments were approved by the Animal Experiments Committee of the Utrecht University.
HSP70 of Mycobacterium tuberculosis (Mt HSP70) was obtained from Dr. Singh, Lionex Diagnostics & Therapeutics GmbH, Braunschweig, Germany. Other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Induction of experimental colitis and treatment with HSP70

Mice were divided in treatment groups of 10 animals and body weight, stool-consistency and behaviour were recorded daily during the experiment. To induce DSS colitis, mice were provided with drinking water containing 3% (w/v) DSS (MP Biomedicals, Aurora, OH; MW 35-50 kD) for 7 consecutive days, followed by normal drinking water until they were sacrificed (fig.1 upper panel). No differences in water consumption between treatment groups were observed. To induce TNBS colitis, mice were sensitized with 1 mg TNBS in acetone on a shaved abdomen for 2 consecutive

days. 6 days later, mice received a rectal administration of 1 mg TNBS in 40% ethanol under isoflurane/O_2 anaesthesia using a vinyl catheter positioned 3 cm from the anus, after which they were held in vertical position for at least 1 minute. Mice were sacrificed on day 9 (fig 1 lower panel).
Oral treatment with HSP70 started 4 weeks before induction of intestinal inflammation by DSS or TNBS. Mice were treated 4 times with 30 µg HSP70 or PBS via intra-gastric gavage, preceded by 2 mg of soy bean trypsin inhibitor (SBTI), dissolved in 0.15 M sodium bicarbonate, pH 8.0 to neutralize stomach content. Intranasal treatment was started 8 days before TNBS sensitization via administration of 10 µl PBS containing 30 µg HSP70 in the nose, and repeated 4 times (fig 1 lower panel).

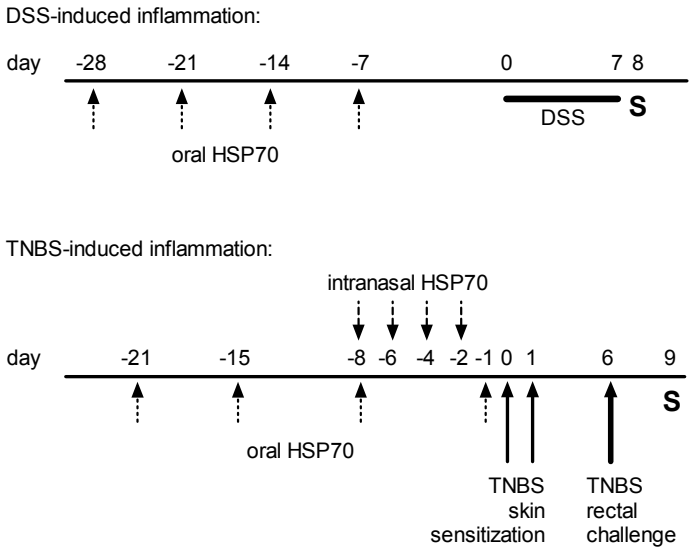

Figure 1: Treatment protocols
Mice were treated with HSP70 either orally or intranasally, after which colon inflammation was induced as indicated in the text. S: section

Evaluation of intestinal inflammation

After mice were sacrificed the colon was dissected from anus to caecum. The colon length was recorded and subsequently the faeces were carefully removed. Colon weight was determined and then cut longitudinally, and the full length of half of the colon was fixed in phosphate-buffered formalin and embedded in paraffin. Routine 5 µm sections

were prepared and stained with haematoxylin-eosin (HE) and evaluated in a blind coded fashion by two independent investigators.
Histological structural alterations were assessed by considering following parameters scored on a 0-3 scale (0 = no alterations; 1 = mild mucosal alterations in less than 1/3 of the colon; 2 = moderate mucosal and submucosal alterations involving up to half of the colon; 3 = severe alterations in more than half of the colon): oedema; crypt loss; loss of goblet cells and leukocyte infiltrations. The histological score was calculated as the sum of the scores for each category and resulted in a maximum score of 12 in the most severe situation. Evaluation of independent investigators (MBS; RB) were averaged and resulted in a histological score for each mouse.

Culture of colon samples and lymphocytes

A distal sample of the dissected colon of 30 to 50 mg (exact weight was recorded), was washed three times in ice-cold PBS, containing 40 mg/ml glucose, 100 IU/ml penicillin-streptomycin and 250 µg/ml fungizone and incubated in 0.5 ml complete RPMI1640 (Invitrogen Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Greiner Bio-one, Germany) for 24h at 37 °C, 5% CO_2. Supernatant was collected and stored at -20 °C until analysis was performed.
Single cell suspensions from colon draining caudal lymph nodes ($3.75*10^5$ per well) were incubated in RPMI1640 supplemented with pen/strep and 10% FCS in the presence of immobilized anti-CD3 (1 µg/ml; clone 45-2C11) and soluble anti-CD28 (1 µg/ml; clone 37.51) in a 96-well plate for 48h at 37 °C, 5% CO_2 after which culture supernatants were harvested and stored at -20°C until analysis.

Cytokine analysis

IFN-γ was determined by sandwich ELISA. IFN-γ capture and biotin antibodies were obtained from BD Pharmingen (Erembodegem, Belgium). Highbond plates (Costar) were coated overnight at 4 °C with 1 µg/ml rat anti-mouse IFN-γ in 0.05M sodium carbonate buffer, pH 9.6, washed with PBS/0.01%Tween 20 (PBS-T) and blocked with PBS-T /3% milk powder for 4h at room temperature. Samples and standard were added in several dilutions in PBS-T/1% (w/v) BSA (PBS-T/BSA) and incubated overnight at 4 °C. After washing, plates were incubated with 0.25 µg/ml biotinylated goat anti-mouse IFN-γ antibody diluted in PBS-T/ BSA for 1h at room temperature. Plates were washed and incubated with streptavidin-HRP (0.1 µg/ml, Sanquin, Amsterdam, The Netherlands) diluted in PBS-T/BSA for 45 min at room temperature. After the final washes, tetramethylbenzidine (TMB) substrate (0.1 mg/ml) was added and the color

reaction was stopped after 15 minutes with 2M H_2SO_4. Absorbance was measured at 450 nm. IL-10 (BD Pharmingen) and TNF-α ELISA (Biosource, Nivelles, Belgium) were performed according to the instructions of the manufacturer. Cytokine levels from colon samples were normalized for weight of colon tissue used in culture.

Statistics

Data are presented as the group mean ± standard error of the mean (SEM) and analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). A Kruskal-Wallis test was performed for all groups together. When appropriate, Mann-Whitney tests were performed to determine differences between two groups. Differences were considered significant when p values were < 0.05.

Results

Treatment with HSP70 did not diminish DSS-induced intestinal inflammation

Exposure of BALB/c mice to DSS resulted in induction of disease characterized by loss in body weight in time (fig 2a) and alterations in stool consistency (data not shown). Histological scoring of the colon (fig 2b) after a 7 day exposure to DSS showed crypt loss and loss of goblet cells accompanied by formation of oedema and influx of neutrophils, indicative for the induction of inflammation. Oral pretreatment with HSP70 did not alter these inflammatory parameters. Furthermore, the levels of both TNF-α and IL-10 in culture supernatants of distal colon sample was comparable in vehicle-treated and HSP70 treated mice (fig 2c).

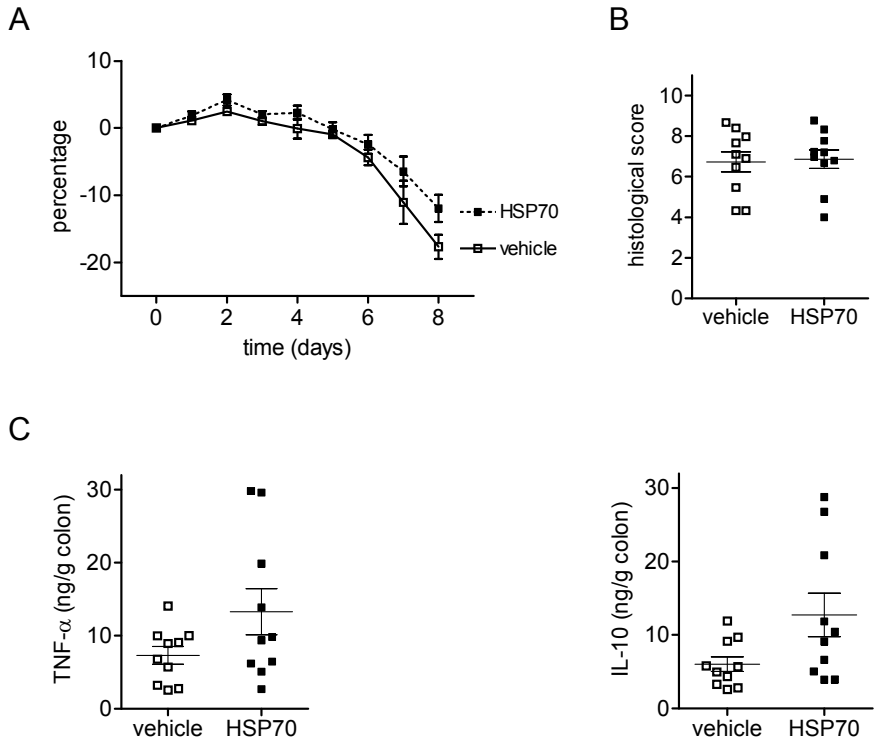

Figure 2: Effect of HSP70 treatment on DSS-induced inflammation in the colon
Mice (n=10) were orally treated with vehicle (open squares) or Hsp70 (closed symbols), followed by exposure to DSS for 7 days, as indicated in materials and methods. Data are depicted as mean ± SEM. **A.** percentage of body weight loss in time. **B.** colon morphology score on day 8, based on oedema formation, loss of crypts and goblet cells, and influx of leucocytes. **C**. distal colon samples were isolated on day 8 and cultured for 24h *in vitro.* TNF-α and IL-10 levels in supernatant was determined by ELISA and presented as ng per g colon sample.

HSP70 lowered weight loss but showed no signs of reduced intestinal inflammation induced by TNBS exposure

Mice that were sensitized to TNBS via the abdomen showed an immediate reduction in body weight after rectal exposure to TNBS, which lasted for 3 days, after which mice were sacrificed (fig.3a). Histological examination of the colons of these mice revealed an influx of granulocytes and lymphoid cells, oedema formation and crypt loss,

indicative of inflammation. In contrast to vehicle-treated animals, mice that were treated with HSP70 recovered very quickly after the initial body weight loss in response to rectal TNBS exposure and their body weight had returned to normal level when mice were sacrificed (fig 3a). In contrast, histological examination of the colons of HSP70 treated mice did not show reduced inflammation compared to vehicle treatment (fig 3b).

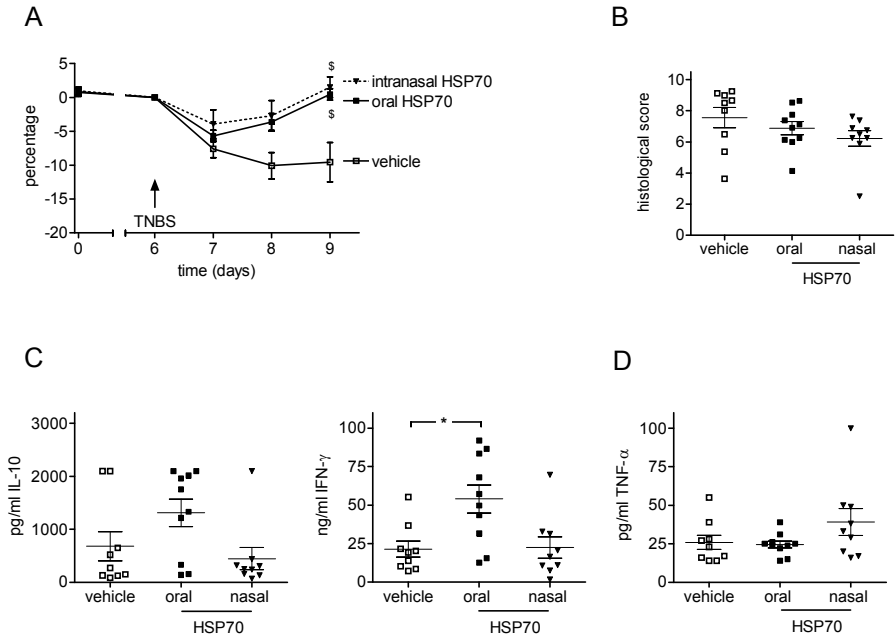

Figure 3: effect of HSP70 treatment on TNBS-induced colon inflammation
Mice (n=10) were orally (closed squares) or intranasally (triangles) treated with HSP70 or with PBS (open symbols) as indicated in materials and methods. Mice were sensitized to TNBS by skin followed by a rectal TNBS challenge 6 days later. **A.** Body weight loss in time after rectal challenge. **B.** morphololgy of colons from 3 days after rectal challenge were scored, based on oedema formation, loss of crypts and goblet cells, and influx of lymphocytes and granulocytes. **C.** IL-10 and IFN-γ levels in CauLN after restimulation with CD3/CD28 for 48h, determined by ELSA. **D**. Levels of TNFα in CauLN after 48h restimulation with LPS. Data are presented as mean ± SEM

As inflammation after exposure to TNBS involves the activation of T cells, lymphocytes from the colon draining caudal lymph nodes (CauLN) were restimulated

in vitro with CD3/CD28 for 48h. Oral pretreatment with HSP70 resulted in an increase in IFN-γ production after restimulation, whereas no differences in IL-10 production were found (fig 3c). Intranasal HSP70 pretreatment did not alter cytokine production in CauLN. Furthermore, no differences in TNF-α production were found between treatment groups after *in vitro* LPS restimulation (fig 3d). Cytokine production in distal colon samples was below detection limit (data not shown).

Discussion

Previous studies in animal arthritis models and in patients suffering from rheumatoid arthritis (RA) showed that exposure to microbial HSP resulted in the induction of self-reactive regulatory T cells (Tregs), that produced IL-10 and were capable of suppressing disease (12, 13, 15, 16). Data from the present study reveal that treatment with HSP70 had only marginal effects in diminishing intestinal inflammation in both DSS and TNBS mouse models for colitis.

Intestinal inflammation due to DSS exposure is known to result in epithelial damage (4), and is accompanied by stress and upregulation of HSP (14). It was therefore considered a suitable model to investigate whether administration of HSP70 and induction of HSP-specific Tregs can also inhibit intestinal inflammation. Inflammation after rectal TNBS exposure also results in epithelial barrier disruption, but the inflammatory response depends on the presence of effector (CD4) T cells (induced via prior sensitization to TNBS). These effector T cells were considered to be probably more responsive to IL-10 that would be present when our intervention had indeed resulted in successful induction of HSP-specific Tregs.

Several reasons can contribute to the minimal HSP-induced protection in these models as observed in the present study. The acute character of the used intestinal inflammation models can be the reason for the absence of protective HSP responses. Both DSS and TNBS exposure result in the induction of inflammation within 24-48h, as a result of epithelial barrier disruption, whereas the onset of inflammation in RA models starts 25 to 30 days after initial induction (17). It is therefore conceivable that the timeframe of the used models is too short to successfully activate regulatory responses. Sun et al. (2007) described that a thymic hormone (Facteur thymique serique, FTS) did not affect the course of acute DSS-induced colitis while a protective effect of FTS was found in chronic DSS colitis, which was based on elevated IL-10

production by T cells in the lamina propria (LP) (18). So, our results do not exclude that HSP pretreatment can be protective in a more chronically inflamed intestine.
The protective effect of HSP administration via the mucosal (oral or intranasal) route depends on the induction of HSP-specific Tregs. Although we did not examine the induction of Tregs in the present studies, it was shown that microbial HSP administered via the same protocol resulted in the induction of IL-10 and IFN-γ producing suppressor T cells in a mouse model for arthritis (17). Whether the observed increase in IFN-γ production in draining CauLN after oral HSP administration in the present TNBS study was attributable to T cell regulation or to the inflammation itself is not clear, but it did not suffice to influence colon inflammation in this model.
The regulatory capacity of microbial HSP depends on cross-reactivity with endogenous self-HSP, that will be upregulated in inflamed tissue (8) as was found in RA (19, 20). In contrast to peripheral tissues, HSP are expressed constitutively in the epithelial cell layer of the intestine and are likely to contribute to epithelial resistance to luminal bacteria (21-23). The expression of the cytoprotective HSP in intestinal epithelial cells is reduced in inflamed parts of the colon of IBD patients (21, 24) and in mouse models for IBD (25, 26). Furthermore, it was shown that transgenic MIF-/- mice were resistant to DSS-induced inflammation, due to high levels of HSP70 expression in intestinal cells (27). Other studies showed that administration of the HSP co-inducer geranylgeranylacetone (GGA) upregulated HSP70 expression in intestine, and resulted in reduced inflammation in both DSS and TNBS colitis (28, 29). Therapeutic interventions in IBD with mesalamine and a probiotic lactobacillus GG demonstrated the upregulation of HSP70 in intestinal epithelial cells (30, 31). Next to that, Debler (2003) found that a HSP70 polymorphism was associated with increased intestinal complications in Crohn's Disease (CD) (32). These data provide compelling evidence that endogenous HSP expression contribute to protection from intestinal inflammation. They may also implicate that beneficial effects are to be expected from strategies that cause upregulation of endogenous HSP expression, rather than boosting the HSP-specific Tregs with exogenous HSP.
Further research needs to be done to investigate the usefulness of HSP administration and HSP-inducing strategies in situations of chronic intestinal inflammation.

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Chapter 4

Changes in intestinal cell populations during cholera toxin-induced sensitization to peanut.

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Abstract

The mucosal adjuvant cholera toxin (CT) is used to induce allergic sensitization to co-administered proteins in mice, and is widely used in animal models to elucidate the mechanisms behind food allergic responses. The present study focuses on the changes in dendritic and lymphoid cell populations in the intestine and draining lymph nodes immediately after exposure to CT with or without peanut extract (PE). Exposure to CT or CT+PE induced a shift in DC subsets in the intestine. Mucosal tolerogenic ($CD103^+$) DCs were decreased in numbers, but conventional inflammatory ($CD11b^+$) DC were increased. Next to changes in DC subsets, we observed a decrease in γδ T cells in the intestine upon exposure to CT. As γδ T cells contribute to immune homeostasis and regulation in the intestine, decreased numbers will render the intestine more susceptible to immune activation. This study thus indicates that CT is able to induce a shift towards an immunogenic environment in the intestine, characterized by an increase in $CD11b^+$ DC and a decrease of γδ T cells. Both effects may be of relevance to the adjuvant capacity of CT.

Introduction

In the western world, food allergy occurs in 1-4% of individuals (1, 2). Understanding of food allergic manifestations has increased over the years, but the underlying mechanisms responsible for allergic sensitization to food proteins remains elusive. Animal models have contributed to more insight in the mechanisms of sensitization to food proteins (3).

The mucosal adjuvant cholera toxin (CT) induces allergic sensitization to co-administered proteins in mice, while feeding the protein alone induces an active state of immunologic non-responsiveness termed oral tolerance (4). CT is therefore widely used to investigate this antigen-specific IgE characterized sensitization to proteins (5-8). However, the immunological mechanisms by which CT induces allergic sensitization is not completely understood (9).

CT is able to induce upregulation of MHCII and co-stimulatory molecules (CD80; CD86) on human monocyte-derived dendritic cells (DCs), resulting in DCs that preferably prime naive CD4 T cells to a Th2 phenotype (10). Furthermore, CT-matured DCs express functional chemokine receptors CCR7 and CXCR4, involved in migration of DC subpopulations from the intestine to the MLN and from the sub-epithelial dome of the Peyers Patches (PP) to T cell areas (11-14). In addition, it is found that CT enhances the production of IL-1 by DCs and of IL-1 and MIP2 (Chapter 6 of this thesis) by intestinal epithelial cells (15, 16). Although induction of DC activation and maturation may indeed be of major importance for the adjuvant capacity of CT, it has been shown that induction of oral tolerance to food proteins also involves maturation and migration of intestinal DCs (17). This indicates that more complicated processes are involved in sensitization by CT.

The present study focuses on the changes in lymphoid and dendritic cell populations in the intestine and draining lymph nodes immediately after exposure to CT alone and in combination with peanut extract (PE) as allergen. Here we show that administration of CT results in profound effects not only on DCs but also on γδ T cells. Other investigators suggested that γδ T cells are involved in the induction of tolerance to orally administered proteins (18-20). Therefore, the present findings may be of relevance to understanding early processes of allergic sensitization.

Materials and Methods

Mice and reagents

Five-week-old specific pathogen-free female C57BL/6J mice purchased from Charles River (France) were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and laboratory food pellets (CMR(E); Special Diet Services, Witham, England) were provided *ad libitum.* The experiments were approved by the Animal Experiments Committee of the Utrecht University. Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Treatment protocol

Peanuts from the Golden Peanut plant (Alpharetta, GA, USA) were kindly provided by Intersnack Nederland BV (Doetinchem, The Netherlands) and peanut extract (PE) was prepared as previously described (21, 22). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). Mice were orally exposed to PBS, 6 mg PE, 15 µg CT or 6 mg PE mixed with 15 µg CT on day 0, 1 and 2, and sacrificed at day 1, 2, 3 and 7.

Cell isolation

For isolation of intestinal epithelial lymphocytes (IEL) and lamina propria cells (LPL) the small intestine was removed from mice, and fecal contents were flushed with 10 ml of HBBS (Invitrogen) supplemented with 15 mM Hepes (Invitrogen). After removing Peyer's patches (PP), the small intestine was cut into 1-cm pieces, and shaken vigorously for 20 min at 37 °C in HBBS containing 15 mM Hepes, 100 U/ml penicillin, streptomycin 100 µg/ml (both Invitrogen), 5 mM EDTA, 1 mM DTT and 10% FCS (Greiner) (HBBS/EDTA). The mixture was passed through a gauze and remaining tissue was used to isolate LPL, whereas the filtrate was washed once in HBBS/Hepes and centrifuged through a 45-67.5% discontinuous Percoll gradient (GE Healthcare) at 600g at 20 °C for 20 min. to collect IEL.

For LPL isolation the remaining tissue was shaken for another 20 min at 37 °C in HBBS/EDTA, and washed in RPMI1640 (Invitrogen), supplemented with penicillin/streptomycin, 10% FCS and 1 mM DTT (RPMI/FCS/DTT). The intestine was digested through incubation for 45 min at 37 °C in RPMI/FCS/DTT supplemented with 1 mg/ml collagenase (Roche) and 20 µg/ml DNAse. Cells were collected via 100

µm gauze and tissue digestion was repeated, after which both cell fractions were pooled and washed once in HBBS/Hepes. LPL were enriched by a 40—100% discontinuous Percoll gradient.
To obtain single cells, the PP were shaken for 20 min at 37 °C in HBBS/ EDTA, washed once in RPMI/FCS, cut in small fragments, and digested in RPMI/FCS containing collagenase/DNAse. Cells were passed through a 70 µm gauze to obtain single cells, and washed once in HBBS/Hepes.
Cells from mesenteric lymph nodes (MLN) and spleen were obtained through squeezing the organs through a 70 µm gauze, after which single cells were washed once in RPMI/FCS. To remove erythrocytes in spleen, cells were incubated with erylysisbuffer (0.16M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA pH 7.4) for 1 min, and washed once in PBS. All cell suspensions were kept on ice, unless stated otherwise.

Flow cytometry

For flow cytometric analysis 2-5*10^5 cells were stained in staining buffer (PBS containing 0.25% BSA, 0.5 mM EDTA and 0.05% NaN_3) with fluorecein isothiocyanate (FITC)-, R-phycoerythrin (RPE)-, Peridinin chlorophyll protein (PerCP) - or allophycocyanin (APC) - labeled antibodies. Cells were incubated for 30 min at 4 °C in the dark. All samples were pre-incubated with anti-CD16/CD32 (2.4G2) to block FcR, and stored in 0.4% formaldehyde until acquisition.
Cell characterization was based on following antibodies (obtained from e-Bioscience or BD-Pharmingen): CD3 (145-2C11); CD4 (RM4-5); CD8α (53-6.7); CD11b (M1/70); CD11c (N418); CD86 (GL1); CD103 (2E7); TCRβ (H57-597); TCRδ (GL3); MHCII (M5-114.15.2)
Data were acquired by means of FACSCalibur (Becton Dickinson). Analysis was performed using Weasel (The Walter And Eliza Hall Institute of Medical Research, Melbourne, Australia). Different cell populations are presented as percentage of lymphocytes based on FFS-SSC pattern.

Tissue staining

Acetone fixed cryostat sections of the small intestine were incubated for 1h with anti TCRδ Ab (GL3, hamster MAb), CD8α (53-6.7, rat MAb) or anti CD11c Ab (N418, hamster MAb) diluted in PBS, 1% BSA. The antibodies were then visualized by first applying biotinylated anti-hamster or anti-rat Ab (DAKO, Denmark) for 1h, followed by 1h incubation with horseradish peroxidase labeled streptavidin (DAKO) and

aminoethylcarbazole (AEC) as a substrate. Sections were counterstained with Mayer's heamatoxylin (DAKO) and mounted with DAKO Faramount Aqueous mounting medium. Images were acquired using an Olympus BX60 microscope with colorview III digital camera and Cell Imaging software.

Statistics

Data are presented as the group mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism software. Two-way ANOVA was performed followed by Bonferroni as a post-hoc test. Differences were considered significant when p values were < 0.05.

Results

Treatment with CT or PE+CT caused a loss of DCs and γδ T cells from the intestine.

Administration of CT+PE (and CT alone, data not shown) resulted in a decrease in $CD11c^+$ and $CD8^+$ cells in intestinal mucosa and lamina propria compared to control treatment as shown by immunohistochemical analysis of the small intestine taken 3 days after initial administration of CT (fig 1). The decrease in CD8α cells was accompanied with a decrease in γδ T cells, in line with the fact that most CD8α cells in the intestine are γδ T cells.

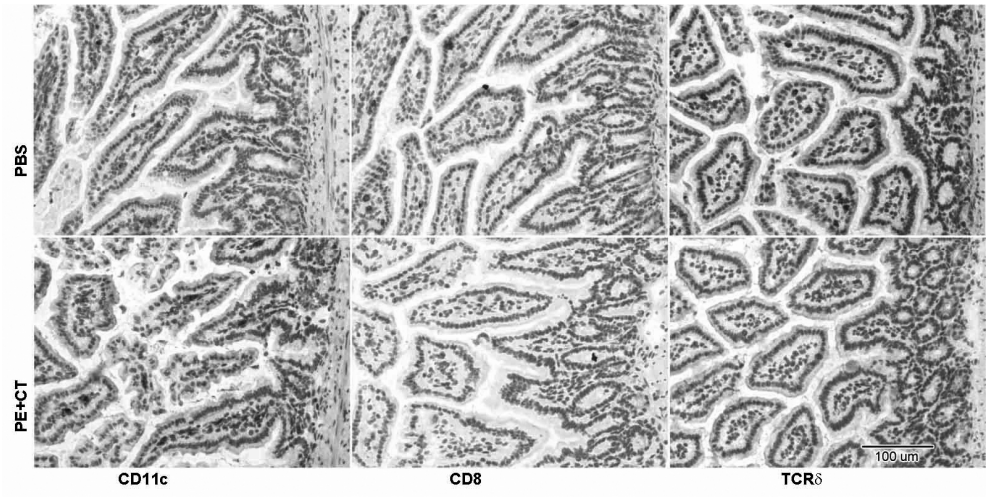


Figure 1: Immunohistochemical analysis of CD11c, CD8 and TCRδ cells in the intestine.
C57BL/6 mice (n=4) were exposed to PBS or PE+CT as described in materials and methods. On day 3 cryosections of the small intestine were stained in red for CD11c (left panel), CD8 (central panel) or TCRδ (right panel) as described in material and methods. Representative micrographs of PBS-treated (upper row) or PE+CT treated mice (lower row) are shown. For color images see page 166.

CT reduced mucosal DCs, accompanied with increase in conventional DCs.

Changes in intestinal $CD11c^+$ dendritic cell populations on days 1 to 3 and 7 were further analyzed by flowcytometry (fig 2a) and revealed a CT-induced decrease in percentage and absolute cell number of mucosal $CD11c^+CD103^+$ DC (fig 2b and 3a). This decrease was most pronounced in the PP. In contrast, CT caused a significant increase in conventional inflammatory ($CD11c^+$ $CD11b^+$) DCs in LPL and IEL but not in PP and MLN (fig 2c and 3b). In addition, the ratio between conventional and mucosal DCs in IEL, LPL, PP and MLN was increased upon treatment with CT or PE+CT (fig.3c). This shift in DC subpopulations was most profound on days 2 and 3, and almost returned to normal on day 7. Furthermore, PE+CT induced a more distinct shift to conventional DCs compared to CT alone in LPL and PP, especially on day 2.

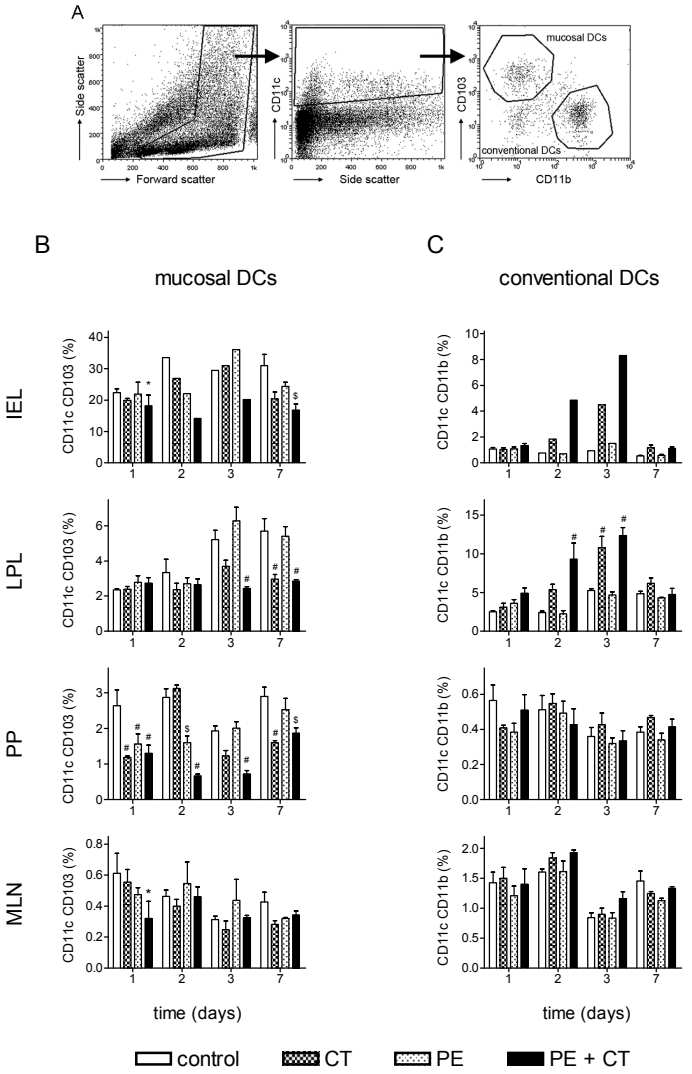


Figure 2: Changes in percentage of DC subsets in the intestines after CT treatment
C57BL/6 mice (n=4) were exposed to PBS, PE, CT and PE+CT during 3 consecutive days as described in materials and methods. Cells were isolated from intestine and MLN were analyzed using flow cytometry **A**. Cells were gated based on FSC-SSC pattern and CD11c staining and subsequently analysed based on CD11b expression (conventional DCs) or CD103 expression (mucosal DCs). Pictures show representative dotplots of DC analysis in LPL of a PBS treated mouse on day 1 after start of exposure. **B.** percentages of mucosal tolerogenic DCs ($CD11c^+$ $CD103^+$ cells). **C.** percentage of conventional inflammatory DCs ($CD11c^+CD11b^+$ cells). Data are represented as mean ± SEM. *: $p < 0.05$, $: $p < 0.01$, #: $p < 0.001$, compared to control of the same day. IEL cell suspensions from day 2 and 3 were pooled per treatment group before analysis.

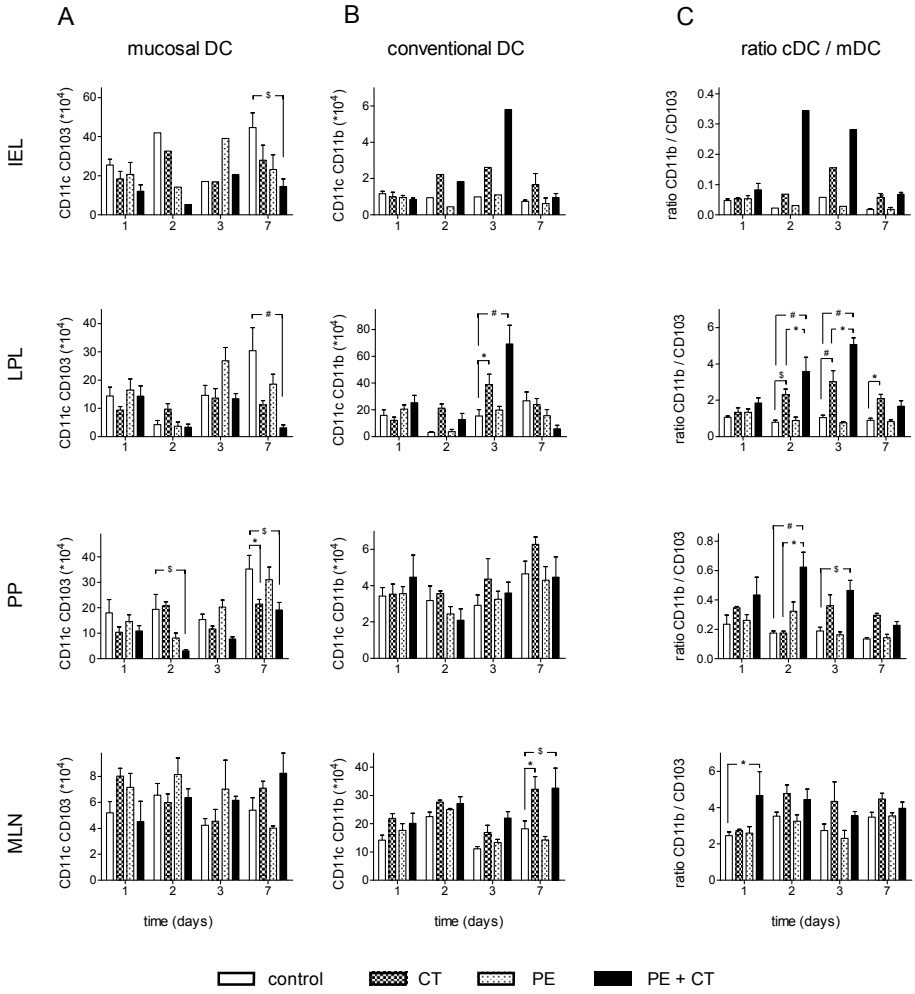


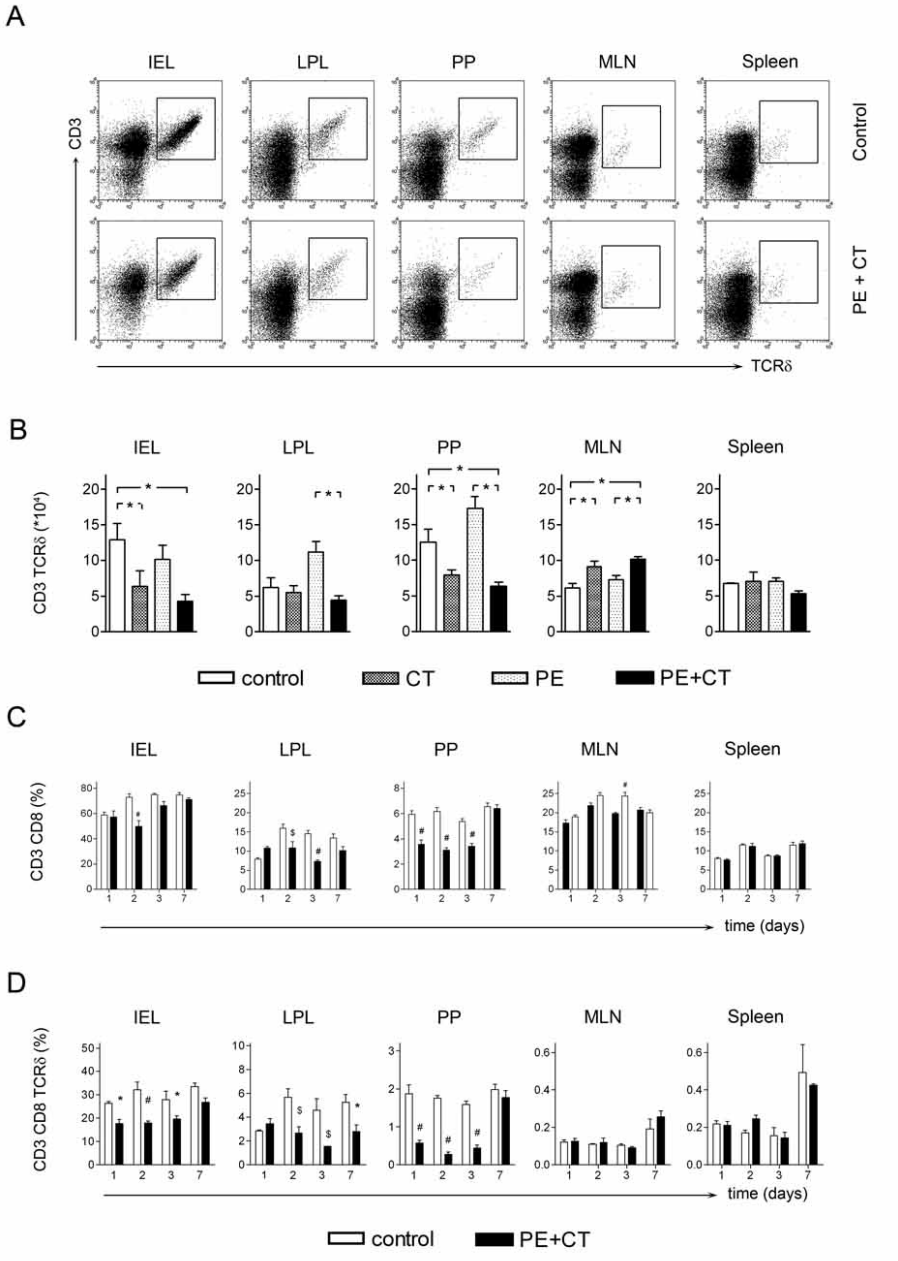
Figure 3: Changes in cell numbers of DC subsets in the intestineafter CT treatment
C57BL/6 mice (n=4) were exposed to PBS, PE, CT and PE+CT during 3 consecutive days as described in materials and methods. Cells were isolated from intestine and MLN were analyzed as indicated in fig 2
A. number of mucosal DCs ($CD11c^+$ $CD103^+$ cells) in various organs. **B.** number of conventional DCs ($CD11c^+$ $CD11b^+$ cells). **C.** ratio of conventional DCs to mucosal DCs. Data are represented as mean ± SEM. *: $p < 0.05$, $: $p < 0.01$, #: $p < 0.001$, compared to control of the same day. IEL cell suspensions from day 2 and 3 were pooled per treatment group before analysis.

CT reduced γδ T cells in intestine but not in MLN and spleen

Changes in the number of γδ T cells (identified by CD3 and TCRδ membrane expression, fig 4a) in the intestine were quantified by flow cytometry and revealed a reduction in γδ T cells after treatment with PE or PE+CT in IEL, LPL and PP, but not in spleen (fig 4b). In contrast, the numbers of γδ T cells in MLN were increased upon CT or PE+CT treatment. The decrease in γδ T cells in the intestine was accompanied with a decrease in CD8 cells, since most γδ T cells are also $CD8\alpha^+$. In addition, this indicates the actual disappearance of γδ T cells, rather than lack of staining or disappearance of the γδ TCR on these cells as suggested by others (23). Timing of changes in $CD8^+$ and γδ T cells in the intestine was further investigated and showed a decrease in percentages of these cell types after PE+CT administration in IEL, LPL and PP fractions compared to PBS treatment (control) (fig 4c-d). This decrease could be detected from day 1 to 2 after administration of PE+CT but also of CT alone (data not shown) and was returned to normal on day 7. Treatment with PE alone did not change the percentages of γδ T cells.

Figure 4: Effect of CT exposure on γδ T cells in various organs

C57BL/6 mice (n=4) were treated with PBS, PE, CT or PE+CT for 3 consecutive days as described in materials and methods after which cells were isolated from intestine, MLN and spleen on indicated time points and analyzed by flow cytometry. **A**. Representative dot plots of γδ T cell analysis in various organs on day 3 after exposure to PBS (control) or PE+CT. Lymphocytes were gated based on FSC-SSC pattern, followed by analysis for co-expression of CD3 and TCRδ. **B.** numbers of CD3 TCRδ double positive lymphocytes on day 3 after indicated treatments. **C.-D.** Changes in percentages of CD8 T cells (C) and CD8 γδ T cells (D) in time after treatment with PBS (control) or PE+CT. Lymphocytes were gated based on FSC-SSC pattern, and analyzed for the co-expression of indicated membrane staining. Percentage are depicted as mean ± SEM. *: $p < 0.05$; $: $p < 0.01$; #: $p < 0.001$ compared to control treatment of the same day.


A
IEL
LPL
PP
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Spleen
CD3
Control
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TCRδ
B
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PE+CT
C
CD3 CD8 (%)
time (days)
D
CD3 CD8 TCRδ (%)
time (days)
control
PE+CT

Remaining γδ T cells in intestine showed elevated membrane expression of MHCII and CD86

It has been reported that activation of γδ TCR can induce the expression of MHCII on the cell membrane of γδ T cells (24). We therefore investigated the expression of MHCII and the costimulatory molecule CD86 on γδ T cells in the various fractions isolated from the intestine of B6 mice. Whereas the number of γδ T cells were decreased, the expression of MHCII on the remaining TCRδ$^+$ cells appeared increased significantly after treatment with PE+CT, especially on day 2 (figure 5a-b), and normalized again on day 7. This increased expression was most pronounced in PP, but was also observed in IEL and LPL. In addition, increased expression of CD86 on γδ T cells was observed in IEL and LPL (figure 5c-d).

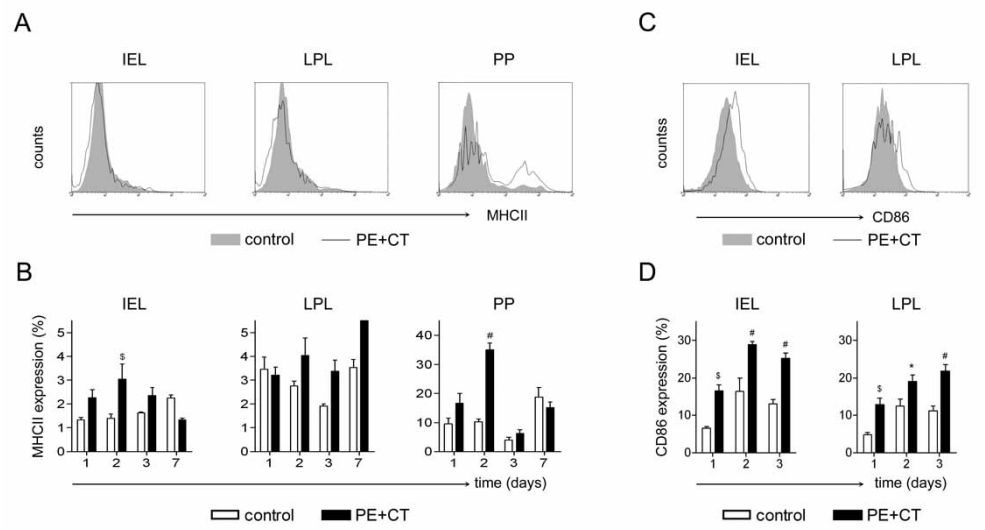

Figure 5: expression of MHCII and CD86 on γδ T cells in intestine
C57BL/6 mice (n=4) were treated with PBS or PE+CT as described in materials and methods. **A.** representative histogram of MHCII expression on γδ T cells in IEL, LPL and PP on day 2 after initial exposure. **B**. percentage of γδ T cells that express MHCII **C.** Representative histogram of CD86 expression on γδ CD8α T cells in IEL and LPL determined on day 2 after first exposure. **D.** γδ T cells in IEL and LPL that express CD86. Data are depicted as mean ± SEM. *: $p < 0.05$; $: $p < 0.01$; #: $p < 0.001$ compared to control of the same day.

Discussion

The mucosal adjuvant CT induces allergic sensitization to co-administered proteins in mice, while feeding the protein alone induces tolerance. Therefore, CT can be used as a tool to model the induction of allergy. In the present study, we show that oral CT exposure in the presence or absence of the antigen PE results in a shift in the balance of DC subsets from mucosal (tolerogenic) to conventional (immunogenic) DCs. In addition we show that CT exposure causes a decrease of γδ T cells in the intestine.
As observed by others (11, 13) we found that CT results in an increase in conventional Th2 priming $CD11c^{+}CD11b^{+}$ DCs (25) in the IEL and LPL but not in PP. Present data shows that in addition, CT causes a decrease of immunoregulatory mucosal $CD103^{+}$ DCs (26) in various intestinal locations (IEL, LPL and PP). This observed shift in intestinal DC subset distribution moving to a more inflammatory DC subtype favors immune activation. It has been shown that exposure to CT results in migration of DCs to MLN (13) and in subsequent T cell activation. It remains to be investigated whether CT affects DC distribution *in vivo* directly or indirectly, for instance by stimulating the production of Th2 skewing thymic stromal lymphopoietin (TSLP) by intestinal epithelial cells (IEC) (4, 9). Together, our data show that the effects of CT on DC subset distribution may have a substantial contribution to its adjuvant capacity.
In addition to effects of CT on DC distribution, we found that exposure to CT in the presence or absence of PE resulted in a significant decrease in γδ T cells in the intestine, whereas remaining γδ T cells had a higher expression of MHCII and the co-stimulatory molecule CD86. Interestingly, γδ T cell numbers in MLN were increased, suggesting that intestinal γδ T cells migrated to the MLN. It remains unclear whether the decrease in γδ T cell number is indeed caused by migration out of the intestine or by apoptosis of these cells.
As described before (27, 28), γδ T cells in mice are most abundant in IEL, and predominantly located in the paracellular space between intestinal epithelial cells on the luminal side of the basement membrane. Due to this location they are ideally situated for crosstalk with intestinal epithelial cells (IEC) and immune cells (DCs, B and T cells) in the LP (28). Several reports indicate that γδ T cells are involved in the induction of oral tolerance to soluble food proteins (18-20, 27, 29, 30) whereas others demonstrate a role in responses to pathogens (31-33) and repair of epithelial damage (34, 35). Furthermore, γδ T cells are able to recognize and eliminate “stressed” cells (36). It is very well possible that exposure to CT results in stressed epithelial cells within the intestine, and thus indirectly trigger γδ T cells. A study by Cardoso et al. described that sensitization to peanut followed by peanut challenge was accompanied by the induction of intestinal epithelial damage and reduced numbers in γδ T cells (37). Of interest as well is the finding that mast-cell deficient mice being highly susceptible

to allergic sensitization in the absence of an adjuvant, had very low percentages of γδ T cells within the IEL and that the number of γδ T cells further decreased when sensitization was established (38). In addition, reconstitution of mast-cell deficient mice with γδ T cells resulted in diminished allergic responses (39).
The observed increase in expression of MHCII and costimulatory molecules on the remaining γδ T cells within the intestine, suggests involvement of γδ T cells in initiation of antigen-specific responses. A recent *in vitro* study showed that mouse peripheral γδ T cells are capable of expressing MHCII molecules, turning them into APC (24). This increase in MHCII expression was accompanied by internalization of the γδ TCR, but whether this also occurs in response to CT exposure *in vivo* remains to be established. Moreover, it is not clear whether CT can serve as a ligand for the γδ TCR or other receptors on γδ T cells such as NKG2D. Expression of MHCII and antigen presenting functions were also found in human (40) and bovine (41) γδ T cells in response to bacterial products. These findings may all link to growing evidence that activated γδ T cells are directly involved in DC maturation (42) and contribute to allergic sensitization.
In conclusion, the process of CT-induced sensitization to oral administered soluble antigens is accompanied by alterations in DC subsets and reduction in γδ T cells. The involvement of these cell subsets in allergic sensitization to orally administered antigens is subject of further research, as extended knowledge on the mechanism of allergic sensitization will contribute to therapeutic intervention.

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Chapter 5

Regulatory function of intestinal γδ T cells in allergic sensitization to peanut

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Abstract

Development of oral sensitization to food proteins involves an abrogation of tolerance induction by the mucosal immune system in the intestine (oral tolerance). In mouse food allergy models, the mucosal adjuvant cholera toxin (CT) is used to induce allergic sensitization to co-administered proteins. In previous studies, we found that exposure to CT resulted in a decrease in the γδ-TCR bearing intestinal epithelial lymphocytes (IEL). γδ IEL are involved in the induction of oral tolerance, and contribute to intestinal homeostasis. We therefore investigated the role of γδ T cells in a mouse model for allergic sensitization to peanut (PE). Treatment with TCRδ specific antibody UC7-13D5 resulted in blockade of the γδ TCR *in vivo.* Upon subsequent exposure to PE+CT, PE-specific IgE responses in both C3H/HeOuJ and C57BL/6 mice were enhanced compared to non-treated mice. Cytokine production in splenocytes after PE-specific restimulation *in vitro* was also elevated upon antibody treatment. In contrast, exposure of γδ -/- mice to CT+PE did not result in altered PE-specific responses in these mice, compared to their wild type littermates.
In conclusion, these results suggest that γδ T cells are involved in regulation of the magnitude of allergic sensitization. Modulation of γδ T cells in the intestine may contribute to strategies to prevent and possibly treat food allergy.

Introduction

Food allergy has emerged as a major health problem in the western world, and affects 4-6% of children less than 3 years of age, and about 2% of the adult population in the US (1-3). At present the only option to prevent clinical manifestations of food allergy is avoidance of the particular food. Allergy to peanuts (PE) and tree nuts can be lifelong and is among the leading causes of fatal and near-fatal food-induced allergic reactions in the US (4).

Development of oral sensitization towards food proteins involves abrogation of tolerance (5). Mouse models to study sensitization towards food components often use the mucosal adjuvant cholera toxin (CT) to overcome intestinal regulation, and enhance local and peripheral Th2 responses (6, 7). We found that exposure to CT in the presence or absence of an antigen resulted in a decrease of γδ T cells in the intestine and in Peyer's patches (PP) (chapter 4 of this thesis).

The major part of intestinal epithelial lymphocytes (IELs) in the mouse consists of γδ T cells which are localized in the paracellular space between intestinal epithelial cells at the luminal site of the basement membrane (8). These γδ T cells are considered to bridge innate and adaptive immunity (9) as they mount early and protective interferon (IFN)-γ-mediated responses towards pathogens (10-12), can activate DCs, and even act as antigen presenting cells themselves (13). In addition, γδ T cells are involved in induction of protective IgA responses (14) and of peripheral tolerance towards orally administered proteins (15, 16). It has been shown that treatment of mice with a TCRδ-specific antibody resulted in impaired oral tolerance induction whereas oral tolerance could be transferred by means of γδ T cells (17). Regulatory capacities of γδ T cells also include suppression of Th2 dependent IgE responses without affecting parallel IgG responses to inhaled antigens (18). However, the role of γδ T cells in the establishment of food allergy is far from understood.

The possible regulatory role of γδ T cells in the intestine together with the CT-induced decrease in γδ T cells shown in the previous chapter prompted us to investigate whether these cells are involved in oral sensitization towards PE. To this end, we used the UC7-13D5 (UC7) antibody, which specifically recognizes the δ chain of the γδ T cell receptor (TCR). Controversy exists on the *in vivo* effects of UC7 on γδ T cells, as some studies reported depletion (10, 12, 19) whereas others described blocking of the γδ TCR, followed by internalization and possible activation of these cells (20-22). The present study shows that treatment with UC7 probably result in blocking of the γδ

TCR, and resulted in elevated PE-specific responses upon sensitization to PE. This provides evidence for involvement of γδ T cells in food allergic responses.

Materials and Methods

Mice and reagents

Five-week-old specific pathogen-free female C57BL/6J and C3H/HeOuJCrl mice were purchased from Charles River (France). TCR δ-chain transgenic mice (B6.129P2-*Tcrd*^{tm1Mom}/J; C57BL/6J background), that lack functional TCR γδ expressing T cells (γδ -/- mice), and their age-matched wild type littermates were purchased from the Jackson Laboratory (Maine, USA), and were 7-10 weeks of age. All mice were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum.* Experiments were approved by the Animal Experiments Committee of the Utrecht University.

Peanuts from the Golden Peanut plant (Alpharetta GA, USA) were kindly provided by Intersnack Nederland BV (Doetinchem, The Netherlands) and peanut extract (PE) was prepared as previously described (23, 24). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Treatment protocols

Oral sensitization towards PE: Mice (n=8) were orally gavaged with 6 mg PE mixed with 15 µg CT for three consecutive days, and dosing was repeated every week for three weeks (exposure on days 0, 1, 2, 7, 14 and 21). Control groups received PBS plus CT. All mice received a challenge of 12 mg PE on day 34, and were sacrificed one day later.

To block γδ TCR function, mice were injected i.p. with 400 µg of the anti-γδ TCR antibody UC7-13D5 (UC7, specific for TCRδ chain) in PBS two days before onset of oral sensitization Injections with 200 µg of the antibody were repeated on days -1, 3, 6, 13 and 20 (fig. 1).

To study the efficacy of UC7 treatment and the subsequent effect of CT exposure, mice (n=4) were treated orally with PBS or PE plus CT for 3 days and sacrificed at day 0, 3,

7 or 14 (fig.1). Blocking of γδ T cells was achieved via two i.p. injections with 200 µg UC7 preceding initial PE exposure.

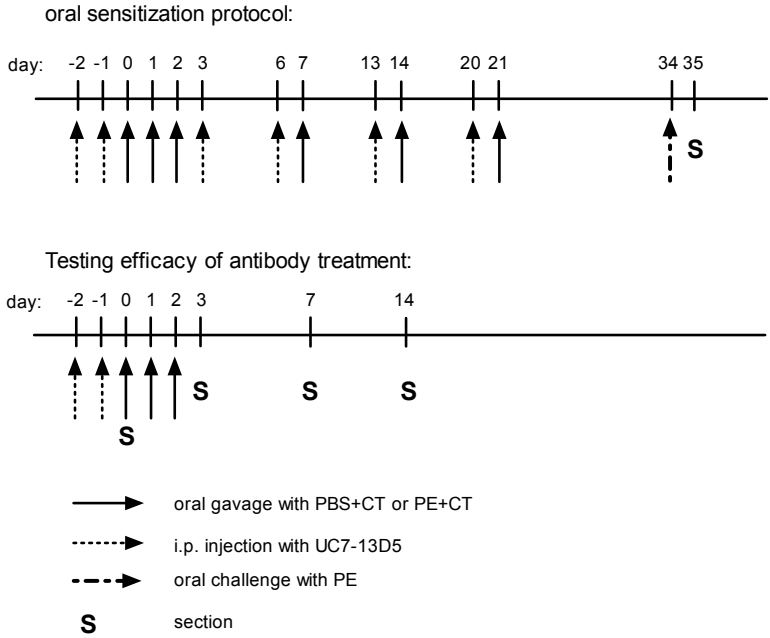


Figure 1: Schematic overview of treatment protocols

Measurement of PE-specific IgG1 and IgG2a in serum

PE-specific antibodies in serum were detected by ELISA. In brief, Highbond plates (Costar) were coated overnight at 4 °C with 10 µg/ml PE in PBS, followed by 1h blocking with ELISA buffer (50 mM Tris buffer, pH 7.2, supplemented with 137 mM NaCl, 2 mM EDTA, 0.05% Tween and 0.5% BSA,). Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (1000 arbitrary units of PE-specific antibodies of various immunoglobulin subclasses) were included on all plates. In order to detect IgG1 or IgG2a, alkaline phosphatase (AP)-conjugated antibodies (Southern Biotechnology Associates) were added and left for 1h at room temperature. Subsequently, p-nitrophenylphosphate (1 mg/ml) in diethanolamine buffer was used as a substrate and color reaction was stopped with 10% EDTA. Absorbance was recorded at 405 nm.

Measurement of PE-specific IgE and IgA in serum

IgE and IgA levels in serum were determined by means of a sandwich ELISA. In brief, Highbond plates (Costar) were coated overnight at 4 °C with 1 µg/ml anti IgE or anti IgA antibody (BD-Pharmingen, Erembodegem, Belgium) in PBS, followed by 1h blocking with ELISA buffer. Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (containing 1000 arbitrary units of PE-specific IgE or IgA) were included on all plates. Subsequently, PE coupled to digoxigenin (DIG) in HPE-buffer (Sanquin, Amsterdam, the Netherlands) was added and left for 1h at room temperature. The coupling of DIG to PE was performed according to the instructions of the manufacturer (Boehringer, Mannheim). Streptavidin-coupled anti-DIG (Roche diagnostics) was added for 1h at room temperature, followed by TMB substrate (0.1 mg/ml). The color reaction was stopped after 15 minutes with 2M H_2SO_4 and absorbance was measured at 450 nm.

Measurement of mouse mast cell protease 1 (mMCP1) in serum

mMCP1 was determined using a specific ELISA kit (Moredun scientific Ltd, Midlothian, Scotland) and performed according to instructions of the manufacturer.

Isolation of intestinal lymphocytes

For isolation of intestinal epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), the small intestine was removed, and fecal contents were flushed with 20 ml of HBBS (Invitrogen) supplemented with 15 mM Hepes (Invitrogen). After removing Peyer's patches (PP), the small intestine was opened longitudinally, cut into 1-cm pieces, and shaken vigorously for 20 min at 37 °C in HBBS containing 15 mM Hepes, 100 U/ml penicillin, streptomycin 100 µg/ml (both Invitrogen), 5 mM EDTA, 1 mM DTT and 10% FCS (Greiner) (HBBS/EDTA). The mixture was passed through a gauze and remaining intestine was used to isolate LPL, whereas the filtrate was washed once in HBBS/Hepes and centrifuged through a 45—67.5% discontinuous Percoll gradient (GE Healthcare) at 600g at 20 °C for 20 min to collect IEL.

For LPL isolation the remaining intestine was shaken for another 20 min at 37 °C in HBBS/EDTA, and washed in RPMI1640 (Invitrogen), supplemented with penicillin/streptomycin, 10% FCS and 1 mM DTT (RPMI/FCS/DTT). The intestine was digested through incubation for 45 min at 37 °C in RPMI/FCS/DTT supplemented with 1 mg/ml collagenase (Roche) and 20 µg/ml DNAse. Cells were collected via 100 µm gauze and tissue digestion was repeated, after which both cell fractions were

pooled and washed once in HBBS/Hepes. LPL were enriched by a 40-100% discontinuous Percoll gradient.
To obtain single cells, collected PP were shaken for 20 min at 37 °C in HBBS/ EDTA, washed once in RPMI/FCS, cut in small fragments, and digested in RPMI/FCS containing collagenase/DNAse. Cells were passed through a 70 µm gauze to obtain single cells, and washed once in HBBS/Hepes.
Lymphocytes from mesenteric lymph nodes (MLN) and spleen were obtained by squeezing the organs through a 70 µm gauze, after which single cells were washed once in RPMI/FCS. To remove erythrocytes in spleen (only when spleen was used in flow cytometry), cells were incubated with erylysis buffer (0.16M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA pH 7.4) for 1 min, and washed once in PBS. All cell suspensions were kept on ice, unless stated otherwise.

Flow cytometry

For flow cytometric analysis $2*10^5$ cells were stained in staining buffer (PBS containing 0.25% BSA, 0.5 mM EDTA and 0.05% NaN_3) with fluorecein isothiocyanate (FITC)-, R-phycoerythrin (RPE)-, peridinin chlorphyll protein (PerCP)- or allophycocyanin (APC)- labeled antibodies. Cells were incubated for 30 min at 4 °C in the dark. All samples were pre-incubated with anti-CD16/CD32 (2.4G2) to block FcR, and stored in 0.4% formaldehyde until acquisition.
Cell characterization was based on following antibodies (obtained from e-Bioscience or BD-Pharmingen): CD3 (145-2C11); CD8α (53-6.7); TCRβ (H57-597) and TCRδ (GL3).
Data were acquired by means of FACSCalibur (Becton Dickinson) and analysis was performed using Weasel (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Lymphocytes were selected based on forward scatter (FSC) and side scatter (SSC) patterns, and cells within this gate were used to calculate the percentage of lymphocytes.

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions ($3.75*10^5$ per well) were incubated in RPMI1640 supplemented with pen/strep and 10% FCS in the presence or absence of 100 µg/ml peanut extract in 96-well plates for 96h at 37 °C, 5% CO_2 after which culture supernatants were harvested and stored at -20°C until analysis.
In the culture supernatants, levels of IFN-γ, IL-4, IL-5, IL-13 and IL-10 were determined by commercially available sandwich ELISA (IL-10 was obtained from BD-

Pharmingen, others were from e-Bioscience), according to the instructions of the manufacturers.

Statistics

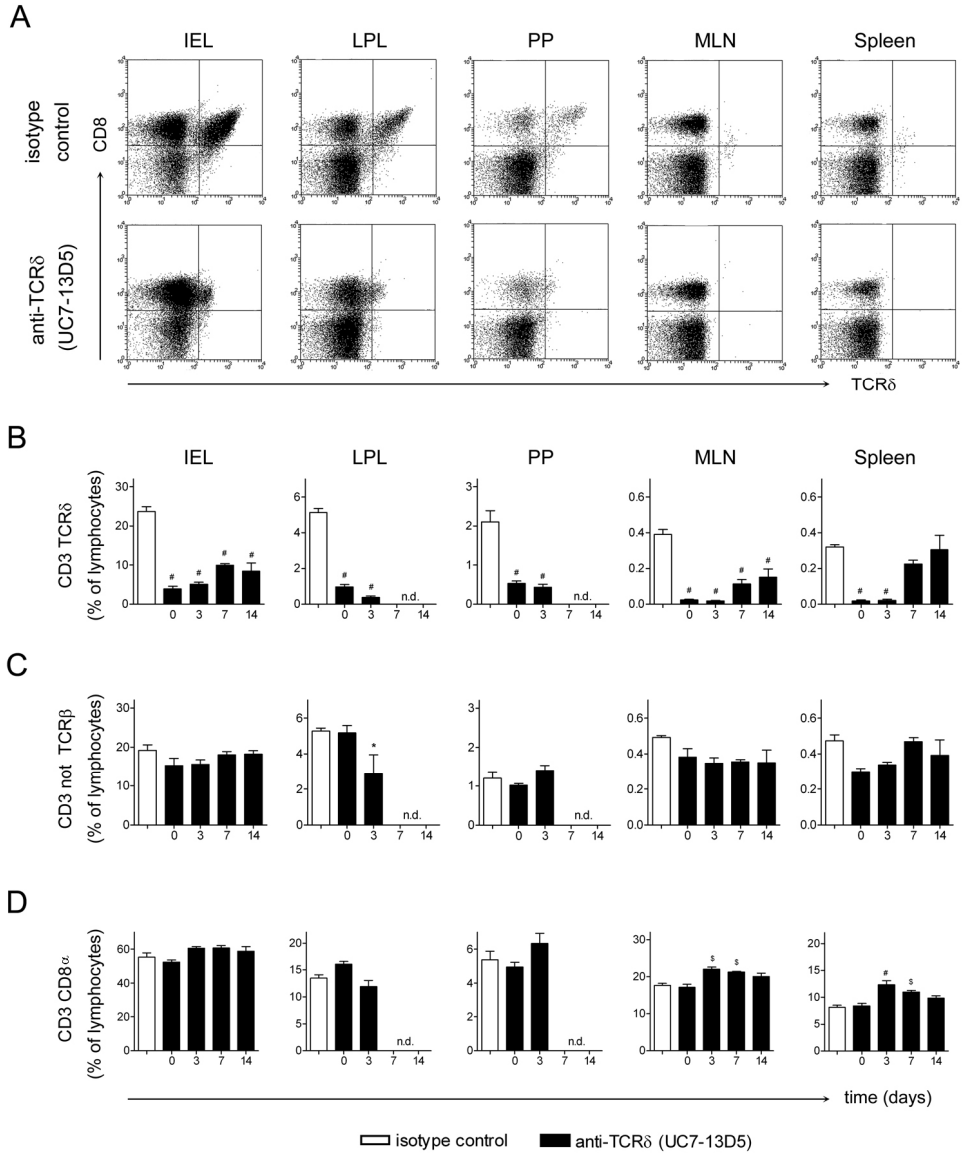
Data are presented as the group mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism software. One-way ANOVA was performed followed by Bonferroni as a post-hoc test. Before testing, data on antibody levels and cytokine production were logarithmic transformed. Differences were considered significant when p values were < 0.05.

Results

Treatment with γδ TCR-specific antibody UC7 resulted in blockade of γδ TCR but not depletion of γδ T cells.

We investigated the effect of UC7 treatment on γδ T cells using the protocol indicated in fig.1. Administration of UC7 antibody during 2 consecutive days resulted in immediate disappearence of detectable γδ T cells in intestine and lymphoid organs (detected with a different TCRδ specific antibody: GL3) which lasted for at least 7 days (figure 2a-b). It is reported that treatment with UC7 results in blocking and internalization of the receptor (20). We therefore analyzed the effect of antibody treatment on CD3-expressing T cells that do not express TCRβ, assuming that this cell population consists merely of γδ T cells. As shown in fig 2c, only a very small decrease in $CD3^+$ $TCR\beta^-$ cells occurred upon treatment with UC7. Moreover, most γδ T cells in intestine are CD8α cells, implicating that a decrease in γδ T cell numbers due to depletion would lead to reduced CD8α T cell numbers, but this was not the case (fig 2d). Together, these findings demonstrated that treatment with UC7 *in vivo* blocks the γδ TCR, rather than depletes γδ T cells.

Figure 2: Effect of administration of UC7 on γδ T cells in the intestine and lymphoid organs.
C57BL/6 mice (n=4) were treated with anti-γδ TCR antibody (UC7) during 2 consecutive days, and T cell subsets were analyzed 1 day later by means of flow cytometry. **A**. Representative dotplots of TCRδ expression (stained with GL3) in various organs after treatment with UC7. T cells were selected based on FSC/SSC pattern and CD3 expression, after which they were analyzed for CD8 and TCRδ. **B**. percentage of CD3 TCRδ-expressing lymphocytes in time. **C**. percentages of CD3 positive lymphocytes that lack TCRβ expression. **D**. percentage of CD3 CD8α-expressing lymphocytes in time. Data are depicted as mean ± SEM of 4 mice; *: $p < 0.05$; $: $p < 0.01$; #: $p < 0.001$ compared to isotype control. n.d. = not determined


A
IEL
LPL
PP
MLN
Spleen
isotype control
anti-TCRδ (UC7-13D5)
CD8
TCRδ
B
CD3 TCRδ (% of lymphocytes)
n.d.
C
CD3 not TCRβ (% of lymphocytes)
D
CD3 CD8α (% of lymphocytes)
time (days)
isotype control
anti-TCRδ (UC7-13D5)

Blockade of γδ TCR by UC7 did not interfere with CT-induced decrease in γδ T cells.
Next, mice were treated with UC7, subjected to the PE+CT protocol (see fig. 1) and the number of T cell subsets was analyzed in the intestine and lymphoid organs. In line with previous findings (chapter 4 of this thesis), exposure of naive mice to PE+CT resulted in decrease in γδ T cells from the intestine and PP, accompanied by a decrease of $CD8\alpha^+$ T cells and of $CD3^+$ $TCR\beta^-$ cells (both of which are mostly γδ T cells in the intestine). Treatment with anti-TCRδ antibodies followed by PE+CT exposure also resulted in loss of $CD8\alpha^+$ T cells and of $CD3^+$ $TCR\beta^-$ in PP (fig. 3b-c), IEL and LPL (data not shown), whereas these populations were not changed upon antibody treatment alone. The percentage of detectable γδ T cells was already low due to blockade of the TCRδ by UC7 (fig 3a). Findings indicate that the effect of PE+CT on γδ T cells is not disturbed by TCR blockade.

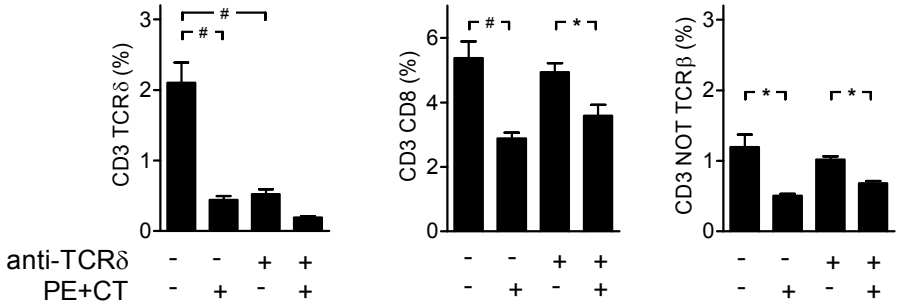


Figure 3: Effect of exposure to PE+CT on γδ T cells in PP after treatment with anti-TCRδ antibodies
C57BL/6 mice (n=4) were treated with anti TCRδ antibodies (UC7) and subsequently exposed to PE+CT for 3 consecutive days. Cell populations in PP were analysed by means of flow cytometry on day 3. Lymphocytes were selected based on FFS-SSC pattern and analyzed for co-expression of CD3 and TCRδ (GL3); CD3 and CD8; CD3 but not TCRβ. Data are represented as mean + SEM. *: $p < 0.05$; #: $p < 0.001$

Treatment with anti-γδ TCR antibodies resulted in enhanced sensitization to peanut.
Exposure to PE+CT resulted in induction of PE-specific IgG1, IgG2a and IgE in both C3H/HeOuJ and C57BL/6 mice (fig 4 a-b). In addition, oral PE challenge induced mast

cell degranulation, measured by mMCP1 levels in serum, in sensitized C3H/HeOuJ mice (fig 4c). Treatment with anti-γδ TCR antibodies increased PE-specific IgE levels in both mouse strains, without affecting IgG1and IgG2a (figure 4a-b). mMCP1 levels after oral challenge were increased as well (fig 4c). Mice that were exposed to PE in the absence of CT did not develop PE-specific antibody responses, irrespective of anti-γδ TCR treatment (data not shown). Together, these data are indicative of increased sensitization due to UC7 treatment and hence of a regulatory effect of γδ T cells on allergic sensitization.

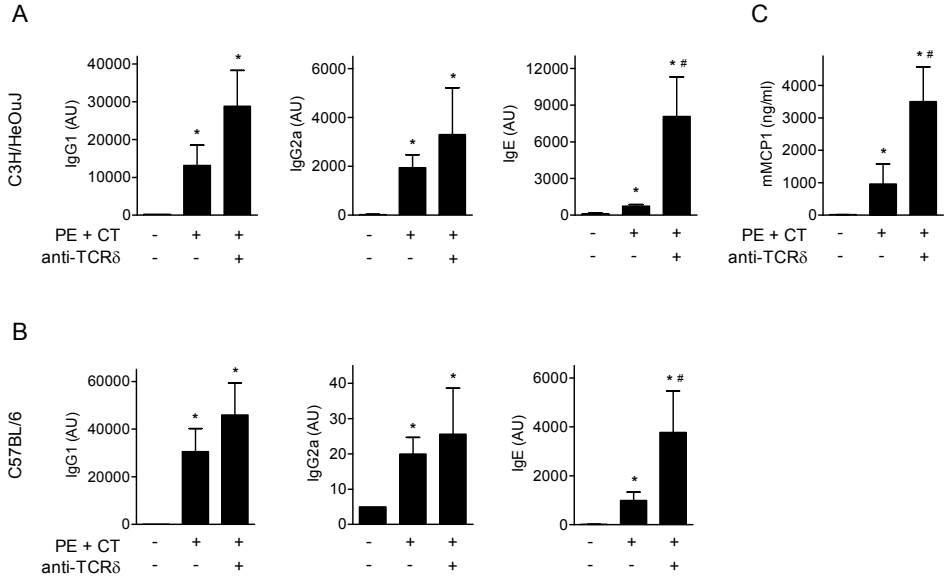


Figure 4: Effect of treatment with anti γδ-TCR antibodies on PE-specific antibody responses in C3H/HeOuJ and C57BL/6 mice.
Mice were exposed to PE+CT during a 4 week period, as described in materials and methods. Prior and during sensitization indicated groups received the anti γδ TCR antibody UC7. PE-specific IgG1, IgG2a and IgE levels in serum of C3H/HeOuJ mice (**A**) and C57BL/6 mice (**B**) were determined by ELISA. **C.** mMCP1 levels in C3H/HeOuJ mice were determined in blood samples that were collected within 45 minutes after oral PE challenge. Data are presented as mean ± SEM from 8 mice after LOG transformation. *: $p < 0.05$ compared to no sensitization; #: $p < 0.05$ compared to no antibody pre-treatment.

PE-specific T cell responses were enhanced after anti γδ TCR pre-treatment.

To investigate PE-specific T cell responses, splenocytes and MLN-derived lymphocytes from sensitized mice were cultured for 96h in the presence or absence of PE. Exposure to PE+CT resulted in induction of IFN-γ, IL-13, IL-10 and IL-5 in C3H/HeOuJ and C57BL/6 mice (table 1). IL-4 levels were below detection limit (4 pg/ml; data not shown). Cytokine levels in spleen and MLN increased significantly in C3H/HeOuJ mice after treatment with anti-γδ TCR antibodies (Table 1). In contrast, no significant differences in cytokine production were found in C57BL/6 mice. Notably, anti-γδ TCR treatment also elevated spontaneous cytokine production, after culture in the absence of PE, in C3H/HeOuJ mice (not in C57BL/6). Concluding, these data show that T cells responses in C3H/HeOuJ mice are increased upon blocking of the γδ TCR.

		IFN-γ		IL-13		IL-10		IL-5	
Ex-vivo restimulation:		*medium*	*PE*	*medium*	*PE*	*medium*	*PE*	*medium*	*PE*
In-vivo treatment									
PE+CT	anti TCRδ								
C3H/HeOuJ									
spleen									
-	-	17 ± 2	30 ± 9	10 ± 2	22 ± 7	<15	29 ± 5	<4	<4
+	-	15 ± 1	1278 ± 544 *	10 ± 2	197 ± 48 *	<15	739 ± 284 *	<4	26 ± 14 *
+	+	2370 ± 1158 *#	3798 ± 1561 *	12979 ± 2470 *#	12288 ± 2271 *#	8513 ± 1391 *#	7795 ± 1211 *#	1110 ± 202	1945 ± 320 *#
MLN									
-	-	<16	<15	<8	<8	<15	<15	<4	<4
+	-	<16	104 ± 56 *	11 ± 3	301 ± 141*	<15	362 ± 273 *	6 ± 2	796 ± 350 *
+	+	<16	73 ± 28 *	1321 ± 343 *#	6389 ± 1531*#	344 ± 141 *#	1031 ± 377 *#	2743 ± 783 *#	2873 ± 601 *#
C57BL/6									
spleen									
-	-	<16	267 ± 247	<8	209 ± 175	<20	88 ± 51	<8	19 ± 11
+	-	<16	465 ± 133 *	<8	411 ± 81 *	21 ± 1	186 ± 46 *	<8	46 ± 20 *
+	+	<16	1107 ± 434 *	33 ± 11	856 ± 235 *	30 ± 5	473 ± 210 *	<8	80 ± 21 *
MLN									
-	-	<16	<16	<8	54 ± 44	<30	30 ± 0	<4	17 ± 11
+	-	<16	42 ± 16	<8	341 ± 124	<30	128 ± 36 *	<4	73 ± 22
+	+	<16	136 ± 74	<8	579 ± 94 *	<30	279 ± 160 *	<4	245 ± 67 *#

Table 1: Effect of anti- γδ TCR antibody treatment on cytokine production by lymphocytes from spleen and MLN.
Mice were exposed to PE+CT for 4 weeks as described in materials and methods. When indicated, mice were treated with antibodies to γδ TCR (UC7) prior to sensitization. Lymphocytes from spleen and MLN from C3H/HeOuJ mice or C57BL/6 mice were cultured in the absence (medium) or presence of PE for 96h. Cytokine levels were determined in supernatant by means of specific ELISA. Data are presented as mean ± SEM for 8 mice per group. *: $p < 0.05$ for C57BL/6 and $p < 0.001$ for C3H, compared to no sensitization; #: $p < 0.001$ (< 0.05 for IL5) compared to no antibody treatment.

Wildtype mice and γδ -/- mice developed comparable PE-specific responses upon sensitization to PE+CT.

In addition to blocking of the γδ TCR using a specific antibody, we used γδ -/- mice to investigate the role of γδ T cells in allergic sensitization. Levels of PE-specific IgG1 and IgE after PE+CT exposure were not different in γδ -/- mice compared to their wildtype littermates (fig. 5a).

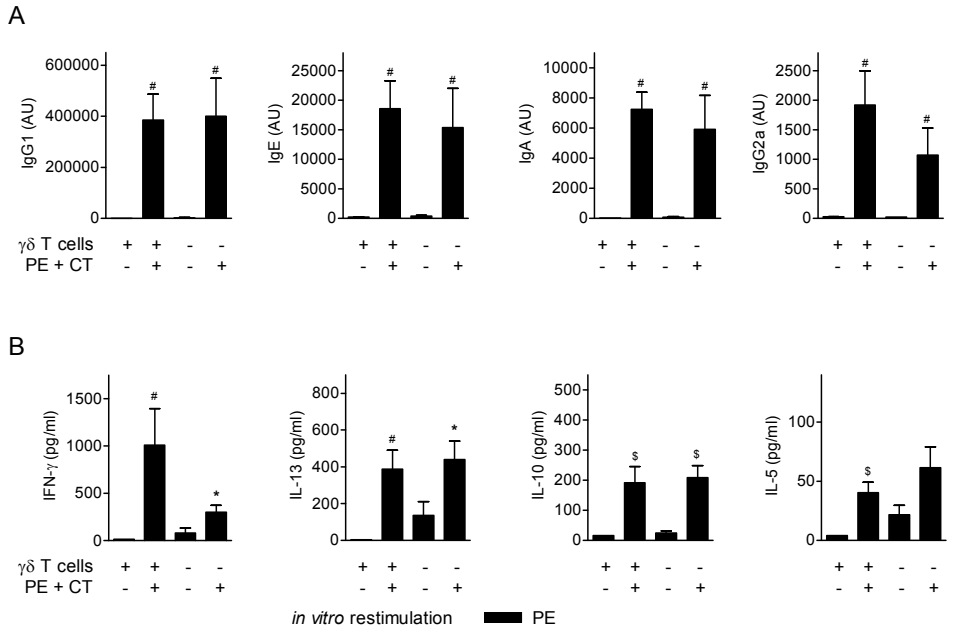


Figure 5: Exposure of γδ T cell -/- mice to PE+CT
Functional γδ T cell -/- mice, and their wild type littermates were exposed to PE+CT for 4 weeks as indicated in materials and methods, and analyzed for PE-specific responses. **A.** PE-specific IgG1, IgE, IgA and IgG2a in serum after 28 days were determined by ELISA. **B.** Splenocytes were cultured for 96h in the presence of PE, and cytokine production in supernatant was determined by means of specific ELISA. No cytokine production could be detected when splenocytes were cultured in the absence of PE. Data are depicted as mean ± SEM from 6 (control) or 8 (PE+CT) mice per group. *: $p < 0.05$; $: $p < 0.01$; #: $p < 0.001$ compared to control within the same strain.

In addition, no differences in PE-specific IgA or IgG2a in serum could be detected between both strains (fig 5a). To investigate PE-specific T cell responses, splenocytes were cultured for 96h in the absence or presence of PE. Levels of IL-5, IL-10 and IL-13 in sensitized mice after PE restimulation did not differ between both strains,

whereas the levels of IFN-γ tended to be lower in γδ-/- mice compared to the wild type although differences were not significant (fig 5b). Levels of IL-4 were again below detection limit (4pg/ml, data not shown). Cytokine levels were also below detection limit when splenocytes were cultured in the absence of PE (data not shown). Concluding, these data indicate that allergic sensitization to PE is comparable between γδ -/- mice and wild type mice. Immunohistochemical evaluation of the intestines of γδ-/- mice confirmed the absence of γδ T cells in these mice. However, $CD8\alpha^+$ cells were not reduced compared to wildtype mice suggesting compensation for the lack in γδ T cells (data not shown).

Discussion

In the previous chapter we have shown that sensitization to PE using the mucosal adjuvant CT was accompanied by a decrease of γδ T cells from the intestine and PP, although the mechanisms by which CT mounted this effect remained unclear. The present study investigated the functional contribution of γδ T cells in sensitization towards food proteins. We found that blocking of the γδ TCR by means of UC7 resulted in enhanced allergic sensitization upon PE+CT exposure, suggesting a regulatory role for γδ T cells.

There is controversy about the effect of *in vivo* treatment with the UC7 antibody on γδ T cells. Initial studies assumed that treatment with this antibody results in depletion of γδ T cells (10, 12, 19). We demonstrate in the present study, that UC7, like GL3, is blocking the γδ TCR, as also suggested by others (20, 21, 25). Whether blockade results in internalization of the TCR, followed by activation of γδ T cells as suggested by others (20) remains unsolved. Further *in vitro* and *in vivo* studies need to be done to investigate possible activation of these cells upon treatment with UC7.

Increased allergic sensitization to PE after functional blocking of the γδ TCR as found in the present study suggests a protective role for γδ T cells in allergic sensitization. This is supported by data on involvement of γδ T cells in oral tolerance. It was shown that transfer of splenic γδ T cells from OVA-tolerized mice resulted in tolerance to OVA in recipients (17, 18), and that pretreatment with anti-TCRδ antibody GL3 resulted in abrogation of tolerance induction (15, 26). Furthermore, oral administration of OVA failed to induce oral tolerance in γδ -/- mice (26). However, our data showed that solely blocking of the γδ TCR is not sufficient to induce sensitization to an orally administered antigen, and that an additional stimulus (like CT) is required to induce

allergic sensitization. These findings are in line with data from previous studies showing that modulation of immune regulation via inhibition of CTLA-4 signaling (24) or via depletion of $CD4^{+}CD25^{+}$ cells (27) was not sufficient to initiate sensitization to PE in this mouse model.

Due to their location, γδ T cells are ideally situated for crosstalk with intestinal epithelial cells (IEC) and immune cells (DCs, B and T cells) in the LP (9). In addition, γδ T cells are able to produce growth factors that promote epithelial cell growth and tissue repair (28, 29). The γδ T cells may recognize and eliminate tumor-, infected- or otherwise "stressed" cells, via interaction with the NKG2D receptor (30) or via other receptors, and contribute to immune surveillance (31, 32). Exposure to CT might result in stressed epithelial cells, and thus indirectly trigger γδ T cells. Whether the γδ TCR itself is involved in responses to stressed cells is not clear. The diversity of the γδ TCR is limited, and it might act as a pattern recognition receptor to mediate innate immune responses (33, 34). Ligands for γδ T cells that have been determined thus far are predominantly of host rather than foreign origin (35) and recognition of these molecules may be a stimulus for γδ T cells to regulate endogenously induced immune responses.

Next to γδ T cells, other innate effector cells, like CD8αα TCRαβ cells, NK and NK-T cells express the NKG2D receptor, and contribute to effective stress-responses and immune surveillance (32). This would implicate that depletion of these innate cells will also result in enhanced allergic sensitization. Indeed, increased PE-specific responses were observed upon depletion of NK cells using anti-asialo-GM in our mouse model (M. Marcondes Rezende, manuscript in preparation). Thus it is very well conceivable that these innate cells partly compensate for the lack of γδ T cells in γδ -/- mice, resulting in a normalized response upon sensitization with PE+CT in these mice, as observed in the present study. The fact that the present study did not show a reduction in CD8α cell numbers in the intestine of γδ -/- mice points towards a compensation for the lack in γδ T cells. Additional blockade or depletion of innate cell populations such as CD8αα cells and NK(T) cells in γδ -/- mice may result in increased allergic sensitization, and give more insight into the contribution of innate cells in food allergy.

The importance of γδ T cells in allergic responses towards ovalbumin as an oral antigen was demonstrated in a study using mast cell-deficient mice that contain very low numbers of γδ TCR-expressing IEL and were found to be highly susceptible to allergic sensitization (37). In addition, upon γδ T cell reconstitution these mast-cell deficient mice displayed diminished allergic responses (38). The role of γδ T cell numbers in allergic sensitization was also confirmed in a mouse study showing that

apple-derived polyphenols (tannins), as possible γδ TCR ligands (39, 40), were able to dose dependently inhibit the induction of allergic sensitization to OVA. This inhibition was accompanied by an increase in the number of γδ TCR-positive IEL (41). Other studies on fruit-derived tannins describe the increase in γδ T cells and the subsequent improvement of innate immune responses in humans (39, 40, 42).
In conclusion, these results suggest that γδ T cells are involved in regulation of food allergic responses. Present data suggest that modulation of γδ T cells (and other innate immune cells) in the intestine may contribute to strategies to prevent and possibly treat food allergy.

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Chapter 6

Diclofenac enhances allergic responses in a mouse peanut allergy model

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Abstract

Diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) interfere with the cyclo-oxygenase (COX)-mediated synthesis of prostaglandins, resulting in inhibition of inflammatory immune responses. It is also known that NSAIDs are able to induce gastrointestinal damage. It was therefore of interest to investigate whether diclofenac is able to enhance the sensitization to food antigens.

For this purpose, mice were sensitized to peanut (PE) by using cholera toxin (CT) in the absence or presence of various concentrations of diclofenac. After 28 days, diclofenac-exposed animals showed increased levels of PE-specific IgG1, IgG2a and IgE in serum compared to vehicle-treated animals. Furthermore, PE-induced cytokine production in spleen was elevated upon diclofenac treatment. Microscopic examination of the small intestine did not reveal extensive intestinal damage in diclofenac-treated mice. Importantly, diclofenac did not induce PE-allergic responses in the absence of CT. Induction of oral tolerance to PE was induced via feeding of PE three weeks before sensitization. Diclofenac was not able to disturb oral tolerance induction as PE-specific IgG1 and IgE were still suppressed in PE-tolerized mice compared to non-tolerized mice. On the other hand, PE-specific T cell responses were significantly higher in diclofenac-treated tolerized mice compared to vehicle treatment. Whether the effect of diclofenac is mediated via COX-derived prostaglandins, or is the result of intestinal damage remains to be elucidated. Together, these data point towards an increased risk for induction of allergic responses by diclofenac, when other circumstances are also in favor of induction of allergy.

Introduction

Food allergy is reported in up to 8% of children below 3 years in the United States, and approximately 2% of the adults experience food allergic reactions (1). However, risk factors that are involved in induction of food allergic responses remain obscure (2). The usual response to orally ingested antigens in the intestine is the induction of tolerance, but the presence of an adjuvant or disturbance of the epithelial barrier function can result in adverse reactions (3, 4). For instance, the use of non-steroidal anti-inflammatory drugs (NSAIDs) occasionally enhances wheat or shrimp-induced anaphylaxis (5), and has been shown to impair oral tolerance induction to β-lactoglobulin (6) and OVA (7). Recent studies show that diclofenac is able to induce sensitization to co-injected antigen (TNP-Ovalbumin) (8, 9) accompanied by antigen-specific antibody responses. It is therefore of interest to investigate whether administration of diclofenac is able to elicit or enhance the response towards food proteins.

NSAIDs, like diclofenac, are originally designed to inhibit inflammation and work via the inhibition of cyclo-oxygenase (COX) enzymes. COX enzymes (constitutive COX-1 and inducible COX-2) catalyze the conversion of membrane-derived arachidonic acid in biologically active molecules (prostanoids) like prostaglandins (for example PGE_2, PGD_2) tromboxanes (10) and the more recently discovered resolvins (11). COX-1 appears to be responsible for basal prostanoid synthesis and COX-2 is especially important in inflammatory processes in peripheral tissues. In general, reduction in prostaglandin production results in control of inflammation, pain and fever (12). However, prostaglandins also have important regulatory effects on APC maturation, migration and secretion of cytokines (10, 13). For instance, PGE_2 inhibits the production of IL-12 by DCs, thereby lowering the Th1-promoting capacity of DCs and shifting T cell responses towards Th2 (14-16). Furthermore, PGE_2 promotes the induction of regulatory T cells via induction of FoxP3 expression and stimulation of TGFβ secretion (13, 17). In the intestine, COX-2 is constitutively expressed by stromal cells (18) and prostaglandins are key factors in physiological regulation of homeostasis (19) and induction of oral tolerance to dietary antigens (14, 20). Another important contribution of COX-2-derived PGE_2 in the intestine is the maintenance of epithelial integrity (21, 22). Recent studies in experimental murine colitis have shown that PGE_2 is involved in repair of mucosal injury via the induction of epidermal growth factor receptor (EGFR) on epithelial cells (21).

So, whereas on the one hand COX-inhibiting drugs are used to limit inflammation and relieve pain, they may, on the other hand, disturb mucosal immunoregulatory processes and intestinal epithelial integrity, resulting in stimulation of inflammatory responses. Ultimately, this can contribute to development of intestinal disorders such as food allergy and inflammatory bowel disease (IBD).
In this study we investigated the effect of diclofenac as a classic NSAID on peanut-induced allergic sensitization and peanut specific oral tolerance induction using established mouse models of food allergy. Results show that oral administration of diclofenac is able to enhance the allergic response towards peanut (PE) in the presence of a mucosal adjuvant (cholera toxin, CT). Whether the effect of diclofenac is based on modulation of the immune response or is related to disturbance of mucosal barrier function, and to what extent COX-inhibition is involved, remains to be elucidated.

Materials and Methods

Mice and reagents

Five-week-old specific pathogen-free female C3H/HeOuJCrl mice were purchased from Charles River (France). All mice were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum*. The experiments were approved by the Animal Experiments Committee of the Utrecht University.
Peanuts from the Golden Peanut plant (Alpharetta, GA, USA) were kindly provided by Intersnack Nederland BV (Doetinchem, The Netherlands) and peanut extract (PE) was prepared as previously described (23). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Treatment protocols

Oral sensitization towards peanut: Mice (n=8) were exposed by intragastric gavage to 6 mg PE mixed with 15 µg CT for three consecutive days, and dosage was repeated every week for three weeks (exposure on day 0, 1, 2, 7, 14 and 21). Control groups received PBS plus CT. Diclofenac (1 mg/kg; 10 mg/kg or 25 mg/kg), dissolved in sterile water (Braun) was administered via intragastric gavage 2h before PE+CT sensitization on days 0, 1 and 2. All mice received a challenge of 12 mg PE on day 26,

and were sacrificed one day later (fig 1 upper panel). In order to investigate the effect of diclofenac on COX-expression in the intestine, groups of mice (n=3) were sacrificed 2h after diclofenac exposure.

Induction of tolerance towards peanut: Mice (n=8) received 1 mg PE or PBS via intragastric gavage for three consecutive days in order to induce oral tolerance. Diclofenac (1 mg/kg) was administered orally 2h before tolerance induction. Immunization was performed i.p. with 100 µg PE in alum (Imject, Pierce) 21 and 28 days after initial exposure, and mice were sacrificed on day 37 (fig 1 lower panel).

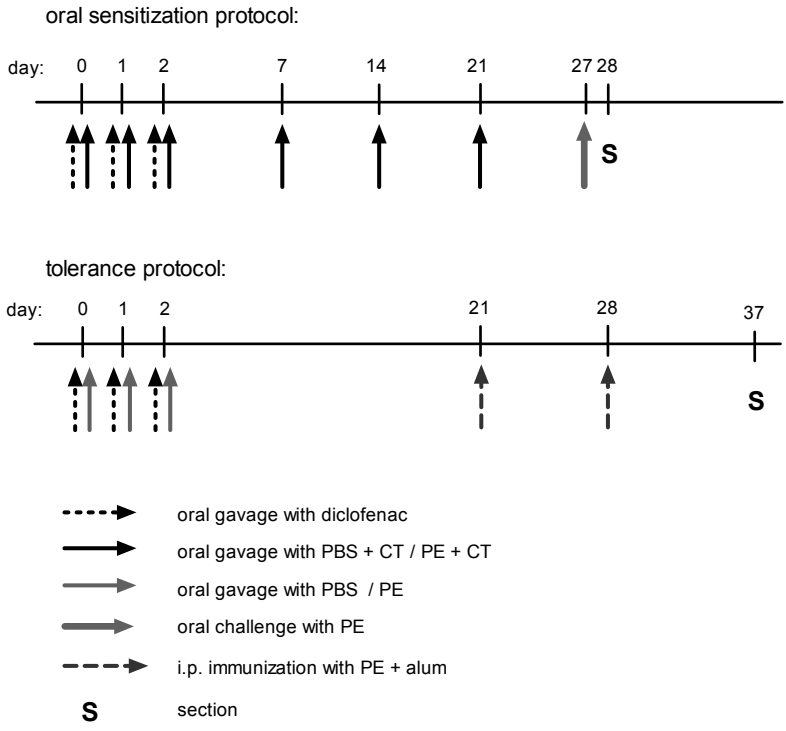

Figure 1. Treatment protocols.

Measurement of serum IgG1 and IgG2a

Blood was collected at several time points and PE-specific antibodies in serum were detected by ELISA. In brief, highbond plates (Costar) were coated overnight at 4 °C with 10 µg/ml PE in PBS, followed by 1h blocking with ELISA buffer (50 mM Tris buffer, pH 7.2, supplemented with 137 mM NaCl, 2 mM EDTA, 0.05% Tween and 0.5% BSA). Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (1000 arbitrary units of PE-specific antibodies of both immunoglobulin subclasses) were included on all plates. In order to detect IgG1 or IgG2a, alkaline phosphatase (AP)-conjugated antibodies (Southern Biotechnology Associates) were added and left for 1h at room temperature. Subsequently, p-nitrophenylphosphate (1 mg/ml) in diethanolamine buffer was used as a substrate and color reaction was stopped with 10% EDTA. Absorbance was measured at 405 nm.

Measurement of serum IgE

IgE levels in serum were determined by means of a sandwich ELISA. In brief, highbond plates (Costar) were coated overnight at 4 °C with 1 µg/ml anti IgE antibody (BD-Pharmingen, Erembodegem, Belgium) in PBS, followed by 1h blocking with ELISA buffer. Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (containing 1000 arbitrary units of PE-specific IgE) were included on all plates. Subsequently, PE coupled to digoxigenin (DIG), diluted in HPE-buffer (Sanquin, Amsterdam, the Netherlands) was added and left for 1h at room temperature. The coupling of DIG to PE was performed according to the instructions of the manufacturer (Boehringer, Mannheim). Streptavidin-coupled anti-DIG (Roche diagnostics) was added for 1h at room temperature, followed by TMB substrate (0.1 mg/ml). The colour reaction was stopped after 15 minutes with 2M H_2SO_4 and absorbance was measured at 450 nm.

Measurement of mouse mast cell protease-1 (mMCP-1) in serum

mMCP-1 was determined using a specific ELISA kit (Moredun scientific Ltd, Midlothian, Scotland) and performed according to instructions of the manufacturer.

Tissue staining

Acetone-fixed cryostat sections of the small intestine were incubated for 1h with anti COX-2 polyclonal Ab (Cayman Ann Arbor, Michigan, USA) diluted in PBS, 1% BSA. Antibody specificity was checked using a COX-2 blocking peptide (Cayman)

according to the instructions of the manufacturer. The antibodies were visualized by first applying biotinylated swine anti-rabbit F(ab')2 fragments (DAKO, Denmark) in PBS, supplemented with 5% normal mouce serum for 1h, followed by 45 min incubation with horseradish peroxidase labeled streptavidin (DAKO, Denmark) and aminoethylcarbazole (AEC) as substrate. Sections were counterstained with Mayer's heamatoxylin (DAKO) and mounted with DAKO Faramount Aqueous mounting medium. In order to determine mucus layer, aceton-fixed cryosections of the small intestine were stained with Periodic Acid Schiff (PAS) followed by staining with Alcian Blue. Images were acquired using a Olympus BX60 microscope with colorview III digital camera and Cell imaging software.

Lymphocyte culture and cytokine measurement

Spleen and MLN single cell suspensions ($3.75*10^5$ per well) were incubated in RPMI1640 supplemented with 100 IU/ml penicillin/streptomycin and 10% FCS in the presence or absence of 100 µg/ml PE in 96-well plates for 96h at 37 °C, 5% CO_2. Culture supernatants were harvested and stored at -20°C until analysis.

In the culture supernatants, levels of IFN-γ, IL-4, IL-5, IL-13 and IL-10 were determined by commercially available sandwich ELISA (IL-10 was obtained from BD-Pharmingen, others were from e-Bioscience), according to the instructions of the manufacturers.

Epithelial cell culture, NF-κB and macrophage inflammatory protein-2 (MIP-2) measurement

The murine small intestinal cell line m-ICc12 stably transfected with a NF-κB luciferase reporter construct, was a kind gift from Dr. A. Vandewalle (INSERM, Paris, France (24, 25)). m-ICc12 cells were cultured in DMEM/F12 (1:1 v/v), supplemented with pen/strep, 60 nM selenium, 5 g/ml bovine transferrine, 2 mM glutamine, 5 g/ml bovine insulin, 2 g/l glucose, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 2% FCS, 50 µg/ml hygromycin and 15 mM HEPES.

Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

To assess activation, m-ICc12 cells were grown to confluence in 96-wells plates (Greiner Bio-one, Germany) and subsequently incubated for 4h with different amounts of diclofenac in the absence or presence of CT (20 µg/ml) or lipopolysaccharide (LPS; 10 ng/ml; O111:B4). Culture supernatant was harvested and stored at -20 °C until determination of MIP-2. Cells were washed once with PBS, and lysed with 30 µl lysis buffer (Promega, Madison WI, USA) Cell-lysates were stored at -80 °C or luciferase

activity was measured immediately by adding luciferase-assaymix (containing 20 mM tricine, 1.07 mM magnesium carbonate hydroxide, 2.67 mM magnesiumsulfate, 0.1 mM EDTA, 33.3 mM dithiotreitol, 261 µM coenzyme A, 470 µM luciferin and 530 µM ATP) to 20 µl sample. Luciferase activity was recorded by means of a luminometer (LUMIstar OPTIMA, BMG Labtech). MIP-2 ELISA (KOMA Biotech., Seoul, Korea) was performed according the instructions of the manufacturer.

Real time quantitative PCR for COX-2 mRNA expression

mICc12 cells were grown to confluence in 6-wells plates (Greiner Bio-one, Germany) and subsequently incubated for 4h with different amounts of diclofenac in the absence or presence of CT (20 µg/ml). Cell were washed with PBS, lysed with RNA Instapure© (Eurogentec, Maastricht, the Netherlands) and stored at -80 °C until isolation of RNA. Approximately 50 mg of the small intestine (without Peyers Patches) and 2 Peyers Patches were dissected and stored at -80 °C until further use. The tissue was homogenized in 1 ml RNA Instapure© by means of a Mixer-Mill (Qiagen). RNA was extracted from the RNA Instapure© samples using chloroform and isopropanol. RNA content was quantified by measuring optical density at 260 nm, and RNA integrity was confirmed by measuring A260/A280 on a SmartSpecPlus (Bio-Rad Laboratories, Veenendaal,the Netherlands). Subsequently, RNA was transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real time quantitative PCR (Q-PCR) was performed using a iCycler iQ system (Bio-Rad), and amplification was done using IQ™ SYBR Green® Supermix (Bio-Rad) with 0.4 µM final primer concentration. Primers used were: COX-2 sense 5'- TGC AAG ATC CAC AGC CTA CC -3', antisense 5'- GCT CAG TTG AAC GCC TTT TG -3'; β-actin sense 5'- ATG CTC CCC GGG CTG TAT -3', antisense 5'- CAT AGG AGT CCT TCT GAC CAT TC – 3'. For each sample mRNA expression was normalized for the detected Ct value of β-actin.

Statistics

Data are presented as the group means ± standard error of the mean (sem) and analyzed using GraphPad Prism software. One-way ANOVA was performed followed by Bonferroni as a post-hoc test. Before testing, logarithmic transformation was performed on antibody and cytokine data from *in vivo* studies to achieve normal distribution. Differences were considered significant when p values were < 0.05.

Results

Administration of diclofenac prior to peanut sensitization resulted in enhanced peanut-specific responses.

To determine whether diclofenac was able to influence peanut-specific responses we administered different doses of this NSAID prior to initial sensitization with PE+CT. Exposure to PE+CT induced PE-specific IgG1, IgG2a and IgE in serum after 28 days (figure 2). Diclofenac exposure resulted in significant increases of serum levels of PE-specific IgG1 and IgE. (figure 2). Furthermore, mMCP-1 as a measure for mast cell release was enhanced upon oral challenge with PE in all cases (figure 3).

When mice were treated with 25 mg/kg diclofenac and PE in the absence of CT, no PE-specific antibodies could be detected (data not shown), indicating that diclofenac is not able to elicit an allergic response in the absence of an adjuvant.

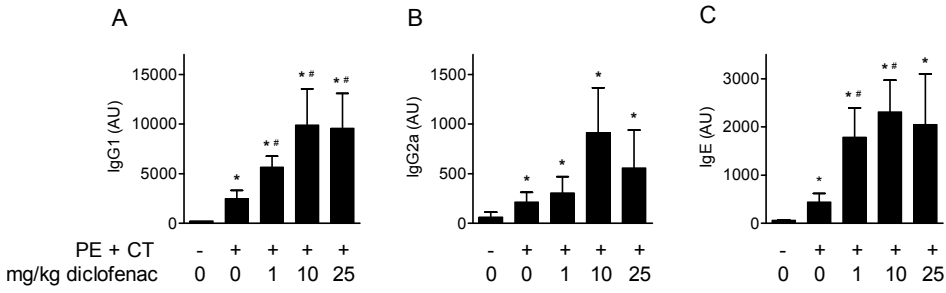

Figure 2: Effect of diclofenac on PE-specific antibody production.
Mice (n=8) were treated with indicated amounts of diclofenac, and subsequently exposed to PBS+CT or PE+CT as described in materials and methods. PE-specific IgG1 (A), IgG2a (B) and IgE (C) in serum were determined by means of specific ELISA. Data are presented as mean ± SEM.
*: $p < 0.05$ compared to control (no peanut sensitization)
#: $p < 0.05$ compared to no diclofenac exposure

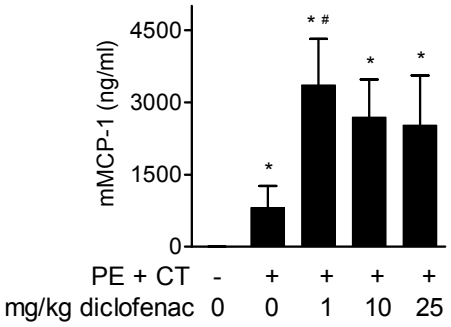

Figure 3: Effect of diclofenac on mMCP-1 release after oral challenge.
Indicated amounts of diclofenac were administered prior to peanut sensitization as described in materials and methods. mMCP-1 was measured by means of a specific ELISA in serum from blood samples taken 30 minutes after oral PE challenge. Data are mean ± SEM from 8 mice per treatment group.
*: $p < 0.05$ compared to control (no peanut sensitization)
#: $p < 0.05$ compared to no diclofenac exposure

Diclofenac resulted in enhanced T cell responses in peanut sensitization.
To investigate the effect of diclofenac on PE-specific T cell responses, splenocytes from treated animals were cultured for 96h in the presence or absence of PE. *In vivo* exposure to PE+CT resulted in detectable levels of cytokines in culture supernatant upon PE stimulation *in vitro* (figure 4). Diclofenac exposure significantly increased levels of IFN-γ and IL-13, but not of IL-10 (figure 4). Levels of IL-4 and IL-5 were below detection limit. These results show that exposure of mice to diclofenac enhanced PE-specific T cell responses.

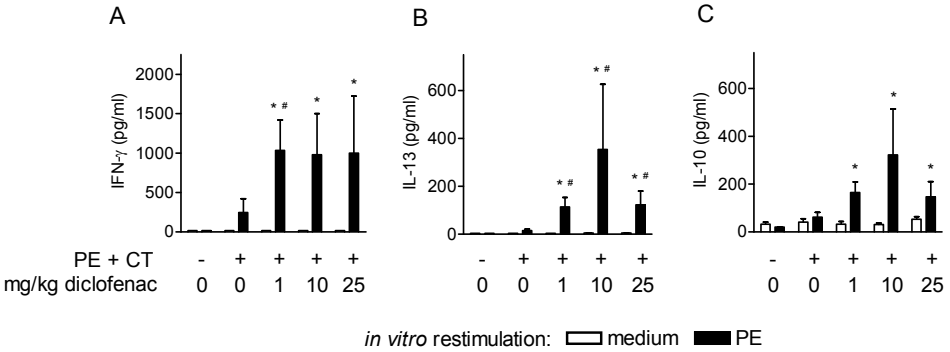

Figure 4: Effect of diclofenac on cytokine production in spleen in PE sensitization model.
Mice (n=8) were treated with indicated amounts of diclofenac and exposed to PE+CT as described in materials and methods. Splenocytes were stimulated *in vitro* with medium or PE for 96h and levels of IFNγ (A), IL-13 (B) and IL-10 (C) were determined in culture supernatant using sandwich ELISA. Data are depicted as mean ± SEM. *: $p < 0.05$ compared to control (no peanut sensitization); #: $p < 0.05$ compared to no diclofenac exposure

Tolerance induction to PE was only slightly altered upon diclofenac exposure.
Next, the effect of diclofenac on the induction of oral tolerance to PE was determined. Mice were orally tolerized by administration of a low dose of PE for 3 consecutive days, and 1 mg/kg diclofenac was administered 2h before oral PE exposure. Mice were immunized 3 weeks later via two i.p. injections with PE/alum at a one week interval (fig. 1). As expected, oral PE exposure prior to immunization resulted in lower PE-specific IgG1 and IgE in serum (fig. 5) and the cytokine production by splenocytes after PE restimulation *in vitro* was diminished (fig. 6) which demonstrated the induction of oral tolerance. Cytokine levels in spleen were below detection limit in all groups after culture in the absence of PE (data not shown).

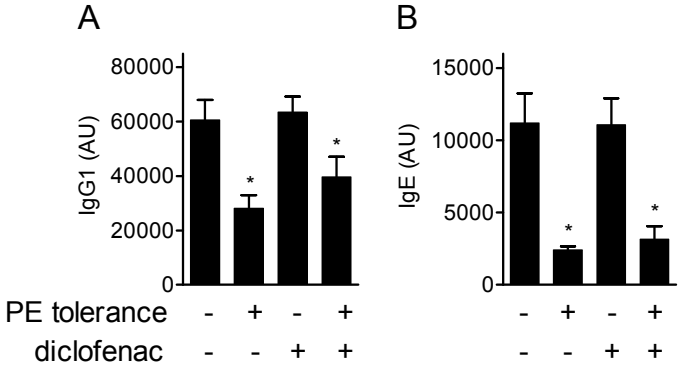

Figure 5. Effect of diclofenac on PE-specific antibodies after oral tolerance induction.
Mice (n=8) were treated with diclofenac (1 mg/kg) prior to oral exposure to PBS or PE, and three weeks later they were immunized i.p. with PE+alum as described in materials and methods. PE-specific IgG1 (A) and IgE (B) were determined in serum. Data are presented as mean ± SEM. *: $p < 0.05$ compared to matched control (pre-treatment with PBS)

Administration of diclofenac prior to oral exposure to PE did not abrogate oral tolerance induction, as PE-specific antibodies in serum were comparably low in diclofenac-treated and vehicle-treated tolerized mice (fig. 5). In addition, cytokine levels in supernatant of cultured splenocytes from PE-tolerized, diclofenac-treated mice were significantly lower compared to non-tolerized mice. Strikingly, the *ex-vivo* PE-specific cytokine levels of diclofenac-treated tolerized mice were significantly higher compared to vehicle-treated tolerized mice, except in case of IL-4 (fig. 6d). Cytokine levels in both non-tolerized groups, one of which received diclofenac, were

comparable, ruling out a non-specific effect of diclofenac on cytokine production. These results point towards a less efficient inhibition of T cell responses after oral tolerance establishment as a result of diclofenac.

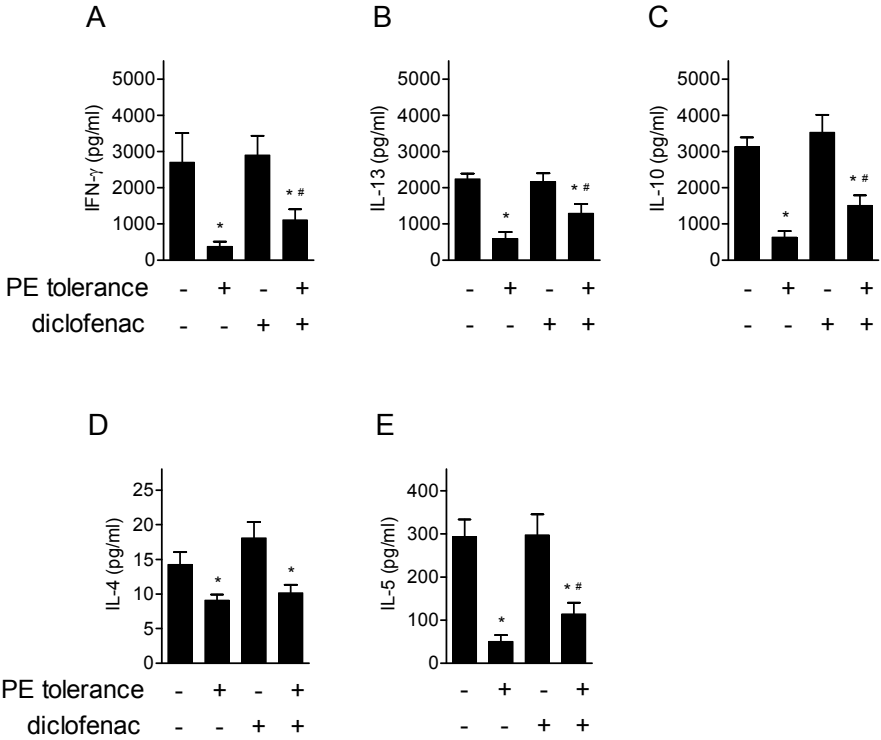

Figure 6: Effect of diclofenac on cytokine levels in spleen after oral tolerance induction.
Mice (n=8) were treated with diclofenac (1 mg/kg) prior to oral exposure to PBS or PE, and three weeks later they were immunized i.p. with PE+alum as described in materials and methods. Splenocytes were stimulated *in vitro* with PE for 96h and levels of IFNγ (A), IL-13 (B), IL-10 (C), IL-4 (D) and IL-5 (E) were determined in culture supernatant using sandwich ELISA. Data are presented as mean ± SEM.
*: $p < 0.05$ compared to matched control (pre-treatment with PBS)
#: $p < 0.05$ compared to no diclofenac exposure

COX-2 expression in the small intestine was not altered upon diclofenac treatment .
COX-2 expression in the small intestine was investigated 2h after diclofenac exposure. Although diclofenac is known to inhibit the activity of COX enzymes, the expression of COX-2 in the small intestine was not diminished as shown by

immunohistochemistry (fig 7a upper panel). These findings were confirmed by RNA analysis as shown in fig. 7b. Furthermore, diclofenac did not result in detectable damage of the small intestine within 2h, as no changes in intestinal morphology or mucus layer (examined with PAS-Alcian Blue staining) could be observed (fig 7a lower panel).

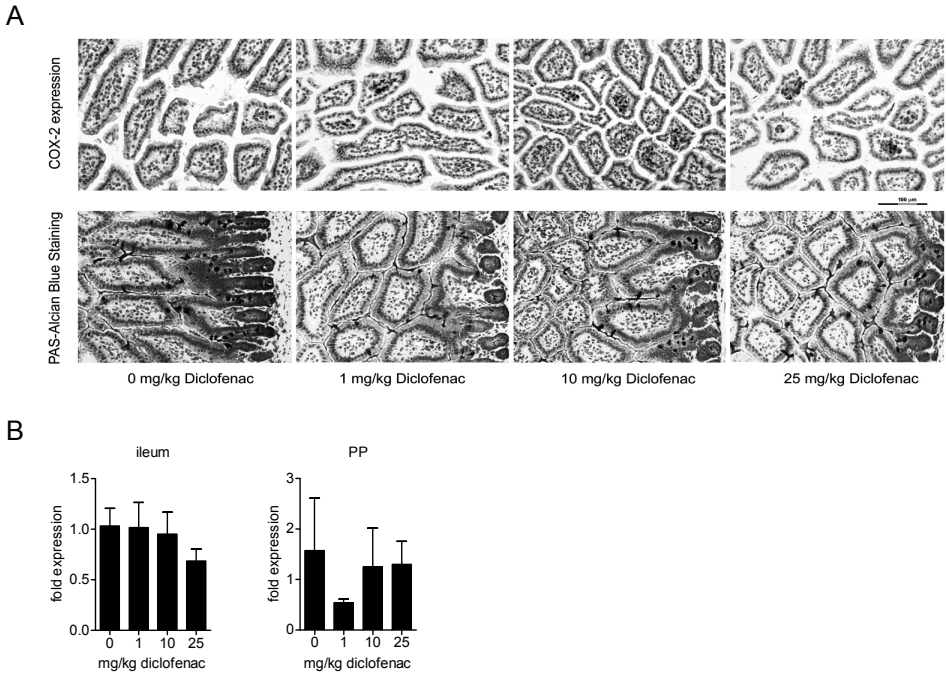

Figure 7: Effect of diclofenac on COX-2 expression in the small intestine
Mice (n=3) were treated orally with 1, 10 or 25 mg/kg diclofenac and small intestine was dissected 2h later. **A**: cryosections of the small intestine were stained in red for COX-2 (upper panel) or with PAS-Alcian Blue (lower panel) as described in material and methods. For color images, see page 167. **B**: RNA in small intestine and PP was determined by rt-PCR as described in material and methods, and is depicted as fold expression, after normalized for β-actin.

Diclofenac did not alter CT-mediated NF-κB activation in epithelial cells in vitro.
In addition, we investigated whether diclofenac could directly modulate epithelial cell responses. Mouse intestinal epithelial cells (m-ICc12), stably transfected with a nuclear factor-κB (NF-κB)-luciferase reporter construct, were incubated with various

concentrations of diclofenac for 4h in the presence or absence of CT or lipopolysaccharide (LPS). NF-κB activation and macrophage inflammatory protein-2 (MIP-2) production were analyzed after 4h of stimulation. Both CT and LPS were able to stimulate m-ICc12 cells to activate NF-κB and to release MIP-2 (fig. 8 a-b). Diclofenac was not able to change CT- or LPS-induced NF-κB activation or MIP-2 release (fig. 8a-b). Conversely, COX-2 mRNA expression was significantly inhibited upon diclofenac exposure (fig. 8c).

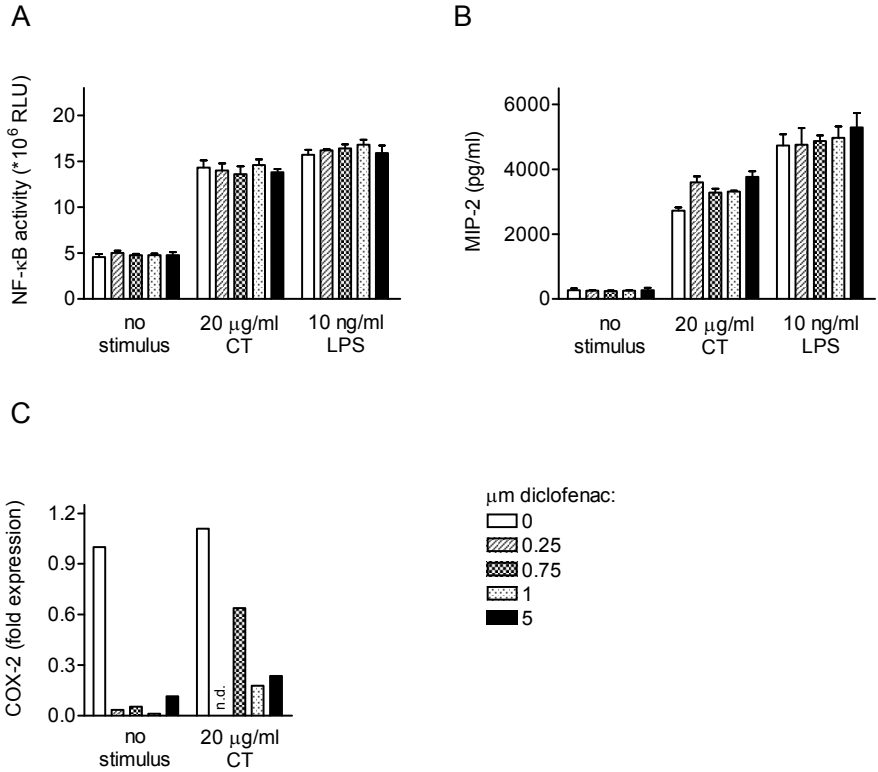

Figure 8: Effect of diclofenac on CT- and LPS-induced activation of mICc12 cells
mICc12 cells were grown to confluence, exposed to 0, 0.25, 0.5, 1 and 5 μM diclofenac, and incubated for 4h with 20 μg/ml CT or 10 ng/ml LPS. **A.**. NF-κB activation is measured using a luciferase reporter construct and expressed as relative light units. **B.** MIP-2 is measured in culture supernatant using ELISA. Data are presented as mean ± sd from a triplicate from one representative experiment out of three. **C.** COX-2 mRNA content, determined by rt-PCR and expressed as fold expression, after normalization for β-actin; n.d.: not determined

Discussion

The current study shows that diclofenac enhanced allergic responses to PE, but did not elicit an allergic response in the absence of CT. Induction of oral tolerance was not abrogated by diclofenac, although T cell responses that are low in case of tolerance were enhanced by diclofenac. We conclude that diclofenac should be considered a risk factor for induction of allergic responses when other circumstances (i.e. genetic susceptibility) are also in favor of allergy induction.

NSAIDs and especially aspirin are connected to urticaria and asthma, although part of the phenomena may be related to hypersensitivity to the drug itself (26-30). Evidence in humans on enhancement of allergic responses comprises mainly of exercise-induced anaphylaxis in combination with aspirin (31). One case study has reported a synergistic effect of NSAIDs in inducing anaphylaxis in a shellfish allergic individual with antigen-specific IgE (32).

In general, treatment with diclofenac will result in the inhibition of COX-activity and hence in modulated production of prostaglandins, with multiple effects. First, prostaglandins are involved in the induction of oral tolerance (7, 14, 20). However, in the present study oral tolerance was only slightly affected by diclofenac. Maybe higher diclofenac concentrations or more continuous exposure is needed to modulate tolerance induction compared to induction of sensitization.

Secondly, as prostaglandins and related leukotrienes are involved in histamine release from mast cells, a direct effect of diclofenac and other NSAIDs on this process is also conceivable, although contradicting results have been obtained (33-35). We did not yet investigate the direct effect of diclofenac on mast cell release in allergic mice.

Third, and probably most relevant to the present study, prostaglandins have been shown to be important in maintaining mucosal homeostasis, and repair of mucosal damage involves the presence of PGE_2 (21, 22). In this context, it is of importance that the use of conventional NSAIDs, like diclofenac, is accompanied with high incidence of mucosal damage, including gastric erosions, ulcers, and perforation and bleeding of the gastroduodenal tract (36-38). Reduced levels of PGE_2 in the intestine as a consequence of COX-inhibition may worsen the outcome due to the absence of appropriate tissue repair (after CT induced damage). We did not observe decreased expression of COX-2 in the small intestine in the present study. Importantly, the absence of decreased expression does not rule out an inhibition of COX activity, and needs the assessment of prostaglandin production. Nevertheless, the absence of diclofenac-mediated inhibition of COX-2 expression in the intestine is in contrast with

our *in vitro* data. Possibly the 2h time point is too early, but the constitutively expression of COX-2 in stromal cells of the intestine (18) may also be an explanation for these finding. Moreover, the fact that we were not able to detect epithelial damage (erosions or ulcers) within 2h after diclofenac exposure, does not exclude that epithelial cells are somehow "stressed" in response to diclofenac, or that damage will occur on later time points. Notably, diclofenac-induced enteropathy has been shown to result in an increased intestinal permeability (36, 39), and the effect of diclofenac on the allergic response may result from increased intestinal permeability for the antigen. This idea is supported by a recent study in which increased serum levels of dietary gliadin were found in healthy subjects after application of aspirin, diclofenac or loxoprofen in representative clinical doses (40). Conversely, the contribution of increased intestinal permeability to food allergic responses in humans remains unclear (41).

Furthermore, diclofenac-induced abrogation of the protective mucus layer may result in increased accessibility of the intestinal epithelial cells (IEC) for diclofenac itself, and also for luminal contents such as bacterial products and food antigens (37). Treatment of IEC with diclofenac *in vitro* resulted in profound inhibition of COX-2, but did not result in activation of NF-κB. In addition, diclofenac was not able to enhance CT- or LPS-induced NF-κB activation in these cells. Therefore, a direct stimulating effect of diclofenac on IEC *in vivo* resulting in induction of a mucosal immune response appears unlikely. The lack in induction of allergic responses by diclofenac in the absence of CT, as found in the present study, also indicates that diclofenac itself is unable to induce a mucosal immune response. In contrast, it has been reported that oral dosing of diclofenac is able to elicit an immune response to an i.p. administered antigen (8, 9) and to high doses of oral antigen (unpublished data). This suggests that (the route of) antigen entrance (peripheral or mucosal) together with antigen concentration are decisive for adjuvant capacity of diclofenac, although the mechanism behind these differences remains to be elucidated.

Studies using more selective COX-2 inhibitors, like NS-398 that lack the induction of intestinal damage, are needed to investigate to what extent COX-2 inhibition or intestinal barrier disruption contribute to the modulation of PE responses by diclofenac.

In conclusion, our data show that usage of NSAIDs can contribute to development of food allergic responses. It is of importance to investigate whether diclofenac and other NSAIDs are indeed able to enhance existing peanut responses in man as this would implicate an important risk factor for the use of these drugs in food allergic individuals.

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Chapter 7

Effect of dietary PUFAs and vitamin E on allergic responses to peanut in a murine food allergy model

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Abstract

Dietary polyunsaturated fatty acids (PUFAs) can be classified in *n-6* PUFAs and *n-3* PUFAs. The *n-6* PUFA arachidonic acid (AA) gives rise to the eicosanoid family of mediators that contribute to immune activation. In contrast, the *n-3* PUFAs decrease the amount of AA in cell membranes and *n-3* PUFA derived eicosanoids antagonize pro-inflammatory effects of *n-6* PUFAs. It is therefore postulated that *n-3* PUFA intake is beneficial in autoimmune and allergic immune responses**.** The present study investigated the effect of saturated fatty acid (SFA)-, *n-6* or *n-3* PUFA-enriched diets on allergic responses in a mouse model of peanut (PE) allergy. To compensate for increased membrane lipid peroxidation with *n-3* PUFAs, the diets were supplemented with low or high amounts of vitamin E. The dietary interventions resulted in altered fatty acid composition of red blood cell membranes and serum vitamin E levels according to the specific contents of the diets. In addition, *n-3* PUFA intervention resulted in reduced (AA-derived) prostaglandin E_2 (PGE_2) production in LPS stimulated splenocytes. In contrast, no differences were induced in PE-specific antibody production by any of the diets. Supplementation with *n-3* PUFA and high vitamin E, however, resulted in elevated PE-induced mast cell responses, probably due to a direct effect of both food components on these cells. Additional studies into the mechanism of increased release of mast cell mediators by vitamin E and *n-3* PUFAs are needed, as increased mast cell activity may lead to adverse allergic responses.

Introduction

During the last half of the twentieth century the incidence of chronic allergic conditions, including allergies, asthma and atopic diseases as well as autoimmune diseases and inflammatory bowel diseases has increased, although a plateau has now been reached especially in northern Europe (1, 2). In the same period major changes in dietary habits occurred within the western population. For instance, the balance of dietary intake of polyunsaturated fatty acids (PUFAs) has altered over the years, resulting in increased consumption of *n-6* PUFA-rich food products (e.g. soybean and rapeseed oil and plant margarines) whereas the intake of *n-3* PUFA-rich food products such as oily fish (3, 4) is low. Since dietary lipids form an important group of immune modulators (5), it is of interest if there is an association between the intake of dietary PUFAs and increased incidence of allergy (6, 7).

PUFAs form a range of immunoactive membrane phospholipids (8, 9), including arachidonic acid (AA) and eicosapentaenoic acid (EPA), which are precursors of eicosanoids, such as leukotrienes and prostaglandins (10). The 4-series leukotrienes and the 2-series prostaglandins are synthesized from *n-6* PUFA AA and are thought to mainly contribute to pro-inflammatory responses (11). For instance, leukotriene B4 (LTB_4) is involved in systemic allergic responses, whereas prostaglandin E2 (PGE_2) mediates inflammatory symptoms like fever, redness and pain (12). PGE_2 may also be able to modulate the Th1/Th2 balance, resulting in decreased production of IFN-γ and increased production of IL-4 and IgE (9, 13-15). On the other hand, *n-3* PUFAs as a source of EPA in cell membranes, will result in decreased amount of AA (16) and consequently of related eicosanoids (e.g. lower levels of PGE_2 and LTB_4). In addition, other eicosanoids possessing immunoregulatory properties are produced (9, 17). In addition to modulation of eicosanoid synthesis, immunoregulation via *n-3* PUFAs involve changes in gene expression, signal transduction pathways, cell death (apoptosis) and altered membrane fluidity (8, 18). It is postulated that an increased *n-6* PUFA intake contributes to an enhanced autoimmune and allergic response, whereas *n-3* PUFA intake is beneficial in these adverse immune responses (10, 19).

Although the concept of the health impact of a controlled balance in dietary intake of PUFAs is mechanistically sound, epidemiological data on the association between *n-6* PUFA intake and increased allergy and on the ability of *n-3* PUFAs to prevent allergic diseases remain inconclusive (4). Some studies report no correlation between dietary habits and the incidence of allergy and asthma in adults and children (20-23), whereas others report beneficial effects of *n-3* PUFA supplementation in the same diseases (24-

26). Beneficial effects on inflammatory disorders such as atopic dermatitis seem more consistent (27, 28), whereas the effect of different PUFAs on food allergy is unknown. It is therefore of interest to investigate whether intervention with differential PUFA containing diets would be a valuable strategy to prevent or diminish allergic sensitization to food allergens.
The outcome of dietary intervention and epidemiological studies may be concealed by a huge number of other dietary nutrients that are also known to contribute to immune functions. It is for instance known that vitamin E is involved in preventing oxidation of tissue PUFAs and accordingly increased intake of PUFAs can result in vitamin E deficiency due to increased lipid peroxidation (29, 30). Moreover, vitamin E is a potent anti-oxidant and immunostimulant (31, 32), and therefore the contribution of vitamin E must be taken into account when the effect of PUFAs is investigated.
The present study aims to investigate the effect of intervention with *n-6* and *n-3* PUFA-enriched diets in combination with low or high vitamin E supplementation on sensitization to peanut (PE) in C3H/HeOuJ mice. Interventions resulted in corresponding alterations in composition of membrane lipids and vitamin E contents. In contrast, no differences were induced in PE-specific antibody production by any of the diets, but supplementation with *n-3* PUFA and high vitamin E-levels resulted in elevated PE-induced mast cell responses.

Materials and Methods

Mice and reagents

Five-week-old specific pathogen-free female C3H/HeOuJCrl mice were purchased from Charles River (France). All mice were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of 23 ± 2 °C, 50-55% relative humidity, and 12 h light/dark cycle. Acidified drinking water and chow pellets were provided *ad libitum.* The experiments were approved by the Animal Experiments Committee of the Utrecht University.
Peanuts from the Golden Peanut, plant (Alpharetta, GA, USA) were kindly provided by Intersnack Nederland BV (Doetinchem, The Netherlands) and peanut extract (PE) was prepared as previously described (33). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, CA, USA). Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Diet intervention

Experimental diets were prepared by Research Diet Services (Wijk bij Duurstede, the Netherlands) and stored at -20 °C until use. Pellets were given *ad libitum* and refreshed twice a week. All mice received a run-in diet identical to the saturated fatty acid (SFA) low vitamin E diet for one week prior to intervention, after which groups of mice (n=8) were maintained on experimental diets until the end of the experiment. A total of six diets were tested based on a combination of different fatty acids (SFA, n-3, n-6) and vitamin E levels (low, high).

All experimental diets contained 18% protein, 60 % carbohydrates and 12% fat, and were based on AIN-93G (Reeves, 1993). Details on composition are summarized in table 1. Fat blends were prepared from palm oil, canola oil (rapeseed oil), corn oil and fish oil. Additional α-tocopherol (vitamin E) was added to the fat blends to obtain high vitamin E diets. The AIN-93 recommended vitamin premix contributed to a level of 75 mg/kg α-tocopherol in the diets.

Incorporation of fatty acids in red blood cell membranes

Blood samples were collected in heparin-coated tubes three weeks after start of diet intervention, just prior to oral sensitization. Samples were centrifuged at 600g, red blood cells (RBC) were washed twice in PBS containing 5 mM EDTA, and stored at -80 °C until analysis. Fatty acids were extracted as previously described (34), using C19:0 as internal standard. In brief, lipids were extracted under liquid nitrogen atmosphere from 1 ml membrane suspension, by adding 2 ml ethanol, 0.9 ml EDTA (1% w/v in MiliQ water) and 1 ml dichloromethane. Phospholipids were separated from other lipids in the extract by means of solid phase extraction (SPE), and methylated using 2% H_2SO_4 in methanol for 1h at 100 °C. After hexane extraction methyl ester fatty acids were dissolved in iso-octane and quantified using gas chromatography with a capillary column (50m x 0.25mm, CP-SIL88-frame). Peaks were identified by reference to commercial standards, and the area under the peak was automatically integrated and compared to the internal standard for quantification.

Table 1: Analyzed composition of diet *

		Fat blend	
Diet:	**SFA**	***n-6***	***n-3***
Fatty acid (g/100g fatty acids)			
C14:0	1.1	0.6	2.9
C16:0	42.1	22.9	21.4
C16:1	0.2	0.2	4.5
C18:0	4.6	3.0	2.6
C18:1	38.3	30.9	20.4
C18:2 *n-6* (LA)	10.1	38.8	14.5
C18:3 *n-3* (ALA)	0.8	0.7	0.6
C20:2 *n-6*	0.2	0.2	0.4
C20:4 *n-6* (AA)			0.8
C20:5 *n-3* (EPA)			14.5
C22:5 *n-3* (DPA)			1.4
C22:6 *n-3* (DHA)			6.1
total SFA	48.8	27.5	27.7
total MUFA	39.6	32.0	27.1
total *n-6* PUFA	10.3	39.1	16.6
total *n-3* PUFA	0.8	0.7	22.6
n-6 / n-3 ratio	13.15	54.77	0.74
Vitamin E (mg/kg diet)			
Low vitamin E diet	76	82	97
High vitamin E diet	830	770	720

* The following ingredients were added at identical levels to all diets (g/kg diet): casein 180; cornstarch 370; maltodextrin 19IT 130; sucrose 100; cellulose 50; mineral mixture 35; vitamin mixture 10; L-cysteine 3; choline bitartrate 2.5, fat blend 120; Mineral and vitamin mixtures were formulated according to AIN-93 recommendations (Reeves et al. 1993). Vitamin mix contributed to a level of 75 mg/kg vitamin E in the diet. High vitamin E diet was supplemented with additional (1 g/kg) vitamin E.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid

Measurement of serum vitamin E (α-tocopherol)

The amount of vitamin E in serum was quantified by measuring its major component, α-tocopherol. Serum samples were mixed with an internal standard (α-tocopheryl acetate), deproteinised with ethyl alcohol, and extracted into n-heptane. Excess solvent was removed under N_2, and residues were redissolved in chloroform-methanol (2:1, v/v). α-Tocopherol content was quantified using an RP C18 column (4 µm, 125 x 3 mm; Bischoff) and isocratic elution with 95% methanol.

Oral sensitization to peanut

Three weeks after start of diet intervention mice (n=8) were orally exposed to 6 mg PE mixed with 15 µg CT for three consecutive days, and dosage was repeated every week for three weeks (exposure on day 0, 1, 2, 7, 14 and 21). Control groups received PBS plus CT. All mice received a challenge of 12 mg PE on day 27, and were sacrificed one day later.

Measurement of serum IgG1 and IgG2a

Blood was collected at several time points and PE-specific antibodies in serum were detected by ELISA. In brief, Highbond plates (Costar) were coated overnight at 4 °C with 10 µg/ml PE in PBS, followed by 1h blocking with ELISA buffer (50 mM Tris buffer, pH 7.2, supplemented with 137 mM NaCl, 2 mM EDTA, 0.05% Tween and 0.5% BSA). Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (1000 arbitrary units of PE-specific antibodies of various immunoglobulin subclasses) were included on all plates. In order to detect IgG1 or IgG2a, alkaline phosphatase (AP)-conjugated antibodies (Southern Biotechnology Associates) were added and left for 1h

at room temperature. Subsequently, p-nitrophenylphosphate (1 mg/ml) in diethanolamine buffer was used as a substrate and colour reaction was stopped with 10% EDTA. Absorbance was assessed at 405 nm.

Measurement of serum IgE

IgE levels in serum were determined by means of a sandwich ELISA. In brief, Highbond plates (Costar) were coated overnight at 4 °C with 1 µg/ml anti IgE antibody (BD-Pharmingen, Erembodegem, Belgium) in PBS, followed by 1h blocking with ELISA buffer. Serum samples were added in several dilutions in ELISA buffer and incubated for 2h at room temperature. Serial dilutions of a reference serum (containing 1000 arbitrary units of PE-specific IgE) were included on all plates. Subsequently, PE coupled to digoxigenin (DIG) in HPE-buffer (Sanquin, Amsterdam, the Netherlands) was added and left for 1h at room temperature. The coupling of DIG to PE was performed according to the instructions of the manufacturer (Boehringer, Mannheim). Streptavidin-coupled anti-DIG (Roche diagnostics) was added for 1h at room temperature, followed by TMB substrate (0.1 mg/ml). The colour reaction was stopped after 15 minutes with 2M H_2SO_4 and absorbance was measured at 450 nm.

Measurement of mouse mast cell protease 1 (mMCP1) in serum

mMCP1 was determined using a specific ELISA kit (Moredun scientific Ltd, Midlothian, Scotland) and performed according to instructions of the manufacturer.

Quantitative rt-PCR for COX-2 mRNA expression

Approximately 50 mg of the small intestine (without PP) were dissected and stored at -80 °C until further use. The tissue was homogenized in 1 ml RNA Instapure© (Eurogentec, Maastricht, the Netherlands) by means of a Mixer-Mill (Qiagen). RNA was extracted from the RNA Instapure© samples using chloroform and isopropanol. RNA content was quantified by measuring optical density at 260 nm, and RNA integrity was confirmed by measuring A260/A280 on a SmartSpecPlus (Bio-Rad Laboratories, Veenendaal, the Netherlands). Subsequently, RNA was transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real time quantitative PCR (Q-PCR) was performed using a iCycler iQ system (Bio-Rad), and amplification was done using IQ™ SYBR Green® Supermix (Bio-Rad) with 0.4 µM final primer concentration. Primers used were: COX-2 sense 5'- TGC AAG ATC CAC AGC CTA CC -3', antisense 5'- GCT CAG TTG AAC GCC TTT TG -3'; β-actin sense 5'- ATG CTC

CCC GGG CTG TAT -3', antisense 5'- CAT AGG AGT CCT TCT GAC CAT TC – 3'. For each sample mRNA expression was normalized for the detected Ct value of β-actin.

Tissue staining

Acetone-fixed cryostat sections of the small intestine were incubated for 1h with anti-COX-2 polyclonal Ab (Cayman Ann Arbor, Michigan, USA) diluted in PBS, 1% BSA. Antibody specificity was checked using a COX-2 blocking peptide (Cayman) according to the instructions by the manufacturer. The antibodies were visualized by first applying biotinylated swine anti-rabbit F(ab')2 fragments (DAKO, Denmark) in PBS, supplemented with 5% normal mice serum for 1h, followed by 45 min incubation with horseradish peroxidase labeled streptavidin (DAKO, Denmark) and aminoethylcarbazole (AEC) as a substrate. Sections were counterstained with Mayer's heamatoxylin (DAKO) and mounted with DAKO Faramount Aqueous mounting medium. Images were acquired using a Olympus BX60 microscope with colorview III digital camera and Cell imaging software.

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions ($3.75*10^5$ per well) were incubated in RPMI1640 supplemented with pen/strep and 10% FCS in the presence or absence of 100 µg/ml peanut extract in 96-well plates for 96h at 37 °C, 5% CO_2 after which culture supernatants were harvested and stored at -20°C until analysis.

In the culture supernatants, levels of IFN-γ, IL-4, IL-5, IL-13 and IL-10 were determined by commercially available sandwich ELISA (IL-10 was obtained from BD-Pharmingen, others were from e-Bioscience), according to the instructions of the manufacturers.

Another fraction of splenocytes ($3.75*10^5$ per well) were stimulated with 2 µg/ml LPS in 96-well plates for 48h at 37 °C, 5% CO_2 after which culture supernatants were harvested and stored at -20°C until analysis. TNF-α in culture supernatant was determined by sandwich ELISA (e-Bioscience), whereas PGE_2 was measured by means of a competitive ELISA (R&D Systems), both according to the instructions of the manufacturer.

Statistics

Data are presented as the group mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism software. One-way ANOVA was performed followed by Bonferroni as a post-hoc test. Before testing, data on antibody levels and cytokine production were logarithmic transformed to achieve normal distribution. Differences were considered significant when p values were < 0.05.

Results

Dietary intervention resulted in altered membrane composition and vitamin E levels in serum

Dietary intervention started three weeks before induction of sensitization, and was continued until the end of the experiment. Fatty acid composition of red blood cell membranes before start of sensitization was measured to verify the effect of the diet. As expected, fatty acid composition was altered significantly in *n-6* PUFA- and *n-3* PUFA-fed mice, irrespective of dietary vitamin E content (figure 1a and table 2). Increase in *n-6* PUFA content was mainly due to linoleic acid (LA) at the expense of oleic acid (AO). Eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) contents in the *n-3* PUFA groups increased significantly compared to SFA-groups, and was accompanied with a 2-3-fold increase in docosahexaenoic acid (DHA), whereas levels of AA were significantly lower.

To examine the efficacy of vitamin E feeding, serum levels of α-tocopherol (the added vitamin E analogue) were determined. α-Tocopherol levels in serum correlated well with vitamin E content of the diet, but mice fed *n-3* PUFA showed 25 to 40% lower serum α-tocopherol levels compared to SFA- and *n-6* PUFA-fed mice, respectively (figure 1b and table 2).

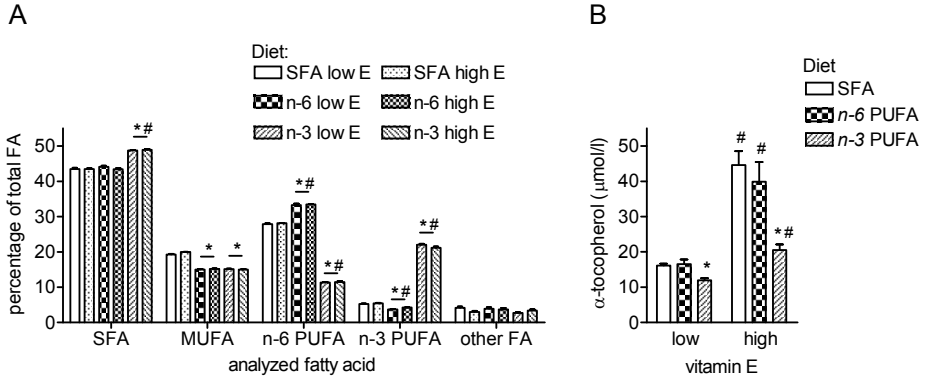

Figure 1: RBC membrane composition and serum α-tocopherol after diet intervention.
A. Mice were fed with SFA-, *n-6* PUFA- and *n-3* PUFA-rich diets supplemented with low or high vitamin E levels. Blood was collected after 7 weeks of diet intervention, and red blood cells (RBC) were isolated and analyzed for fatty acid contents as described in Materials and methods. Relative contents of various fatty acids in RBC are depicted as mean ± SEM of 6 to 8 mice per group. *: $p < 0.001$ compared to SFA irrespective of vitamin E; #: $p < 0.001$ compared to other PUFA irrespective of vitamin E.
B. Serum was collected at the end of the experiment, and analyzed for the amount of the main vitamin E component α-tocopherol as described in materials and methods. The graph shows α-tocopherol concentrations in µmol/l (mean ± SEM) from 8 mice per group. *: $p < 0.05$ compared to SFA within same vitamin E diet level. #: $p < 0.001$ (0.05 for *n-3*) compared to same PUFA with low vitamin E.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 2: RBC fatty acid contents and serum α-tocopherol levels

	Diet					
	SFA		*n-6*		*n-3*	
	low	high	low	high	low	high
Fatty acid (% of total fatty acids) *in RBC*						
C14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C16:0	26.8 ± 0.2	26.8 ± 0.2	26.5 ± 0.2	26.3 ± 0.2	31.4 ± 0.2	31.3 ± 0.1
C16:1	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.5 ± 0.0
C18:0	11.1 ± 0.3	10.8 ± 0.2	12.4 ± 0.3	12.1 ± 0.2	11.3 ± 0.2	11.7 ± 0.3
C18:1 (OA)	14.9 ± 0.1	15.2 ± 0.1	11.9 ± 0.1	12.1 ± 0.1	11.9 ± 0.1	11.9 ± 0.1
C18:2 *n-6* (LA)	5.0 ± 0.1	5.2 ± 0.1	8.0 ± 0.2	8.6 ± 0.1	4.5 ± 0.1	4.7 ± 0.1
C18:3 *n-3* (ALA)	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C20:2 *n-6*	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C20:4 *n-6* (AA)	17.0 ± 0.2	17.3 ± 0.1	17.5 ± 0.2	17.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1
C20:5 *n-3* (EPA)	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	8.6 ± 0.2	8.2 ± 0.2
C22:5 *n-3* (DPA)	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	3.1 ± 0.1	3.1 ± 0.0
C22:6 *n-3* (DHA)	4.7 ± 0.1	4.9 ± 0.1	3.5 ± 0.1	3.9 ± 0.0	10.4 ± 0.1	10.0 ± 0.1
total SFA	43.5 ± 0.3	43.5 ± 0.2	44.1 ± 0.3	43.4 ± 0.3	48.7 ± 0.1	48.9 ± 0.2
total MUFA	19.3 ± 0.2	20.0 ± 0.1	15.0 ± 0.2	15.2 ± 0.1	15.1 ± 0.1	15.0 ± 0.2
total *n-6* PUFA	27.9 ± 0.2	28.1 ± 0.1	33.3 ± 0.4	33.4 ± 0.2	11.3 ± 0.2	11.5 ± 0.3
total *n-3* PUFA	5.3 ± 0.1	5.4 ± 0.1	3.7 ± 0.1	4.2 ± 0.0	22.1 ± 0.2	21.2 ± 0.3
α-tocopherol (µmol/l) in serum						
	16.1 ± 0.5	44.6 ± 3.9	16.5 ± 1.3	39.9 ± 5.6	11.9 ± 0.6	23.8 ± 1.6

Data are represented as mean ± SEM of 8 animals per group.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid

COX-2 expression in intestine was altered as a result of diet intervention.

Next, we investigated whether the observed changes in fatty acid composition of membranes resulted in altered expression of the COX-2 enzyme, responsible for conversion of AA into various prostaglandins. COX-2 expression was decreased in mice fed *n-3* PUFAs, but in *n-6* PUFA-fed mice higher levels of COX-2 were found than in SFA-fed animals. These effects were most pronounced in low vitamin E-fed mice (figure 2). No significant alterations in COX-2 mRNA levels in small intestinal tissue were found (figure 3).

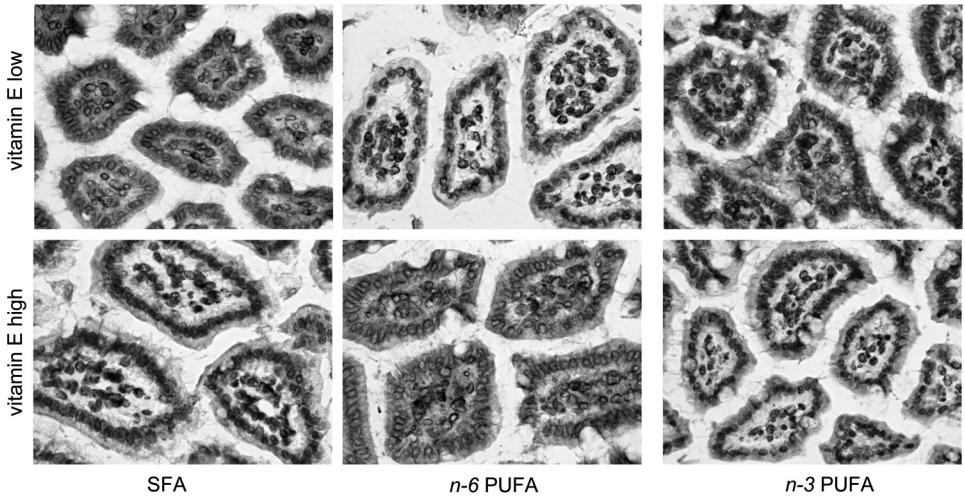

Figure 2: COX-2 expression in small intestine

Mice were fed with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E, and sensitized to PE+CT as described in Materials and methods. At the end of the experimental intervention, mice were dissected and cryosections of small intestine were stained for COX-2 (in red) as described in material and methods. Representative sections of one mouse per group are shown (original magnitude *200) **A-C**: vitamin E low diet; **D-F**: vitamin E high diet. **A** and **D.** SFA diet; **B** and **E**: *n-6* diet; **C** and **F**: *n-3* diet. For color images see page 167.

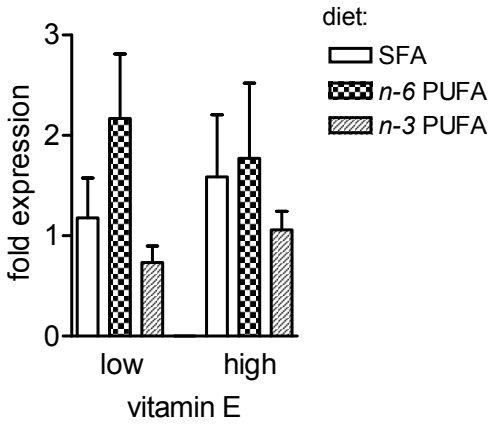

Figure 3: mRNA levels of COX-2 in small intestine with different PUFA and vitamin E containing diets
Mice were fed with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E, and sensitized with PE+CT. mRNA was isolated from small intestine at the end of the study as described in Materials and methods, and rt-PCR was performed for COX-2 expression. Data are presented as mean ± SEM from 3 to 4 mice per group.

Dietary n-3 PUFA modulated the LPS-induced prostaglandin-E_2 and TNF-α response in spleen.

In addition, it was of interest to investigate whether the changes in RBC membrane fatty acid composition and COX-2 expression would result in altered innate immune responses. As shown in figure 4, feeding *n-3* PUFAs resulted in diminished PGE_2 production in response to LPS in splenocytes compared to SFA and *n-6* PUFA fed mice, which was independent of vitamin E content (figure 4a). On the other hand, a small but significant increase in TNF-α production by LPS-stimulated splenocytes was found in *n-3* PUFA-fed mice (figure 4b).

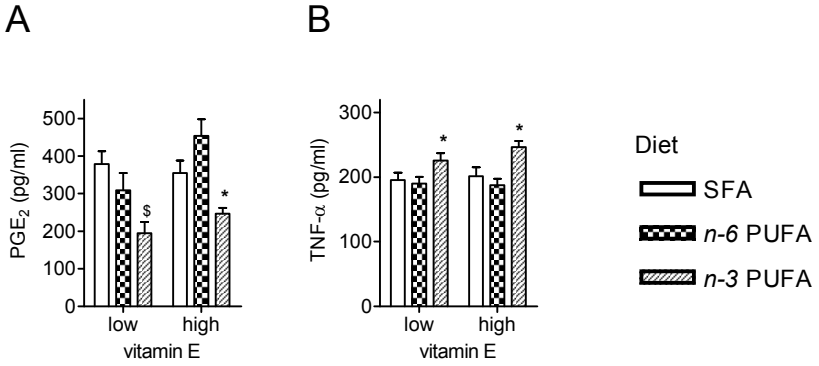

Figure 4: **Effect of dietary fatty acids and vitamin E on PGE_2 and TNF-α production in splenocytes after LPS restimulation**
Splenocytes from mice fed with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E were restimulated with LPS for 48h as described in materials and methods, after which PGE_2 (**A**) and TNF-α (**B**) were determined in culture supernatant. Data are shown as mean ± SEM from 6 (PGE_2) or 8 (TNF-α) mice per group. *: $p < 0.05$; $: $p < 0.01$ compared to SFA given the same level of vitamin E.

PE-specific antibody responses were not altered with different diets, but mMCP1 was elevated with n-3 PUFA and high vitamin E.

Sensitization to PE + CT resulted in induction of PE-specific IgG1, IgG2a and IgE in serum after 28 days, whereas no PE-specific antibodies could be detected after administration of either PE or CT alone (data not shown). Diets enriched in *n-3* or *n-6* PUFAs did not cause a change in levels of PE-specific antibodies, irrespective of vitamin E intake (figure 5a). In addition, vitamin E supplementation did not significantly alter PE-specific antibody levels. Remarkably, mMCP1 release, being a marker for mast cell degranulation *in vivo*, was increased significantly upon oral challenge with PE in *n-3* PUFA-fed mice compared to SFA- and *n-6* PUFA-fed mice at low vitamin E (figure 5b). The presence of high vitamin E in diet also resulted in elevated mMCP1 release, but independent of PUFA content.

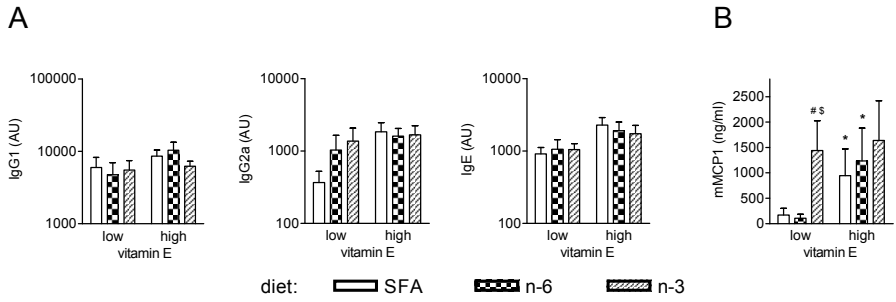

Figure 5: Effect of dietary fatty acids and vitamin E on PE-specific responses
After 3 weeks of dietary intervention with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E, mice were sensitized to PE+CT for 4 weeks as described in materials and methods. **A**. At day 28 PE-specific antibodies in serum were determined by ELISA. **B.** mMCP1 was measured in blood samples, collected 30 min after an oral challenge with PE. Data are presented as mean ± SEM of 8 mice per group. *: $p < 0.05$ compared to the other vitamin E group. #: $p < 0.05$ compared to SFA low vitamin E diet, $: $p < 0.05$ compared to *n-6* PUFA low vitamin E diet.

High vitamin E in diet resulted in elevated T cell responses in MLN.

To investigate PE-specific local and peripheral T cell responses, MLN and spleen cells were restimulated with PE for 96h. Sensitization to PE+CT resulted in IFN-γ, IL-13 and IL-10 in culture supernatant of spleen and MLN cells. No significant differences between treatment groups were found in these cytokines (figure 6). However, cytokine responses were elevated in MLN-cultures of mice that had received vitamin E high diets. Culture of cells in the absence of PE did not result in detectable cytokine levels (data not shown) and IL-4 and IL-5 levels were below detection limit (IL-4: 4 pg/ml; IL-5: 12 pg/ml) after PE restimulation in spleen cells.

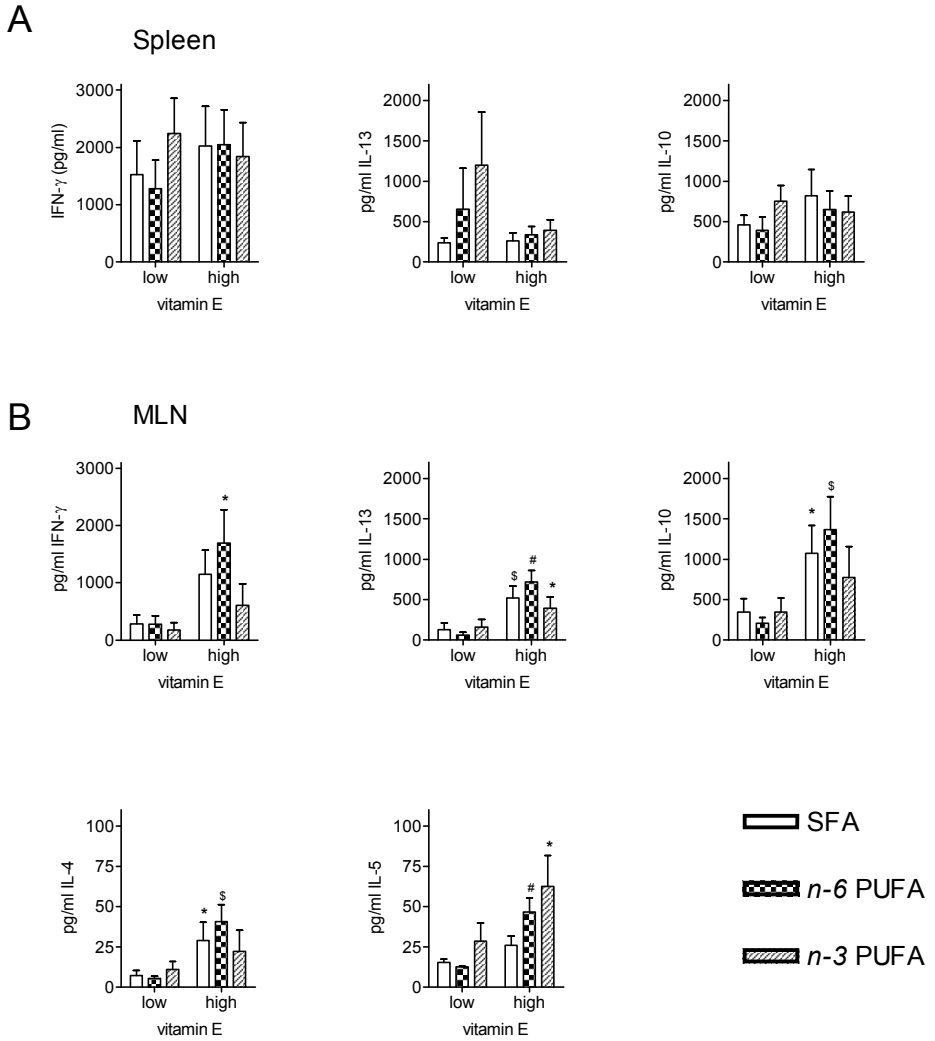

Figure 6: Effect of dietary intervention of PE-specific T cell responses in MLN and spleen.
After 3 weeks of dietary intervention with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E, mice were sensitized to PE+CT for 4 weeks as described in Materials and methods. Diet intervention started three weeks prior to sensitization. After 28 days mice were sacrificed and MLN and spleen cells were restimulated with PE for 96h. Cytokine levels in supernatant of spleen (**A**) or MLN (**B**) were determined by ELISA. Mean ± SEM from 8 mice per group are shown. *: $p < 0.05$; $: $p < 0.01$; #: p, 0.001 compared to corresponding fatty acid treated group within the other vitamin E diet.

Discussion

In the present study, we used an established mouse model of peanut allergy to evluate the effect of PUFA-enriched diets with low or high vitamin E content on the induction of food allergy. As expected, *n-3* PUFA-enriched diet resulted in significantly increased levels of EPA, DPA and DHA and decreased levels of LA and AA in red blood cell membranes, in comparison to *n-6* PUFA- or SFA-enriched diets. No major differences in fatty acid content of membranes were found between *n-6* PUFA- and SFA-fed mice. Levels of α-tocopherol in serum were significantly increased in mice that consumed vitamin E-high diets. These data demonstrate that the dietary interventions had resulted in altered fatty acid and vitamin E composition according to the specific contents of the diets.

In general, immunological changes were observed only in case of increased *n-3* PUFA intake and in all experimental groups that received high vitamin E diets, irrespective of fatty acid content. In addition, changes appeared largely limited to LPS-dependent responses and antigen-specific mast cell responses, although cytokine production by MLN cells was increased in case of high vitamin E intake.

The finding that LPS-induced TNF-α production by splenocytes from *n-3* PUFA-fed mice was increased is in line with previous observations by our laboratory (35) and by others (36, 37). Yet, a reduction in TNF-α production would be more in line with the assumed anti-inflammatory capacity of *n-3* PUFAs. Indeed, application of n-3 PUFA infusions in sepsis patients resulted in decreased levels of pro-inflammatory cytokines, including TNF-α, in serum (38). Moreover, piglets fed with fish oil showed reduced TNF-α production in splenocytes after LPS restimulation in vitro (31). A study by Ishihara et al. (39) has shown that blood-derived cells, but not splenocytes, from *n-3* PUFA-fed rats showed reduced TNF-α responsiveness to LPS. To complicate this issue further, an intervention study in healthy humans with *n-3* and *n-6* PUFA-enriched diets showed no differences in TNF-α in PBMC after LPS stimulation (40). These conflicting data on TNF-α production under influence of FA-diet interventions suggest that modulation not only depends on diet, but possibly also on species- and cell-specific factors.

The reduced capacity of *n-3* PUFA-fed mice to produce PGE_2 (using LPS-stimulated splenocyte-cultures as read-out system) is also in line with our (35) and previous findings (10, 16, 19). Since the intestine of *n-3* PUFA-fed mice displayed lower levels of COX-2, it is expected that also in the intestine PGE_2-production will be reduced due to the particular diet. Unlike the concept that PGE_2 is involved in driving toward Th2

responsiveness, the decreased PGE_2 production (and of AA content) as observed here did not result in decreased PE-sensitization. But, although some reports describe beneficial effects with *n-3* PUFA-enriched diets (26, 41), the lack of modulation found in the present study confirms the results from human studies, that did not show beneficial effects of *n-3* PUFA-enriched diets on asthma in adults (23, 42). In addition, epidemiological studies indicate a inverse relation between AA and allergy as they show a positive correlation between low AA levels and atopic allergies (reviewed by Sala-Vila (42)). On the other hand, promising data have been described on the prevention of allergy and enhancement of immune functions when intervention took place prenatally (via the maternal diet) or early in life (4, 9).

The increased mast cell response to the PE challenge in the *n-3* PUFA and high vitamin E intake groups is remarkable, since IgE levels (as well as other isotypes) were not altered by any of the diets. Certain cytokines, including IL-4 may induce a high mast cell response (43) but that does not explain the present results, since PE-mediated cytokine production was only increased in case of high vitamin E intake. Rather, these findings point to direct effects of both *n-3* PUFAs and vitamin E on mast cell function. Membrane soluble vitamin E is used to protect for peroxidation of *n-3* PUFA (30), and thus to preserve membrane quality. Vitamin E also enhances innate and adaptive protective immune responses (32) (44). But the exact molecular mechanism of vitamin E action is still a matter of debate (45-47) and it is suggested that vitamin E may not only function as an anti-oxidant but also interact with not yet identified specific cellular targets. *n-3* PUFA may affect mast cell function by decreased PGE_2 production (and COX-2 inhibition) as it has been demonstrated that PGE_2 can inhibit mast cell release in asthma patients (48). Furthermore, *n-3* PUFAs strongly affect membrane fluidity (49) and changes in membrane properties may result in an altered threshold for mast cell degranulation.

In conclusion, the present study shows that intervention with *n-3* PUFA-enriched diet or high vitamin E diet resulted in an enhanced antigen-specific mast cell response, but allergic responses were not altered upon intervention with *n-3* or *n-6* PUFA rich diets. Studies into mechanism of increased release in mast cell mediators by vitamin E and *n-3* PUFA's are warranted, as increased mast cell activity may lead to exacerbation of allergic responses.

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Chapter 8

Summarizing Discussion

The challenge of the mucosal gut associated immune system is to remain unresponsive to food products and commensal microbiota, while mounting an appropriate immune response towards pathogens (1, 2). Paradoxically, the gut associated lymphoid tissue (GALT) needs the interaction with commensal microbiota and food proteins to develop properly (3-6). This implicates the necessity of tight immune regulation within the intestine (7). Imbalance between tolerance and immunity (e.g. intestinal homeostasis) contributes to the pathogenesis of intestinal diseases like inflammatory bowel disease (IBD), food allergies and intestinal cancer (8-10).
Epidemiologic data reveals a steady increase in the incidence of IBD and food allergies during the last half of the twentieth century, although a plateau has now been reached in high-incidence areas such as northern Europe and North America (11, 12). Several ideas have been postulated to explain this increased prevalence, including western lifestyle, changes in microbial exposures due to improved hygiene (13), widespread use of antibiotics (14) and changes in diet (15).
The aim of this thesis was to gain more insight in the role of environmental factors (bacterial products, food components, drugs) in intestinal immune regulatory processes contributing to intestinal inflammation and food allergy.

1. Summary

Intestinal epithelial cells (IEC) are important in the maintenance of mucosal homeostasis, as these cells constitute the physical barrier between the body and the intestinal lumen. Disruption of this physical barrier can result in the activation of nuclear factor-κB (NF-κB) in IEC and intestinal antigen presenting cells (APC), and in the entrance of luminal bacterial products like lipopolysaccharide (LPS) into the intestinal tissue. These effects may subsequently contribute to induction of (chronic) intestinal inflammation (16-18). The extent and severity of mucosal lesions associated with intestinal inflammation are dependent on the balance between aggressive, cell-destructive processes and counteracting cytoprotective and wound reparing mechanisms (19). One of these protective mechanisms is the expression of the enzyme alkaline phosphatase (AP) by differentiated IEC. AP is able to detoxify LPS, and contributes to the maintenance of gut homeostasis (20, 21). **Chapter 2** shows that administration of AP to mice exposed to dextran sulfate sodium (DSS) resulted in reduced intestinal inflammation, but only in situations of severe intestinal damage. The administration of AP to mice with a moderate inflamed intestine did not alter disease outcome. Interestingly, rectal administration of LPS did not aggravate moderate intestinal inflammation, implicating that in general, regulatory capacities in the

intestine, including AP expression, are sufficient to counteract luminal LPS even in situations of moderately damaged epithelium.

One of several other factors belonging to the intrinsic capacity of the epithelium to maintain resistance to bacterial toxins is the constitutive expression of stress proteins, especially heat shock proteins (HSP) (19, 22, 23). HSP are able to induce anti-inflammatory T cell responses and are possible targets for regulatory T cells (Tregs) (24). It has been shown that administration of mycobacterial HSP resulted in the induction of interleukin 10 (IL-10) producing Tregs in animal models for rheumatoid arthritis, and that these Tregs ameliorated disease onset (25). In fact, mycobacterial HSP may increase the pool of HSP-specific Tregs that may also respond to endogenously upregulated HSP under stressed and inflamed conditions (26). C**hapter 3** shows however, that mycobacterial HSP was not able to ameliorate intestinal inflammation in DSS and trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. This lack in protective effect could be due to the acute character of both models.

In addition to Tregs, it is known that innate cells, like γδ T cells contribute to the responses to stressed cells (27-29) and promote intestinal epithelial cell growth and tissue repair (30, 31). Strikingly, **chapter 4** shows that γδ T cells within the mouse intestine were decreased during establishment of allergic sensitization using cholera toxin (CT). CT is widely used as a mucosal adjuvant in mouse models to induce allergic IgE sensitization to co-administered antigens (32, 33). The mechanisms underlying the decrease in γδ T cells remains unclear, although the response of γδ T cells to CT-induced cell stress might be involved. Next to an effect on γδ T cells, we also observed that CT caused a shift in DC subsets from mucosal $CD103^+$ (tolerogenic) DCs to conventional $CD11b^+$ DCs. Data from **chapter 5** reveals that blockade of the TCR on γδ T cells using an antibody (UC7-13D5) resulted in increased sensitization to orally administered peanut extract (PE) plus CT. This implicates that intestinal γδ T cells are involved in regulation of the food allergic responses.

The contribution of cell stress or injury of the intestinal barrier in food allergic responses is also suggested from a study using diclofenac, described in **chapter 6**. It shows that exposure to diclofenac resulted in enhanced allergic sensitization to PE+CT. Induction of intestinal epithelial damage by diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) is known for a long time (34). This is partly attributable to the inhibition of cyclooxygenase (COX) enzymes which are important in repair of intestinal damage (35, 36). Another factor of importance to diclofenac-induced enteropathy is the chemical interaction of NSAIDs with the mucus layer (37, 38). Disturbance of the mucus layer results in increased accessibility of IEC for diclofenac

itself, and also for luminal contents, including bacterial products and food antigens, which may result in increased cell stress. We failed to detect enteropathy (disruption in mucus, or intestinal pathology) upon diclofenac exposure. Strikingly COX-2 expression in the intestine was also not inhibited by diclofenac, although we did not assess COX-2 activity. Further studies are needed to reveal the exact mechanism behind the diclofenac-induced increase in allergic sensitization.
Much research has been done over the years to enhance the regulatory capacity of the (mucosal) immune system (39), and hereto dietary interventions are investigated (40, 41). The precursors of immunomodulating eicosanoids, the dietary *n-6* and *n-3* polyunsaturated fatty acids (PUFAs) are of special interest (42). From the eicosanoids, prostaglandin E_2 (PGE_2), synthesized from *n-6* PUFAs, possesses the most prominent immunomodulatory capacities. **Chapter 7** shows however, that hypersensitivity responses to PE+CT were not altered as a result of differences in dietary fatty acids, although PGE_2 production in splenocytes was significantly changed. Variations in dietary lipids alter membrane composition, and need the presence of antioxidants, like vitamin E to prevent oxidative stress. This prompted us to combine the various PUFA diets with low and high dietary vitamin E. Remarkably, increased release of mast cell protease (mMCP1), a marker of mast cell degranulation *in vivo*, was found in the presence of high vitamin E or *n-3* PUFAs upon oral challenge. Whether this is the consequence of changes in membrane fluidity, resulting in a lower threshold to release cellular mediators, remains to be elucidated. Increased mast cell release in the high vitamin E groups and the *n-3* PUFA group did not likely result from enhanced sensitization as other parameters of allergic sensitization were not altered.

2. Intestinal barrier function

The homeostatic balance between host and commensal flora is maintained through the cooperation of various regulatory mechanisms that prevent the immune system from reacting towards harmless exogenous antigens present in the intestine (8).
Alterations in barrier integrity or disturbances in regulatory immune processes will lead to loss of intestinal barrier function. As a consequence, bacteria and antigens get access to the intestinal tissue and this may result in chronic intestinal disease (8). The studies in this thesis investigated aspects of barrier disruption and impaired regulation in relation to intestinal inflammation and allergic sensitization. The results of these studies are discussed in more detail below, and summarized in figure 1 and 3.

2.1 Epithelial integrity and intestinal permeability

In case of IBD, the loss in epithelial barrier function may allow (bacterial) antigens to enter the lamina propria, triggering T cell activation, and break tolerance mediated by immunosuppressive cytokines and Tregs. Pro-inflammatory cytokines, like tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) further increase epithelial permeability, creating a vicious cycle of chronic inflammation (10, 43). Several lines of evidence suggest that increased intestinal permeability has a central role in the pathogenesis of IBD (43, 44). For example, in clinically asymptomatic Crohn's disease patients, increased intestinal permeability precedes clinical relapse (45). Therefore, strategies to overcome loss in epithelial integrity will contribute to therapies of IBD (46).

Food-related effector responses (anaphylaxis in sensitized individuals) also require breaking the epithelial barrier to reach effector cells such as mast cells and basophils (47). Loss in barrier integrity is probably less relevant in allergic sensitization to food proteins (44). However, during establishment of allergic sensitization mimicked by the use of mucosal adjuvants like CT, induction of "stress" in epithelial cells (48) may be of importance (chapter 4). The response to the stressed cells can eventually result in activation of distinct DC subsets in the LP or PP (discussed in more detail below). It has been shown that *in vitro* exposure of IEC to microbial products such as CT results in NF-κB activation, and the production of pro-inflammatory cytokines, such as IL-8 and TNF-α ((49) and chapter 6). TNF-α may in turn potentiate IFN-γ-mediated increase in intestinal permeability and thus also contribute to the overall adjuvant properties of CT.

Furthermore, enhanced allergic responses were found when diclofenac was administered prior to PE+CT sensitization (chapter 6). Diclofenac (through COX inhibition) interferes with synthesis of immune modulating prostaglandins, but also mediates intestinal damage (38) which may contribute to enhanced sensitization.

Another example of the role of barrier integrity in allergic sensitization was illustrated by an experiment in which rats were exposed to psychological stress through water avoidance, resulting in increased intestinal permeability. Exposure of these rats to antigen plus adjuvant (oral administration of antigen + alum) resulted in sensitization to the fed antigen, whereas non-stressed rats did not become sensitized. Together, these examples reveal that manipulation of barrier function contributes to allergic sensitization but still needs the presence of an adjuvant (50).

2.2 Immune regulation

Tolerance in the intestine depends on a wide range of immunosuppressive mechanisms with overlapping functions (8). The mechanisms that are of relevance in this thesis include IEC-dependent pathways such as the expression of AP and HSP. Furthermore, the contribution of DCs and γδ T cells and of dietary fatty acids in modulation of immune regulation are discussed in more detail.

AP expression by IEC

The expression of AP by IEC, facilitating the detoxification of LPS, is one of many mechanisms that can preserve bacterial unresponsiveness in the intestine (20, 21). The expression of iAP coincides with the establishment of the intestinal flora, whereas loss of nutrition or generation of germ-free conditions result in reduced iAP expression, strongly suggesting a role of iAP in maintenance of intestinal homeostasis (21). Nonetheless, fortification of this system by adding exogenous AP did not reduce inflammation in situations of moderate intestinal damage (chapter 2). Addition of high levels of LPS to a moderately inflamed intestine as a result of DSS-induced epithelial cell damage did not aggravate disease outcome. Apparently, the intrinsic epithelial AP production was sufficiently protective in situations of mild inflammation. However in case of severe epithelial damage, addition of AP may help to counteract inflammatory disease. These results also emphasize the need of therapies aiming at restoring epithelial integrity.

HSP response in IEC

The constitutive expression of HSP in the epithelial cell layer of the intestine add to an effective stress response, which also contributes to epithelial resistance to luminal bacteria (19, 22, 23). Endogenous HSP constitute a group of 'autoantigens' with the potential to trigger immunoregulatory pathways, including the induction of IL-10-producing Tregs, which can suppress inflammation (26). It has been shown that bacterial fermentation products, including short-chain fatty acids (SCFA) like butyrate as well as bacterial components, such as LPS are potent physiological inducers of HSP in the colon (23, 51). Contrary to colonic tissue, small intestinal tissue is not exposed to such stimuli and does not show the same HSP distribution (23, 52). This illustrates another example of the contribution of the commensal flora to an appropriate intestinal homeostasis. Strikingly, HSP expression in chronically inflamed intestines is low (19, 53, 54). Therefore, strategies to enhance HSP expression in the inflamed intestine may contribute to disease amelioration.

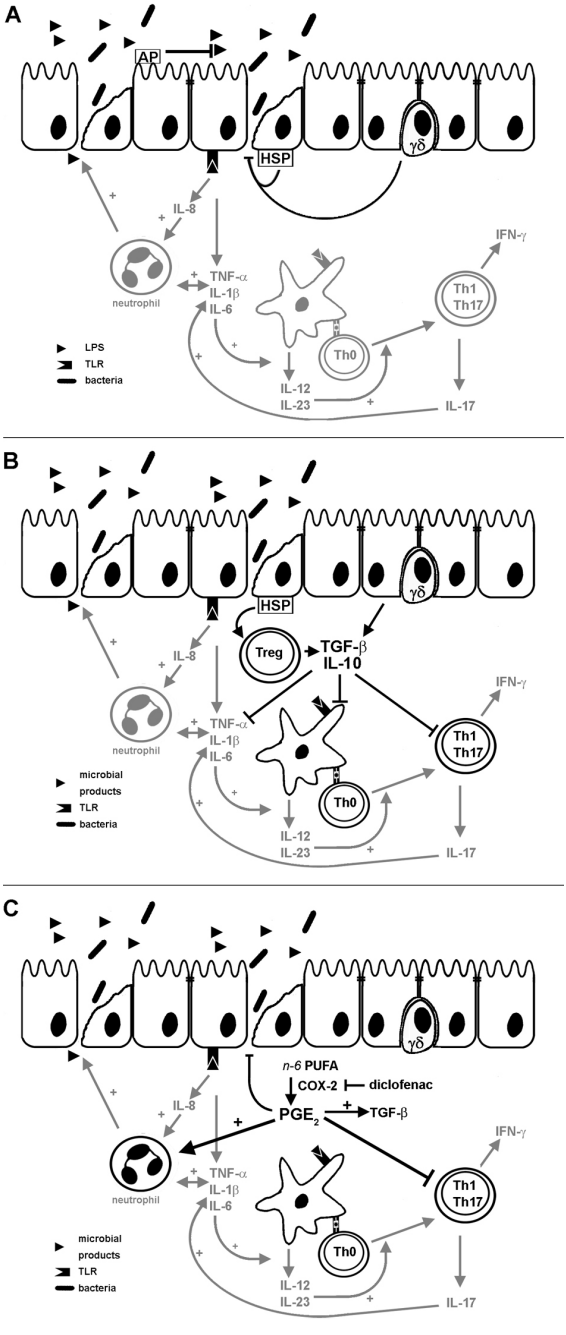

Figure 1: Schematic overview of regulatory mechanisms that are described in this thesis in relation to intestinal inflammation.
Epithelial barrier disruption will attract neutrophils to the intestine, and result in the production of proinflammatory cytokines followed by activation of Th1/Th17 cells. In turn Th1-derived IFN-γ will enhance intestinal permeability whereas IL-17 enhances proinflammatory cytokine production, together leading to a vicious circle of intestinal inflammation. Interference with this vicious circle may result in resolving inflammation. **A.** Prevention of innate immune activation can be achieved via dephosphorylation of luminal LPS by AP, thereby reducing TLR-signaling in the intestine. Upregulation of HSP-expression may result in a more effective clearance of stressed cells and restoration of damaged tissue. In addition, γδ Tcells will contribute to tissue repair. **B.** HSP-expression by epithelial cells may stimulate Treg induction. The regulatory cytokines IL-10 and TGF-β will inhibit DC activation and the subsequent induction and activation of Th1/Th17 cells. **C.** Inhibition of the pro-inflammatory effect of PGE_2 via inhibition of COX-2 or via replacing *n-6* PUFAs by *n-3* PUFAs may result in a decrease in the number of inflammatory cells, such as neutrophils. In contrast, an increase in PGE_2 production inhibits IL-12/IL-23 production by DCs and the subsequent induction of Th17 cells. Furthermore PGE_2 stimulates TGF-β production by Tregs and IEC, and stimulates tissue repair.

Indeed, efforts to upregulate endogenous HSP expression resulted in inhibition of inflammation (55-58). Administration of exogenous (mycobacterial) HSP may help to trigger endogenous HSP production and to induce the development of IL-10-producing HSP-specific Tregs (25, 59). Our efforts to induce HSP-specific Tregs via the administration of mycobacterial HSP were not successful in the used DSS and TNBS colitis models. Possibly the acute character of these inflammatory models leaves no time to activate sufficient regulatory capacity of HSP (chapter 3).

Dendritic cells (DC)

The interaction between IEC and DCs is of major importance in immune regulation (7, 60). The presence of IEC-derived transforming growth factor-β (TGF-β), together with retinoic acid (RA, a metabolite from vitamin A) contribute to the development of tolerogenic $CD103^+$ DCs (60-64). Production of thymic stromal lymphopoietin (TSLP) by IEC imprint $CD11b^+$ DCs to promote T helper 2 (Th2) responses (65). Interestingly, exposure to CT resulted in a decrease in $CD103^+$ DC subset in the intestine accompanied with an increase in conventional $CD11b^+$ DCs (chapter 4), thereby altering the intestine in a more susceptible environment for immune activation. This shift in DC populations may be the consequence of a direct effect of CT on IEC thereby altering the production of IEC-derived factors.

Moreover, the presence of PE together with CT further enhanced the influx of $CD11b^+$ DCs. Probably PE (and other food allergens) possess properties that actually can activate DCs as shown by others (66). Noteworthy, only a minority of several thousand ingested proteins are able to elicit allergy, and despite extensive research, it is still unknown which structural, functional or biochemical features explain their allergenic potential (67). Food processing may for instance alter allergens in a way that they cross the epithelial barrier instead of entering via M cells (68). Therefore, the capacity of CT to enhance intestinal permeability (49) will contribute to increased allergen entrance across the epithelial barrier, and this may indirectly result in increased activation of conventional DC. Furthermore, it has been shown that CT directly stimulates DC for instance by upregulating Th2 driving co-stimulatory molecules OX40L and Jagged2 (69). Together, both direct and indirect effects of CT may cause stimulation of DC. The CT effects that may contribute to allergic sensitization are summarized in fig. 2.

Whereas CT is probably not involved in induction of human food allergic responses under normal circumstances, other enterotoxins such as enterotoxin B from *Staphylococcus aureus* (SEB) and thermolabile enterotoxin from *Escherichia coli* (LT) that are found in humans, have comparable adjuvant capacities in mouse models for

allergic sensitization (33, 70), and exert comparable effects on DCs (71). Therefore CT can be a useful tool to gain more insight in the role of DCs in allergic sensitization.

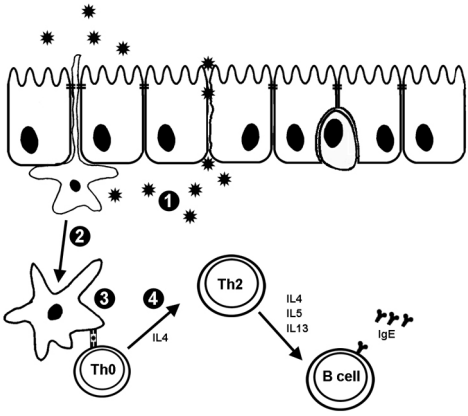

Figure 2: effects of CT that contribute to its adjuvant capacity
The mucosal adjuvant CT can affect antigen availability by an increase in intestinal permeability and enhancement of antigen uptake by DCs (1). It can increase DC recruitment and migration (2), as well as DC maturation by increasing stimulatory molecules (3). CT can also promote Th2 polarization (4).

γδ T cells

Due to their location in the epithelial layer, γδ T cells are ideally situated for crosstalk with IEC. Among other things, γδ T cells recognize and eliminate tumor-, infected- or otherwise "stressed" cells (27), and contribute to immune surveillance and first line of defense (72). As indicated above, exposure to CT may result in cellular stress in IEC (48, 73, 74) and subsequent activation of IEC. It is very well possible that induction of cellular stress in IEC indirectly triggers γδ T cells, and that this process contributes to the observed decrease in γδ T cells upon CT exposure (chapter 4). Supportive to this idea is the finding by others that sensitization to peanut followed by peanut challenge was accompanied by the induction of intestinal damage and reduced numbers in γδ T cells (75). Whether the decrease in γδ T cell numbers observed in this paper is facilitating food allergic sensitization or is the result of the observed pathology remains unclear. It also remains unclear whether the IEC-mediated stress response will directly result in diminished numbers of γδ T cells. The reduction in γδ T cells may also be a primary effect of CT. Subsequently, reduced γδ T cell numbers will result in a decreased capacity to respond to cellular stress in IEC, and this may contribute to increased intestinal permeability.

In addition, γδ T cells possess a variety of protective capacities that are linked to maintenance of mucosal homeostasis (76). Comparable to AP and HSP-expression, IEL numbers highly depend on the microbial colonization of the gut and changes in

antigen load and composition. They are an important source of TGF-β (77), which contributes to immune regulation. Several studies showed the involvement of γδ T cells in induction and maintenance of oral tolerance (78-80). In chapter 5 we showed that blocking of the γδ TCR using an antibody (UC7-13D5) resulted in enhanced allergic responses after sensitization with PE+CT. A decrease in γδ T cells due to CT exposure, as observed in chapter 4, would implicate a reduction in TGF-β production and a diminished suppressive environment in the intestine, thereby facilitating sensitization to co-administered allergen (e.g. PE). This may explain the increased sensitization in the absence of functional γδ T cells, as observed in chapter 5.
Another function of γδ T cells is the regulation of the continuous turnover of IEC and the epithelial restitution upon intestinal injury through the production of both keratinocyte growth factor (KGF) and TGF-β (29, 31, 81). Several studies indeed report the beneficial effects of γδ T cells in animal models for IBD (29, 77, 82, 83). These findings confirm the importance of restoring intestinal barrier intergrity as a strategy to overcome intestinal inflammation (see also paragraph 2.1 of this discussion). Maintenance of barrier integrity would also help to protect from food allergic sensitization and prevention of anaphylactic effector responses (see also paragraph 2.1).
Together, the importance of γδ T cells in maintenance of intestinal homeostasis is apparent, but further research is needed to elucidate their exact role in sensitization to food proteins and their possible contribution in therapeutic interventions.

Polyunsaturated fatty acids and prostaglandins
Polyunsaturated fatty acids (PUFAs) can be classified in *n-3* fatty acids and *n-6* fatty acids. Both types of fatty acids are precursors of signaling molecules (eicosanoids) with opposing effects (84). The predominant *n-6* PUFA is arachidonic acid (AA), which is converted to prostaglandins and leukotrienes through cyclo-oxygenase (COX) and lipoxygenase (LOX)-enzymes (42). PGE_2 is the best characterized AA-derived prostaglandin, and possesses a number of pro-inflammatory functions, including induction of fever, pain and oedema, and it promotes the production of IgE by B-cells. In contrast, it can be immunosuppressive in other circumstances (41). The constant levels of PGE_2 in the intestine as a result of constitutive expression of COX-2 contributes to the maintenance of epithelial integrity (85), and has a role in the induction of oral tolerance (86).
Modulating PGE_2 production can be achieved by replacing the membrane *n-6* PUFA AA by *n-3* PUFAs that will result in the synthesis of other eicosanoids (87). The

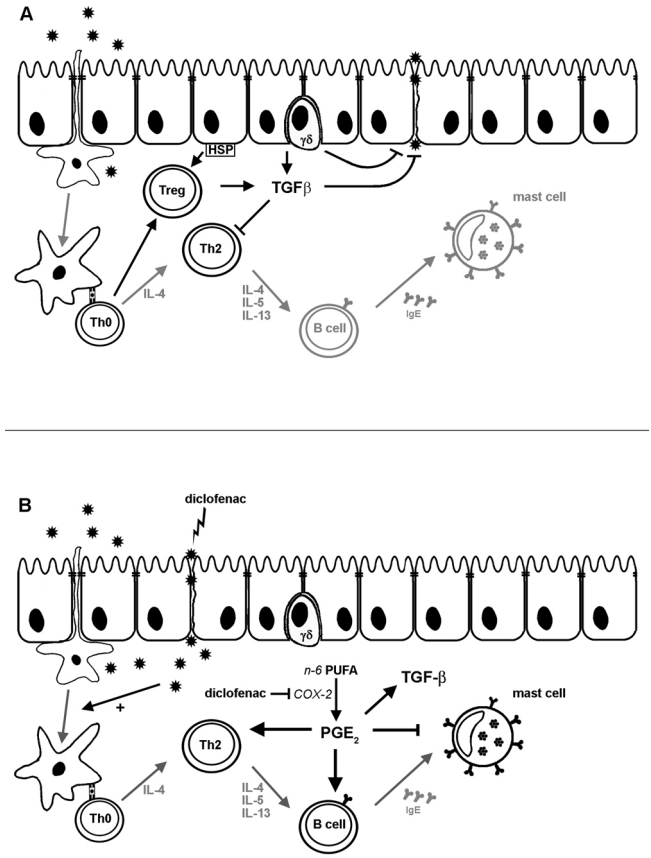

Figure 3: Schematic overview of regulatory mechanisms investigated in this thesis in relation to allergic sensitization.
Prevention of allergic sensitization can be achieved via inhibition of Th2 induction or via inhibition of antigen entrance. **A.** Effective clearance of stressed cells and restoration of damaged parts of the epithelial lining can be directly mediated by γδ T cells or via the induction of the regulatory cytokine TGF-β and will reduce antigen entrance. TGF-β produced by either Tregs or γδ T cells may also inhibit Th2 cells. Additionally, expression of HSP by epithelial cells may stimulate Treg induction. **B.** PGE_2 has Th2 promoting capacities and stimulates B cells to produce IgE. In contrast, PGE_2 inhibits mast cell degranulation. Reduction in PGE_2 production by replacing *n-6* PUFAs by *n-3* PUFAs or by diclofenac-mediated inhibition of COX-2 may inhibit allergic sensitization, but enhances mast cell degranulation. On the other hand, diclofenac may enhance intestinal permeability, thereby enhancing allergen entrance and allergic sensitization.

reduced production of PGE_2, accompanied by a simultaneous increase in regulatory prostaglandins will contribute to anti-inflammatory and regulatory effect of *n-3* PUFAs. Furthermore, *n-3* PUFAs are directly modulating gene expression (especially NF-κB), signal transduction pathways, cell death (apoptosis) and membrane fluidity (84). Together, the application of fish oil and other dietary products rich in *n-3* PUFAs has therefore been proposed in the approach to reduce adverse immune responses (87, 88).

More recently, it has become clear that AA-derived prostaglandins are needed to guarantee a proper immune function and that care must be taken by replacing all *n-6* PUFAs by its *n-3* equivalent (89). Outcomes of dietary intervention studies with *n-3* PUFAs in humans in both IBD and allergies were inconsistent. In line with human intervention studies, we were not able to demonstrate a beneficial effect of *n-3* PUFAs on allergic sensitization in mice (chapter 7). In contrast, we observed increased release of mast cell mediators in response to oral antigen challenge after a *n-3* diet. So, the complex network of prostaglandins and other eicosanoids are a difficult target because of the variety of their immuno modulating effects.

3. Risk factors

'Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are more in danger from them than from the invaders. We live in the midst of explosive devices; we are mined'. Thomas, 1972 (90)

An enormous amount of bacterial products and nutrients are challenging the intestine. To achieve tolerance to food antigens and commensal flora, intestinal homeostasis relies on a network of different regulatory cell populations and additional mechanisms (8). Due to this redundancy the intestine stays rather insensitive to disease. Development of adverse responses in the intestine need the combination of a susceptible genetic make-up, break of intestinal barrier function, and presence of specific adjuvant signals. On the other hand, epidemiologic data still reveals a considerable incidence of intestinal disease: approximately 4% of individuals suffers from food allergies in the westernized countries (91, 92), and up to 0.4% of the general population is affected by IBD (93). Defined risk factors in IBD include genetic polymorphisms (93), diets (94), bacterial infections (9, 95) and drugs such as NSAIDs (96), all of which are more or less related to intestinal permeability.

NSAIDs such as diclofenac, that inhibit COX-enzymes, result in decreased levels of PGE_2 and reduction of proinflammatory cell influx such as neutrophils (fig. 1C). In theory, this would be beneficial in inflammatory conditions like IBD. On the other hand, PGE_2-mediated repair of mucosal injury (mediated via the induction of epidermal growth factor receptor (EGFR) on epithelial cells) may also be impaired due to decreased PGE_2 levels (35). In addition, NSAIDs are able to directly induce enteropathy by disturbing the protective mucus layer (38). Therefore, the therapeutic capacity of NSAIDs in IBD remain unpredictable.
In theory, the diclofenac-mediated decrease in PGE_2 production would result in diminished Th2 responses, and reduction of IgE formation by B cells. Our data however, show that diclofenac can enhance allergic sensitization to peanut (chapter 6) which signifies a relevant risk factor for the human situation. Several factors contribute to this risk. First, diclofenac is normally used to treat fever, pain or inflammation (97), and diclofenac exposure may coincide with a range of inflammatory, possibly adjuvating mediators. Second, diclofenac-associated enteropathy may result in breaking of the intestinal barrier, enabling the entrance of food allergens in the intestine. Third, as prostaglandins and related leukotrienes are involved in histamine release from mast cells, a direct effect of diclofenac and other NSAIDs on this process is also conceivable, although contradicting results have been obtained (98-100). For this reason, it is of importance to investigate whether diclofenac and other NSAIDs are able to enhance food allergic effector responses.

4. Prophylactic and therapeutic opportunities

Reduction of intestinal inflammation via suppression of the immune response is the main focus of therapeutic intervention in IBD (101). This harbors a risk in simultaneously suppressing regulatory immune processes (8). Strategies that stimulate regulatory mechanisms, together with preserving barrier integrity may contribute in resolving disease and in preventing disease flare up (46).
Stimulation of immune regulation is part of strategies aiming at curing of both IBD and allergic sensitization. For instance, the administration of helminths results in the production of regulatory cytokines, and is protective in inflammatory and allergic disorders (102, 103). More conflicting data have been described on dietary intervention with *n-3* PUFAs. Beneficial effects have been found in IBD (104) and allergy (105, 106), whereas other studies reported no effects of dietary intervention on disease outcome (107, 108). On the other hand, prevention of allergy and enhancement of

immune functions have been described when intervention with *n-3* PUFAs was initiated prenatally (via the maternal diet) or early in life (109, 110).
Targeting regulatory γδ T cells may hold a promising strategy. Enhancement of γδ T cell numbers using fruit-derived polyphenols (tannins) resulted in improvement of innate and regulatory immune responses in humans (111-113). Tannin-induced increase in γδ T cells in a mouse model for food allergy resulted in reduced allergic sensitization (114). In addition, the red wine-derived polyphenol resviratrol has been of benefit in reduction of experimental colitis (115) although it is unclear whether this effect is contributable to γδ T cells.
This short and very incomplete list of prophylactic and therapeutic opportunities mirrors the factors that contribute to intestinal barrier function. Research on the mechanisms underlining adverse immune responses therefore contributes to target finding for therapies. In addition, animal studies can also reveal limitations in relation to targeting specific regulatory mechanisms. Some examples described in this thesis illustrates drawbacks of possible intervention. This includes the observed increase in mast cell release with n-3 PUFA or vitamin E (chapter 7). Also the intervention study with AP showed anti-inflammatory activity only in case of severely inflamed intestinal disease. (chapter 2). Together, our findings emphasize the need of an approach to reveal the underlying disease cause before randomly targeting a regulatory process.

5. Future directions

Together, the data described in this thesis shows a prominent involvement of epithelial integrity in adverse immune responses in the intestine. Gaining more knowledge on IEC function and related neighbouring cells such as γδ T cells and DCs will contribute to definition of therapeutic targets and to identification of risk factors.
Part of this approach can include mechanistic studies on IEC function *in vitro*, their response to bacterial components such as LPS or CT and modulation with dietary products (for example PUFAs, vitamin E) or drugs (like diclofenac and other NSAIDs). In addition, the interaction with other cell types (γδ T cells, DCs) can be assessed *in vitro* by developing coculture and transwell systems. These *in vitro* obtained data can contribute to a more refined use of animal studies to reveal mechanisms and therapeutic targets.
Furthermore, the contribution of HSP-expression in the protection of the intestinal barrier is of interest. Future research may reveal strategies to upregulate HSP expression, for instance by using co-inducers such as carvacrol in animal models for

intestinal inflammation and allergy. Also here, the use of *in vitro* IEC cell-lines may contribute to unravel the role of HSP in immune activation.
The mechanisms behind the effect of CT on γδ T cells and the exact role of these innate cells in allergic sensitization need to be addressed further. Questions that remain to be elucidated include the cytokines produced by γδ T cells (IFN-γ; TGF-β; IL-17 (116)) and the role of γδ T cells in immune surveillance (clearance of stressed cells) in regulation of allergic sensitization. Moreover, it will be of interest to investigate whether γδ T cells can be a target for therapeutic intervention.
Identification of risk factors for both intestinal inflammation and allergic sensitization and the mechanisms underlying these risks will contribute to improvement of prophylactic and therapeutic approaches in humans. It is therefore worthwhile to further elucidate the mechanism behind the diclofenac-induced increase in allergic sensitization. For instance, when increased sensitization is solely dependent on induction of intestinal damage, the use of a mucus-protecting drug may be sufficient to substantially reduce this risk. However, if the effect of NSAIDs involves COX-derived mediators more careful use of these drugs is recommendable in genetically predisposed individuals.
Further research is also warranted to clarify the observed increase in mast cell release with *n-3* PUFAs and vitamin E as described in chapter 7. The use of mast cells *in vitro* can probably reveal whether this effect is directly attributable to membrane alterations, or that other factors are involved.
Together, a translational approach, using both *in vitro* techniques and refined *in vivo* and *ex-vivo* animal studies may contribute to gain more insight in the mechanisms underlying the maintenance of intestinal homeostasis, and may find new targets to treat IBD and food allergy.

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Nederlandse Samenvatting

De darm heeft als belangrijkste taak om voedingsstoffen op te nemen. Maar met het voedsel komen ook andere stoffen (bijvoorbeeld bestrijdingsmiddelen, conserveringsmiddelen en medicijnen) en ziekteverwekkers (bacteriën, schimmels en virussen) naar binnen. Bovendien wordt het laatste stuk van de dunne darm en de gehele dikke darm bevolkt door een groot aantal gunstige bacteriën, de zogenaamde darmflora. In feite huizen er minstens tien keer meer bacteriën in de darm, dan dat er eigen cellen in het menselijk lichaam zijn.
Het is duidelijk dat het afweersysteem van de darm (in het Engels: Gut Associated Lymphoid Tissue, of GALT) de complexe taak heeft om een goede afweerreactie tegen ongewenste ziekteverwekkers te verzorgen, zonder te reageren tegen voedsel-bestanddelen, medicijnen of darmflora. De GALT kan vergeleken worden met een portier van een uitgaansgelegenheid: hij bepaalt welke mensen naar binnen mogen (voedselbestanddelen, medicijnen), van wie het gezellig is als ze voor de deur blijven hangen (darmflora), en voor wie je meteen knokploegen moet alarmeren (ziekteverwekkers). Deze portiersfunctie van de GALT is een complex gebeuren, waar meerdere celtypen bij betrokken zijn en waarbij een goede regulatie nodig is. Wanneer de regulatie in de GALT niet goed verloopt kan dit leiden tot het ontstaan van chronische darmontstekingen, voedselallergie of kanker.

Het onderzoek dat in dit proefschrift is beschreven is gericht op het verkrijgen van meer inzicht in het effect van omgevingsfactoren (bacteriële producten, voedingsbestanddelen en medicijnen die in de darm aanwezig kunnen zijn) op de functie van de GALT. Met andere woorden: welke omgevingsfactoren kunnen bijdragen aan de regulatie door de GALT, waardoor chronische darmontsteking en voedselallergie voorkomen of genezen kunnen worden? Welke omgevingsfactoren zijn juist aanleiding voor verslechtering van de GALT functie? Meer kennis over de regulatie in de GALT kan bijdragen aan het begrip van de GALT en daarmee aan het voorspellen van nadelige effecten van stoffen op darmgezondheid maar ook aan de ontwikkeling van therapieën voor chronische darmontstekingen en voedselallergie.

Hoe werkt de GALT
De eerstelijns bescherming van de darm bestaat uit één enkele laag cellen (epitheelcellen), die via eiwitten aan elkaar verbonden zijn en bedekt zijn met een slijmlaag (mucus). Op deze manier vormen de epitheelcellen een ondoordringbare laag voor bacteriën. Daarnaast produceren epitheelcellen enzymen die bacteriële producten

onschadelijk maken. Tegelijkertijd zijn de epitheelcellen verantwoordelijk voor een groot deel van het transport van voedselbestanddelen.
De barrière functie van de epitheellaag wordt versterkt door de aanwezigheid van bepaalde witte bloedcellen die behoren tot het aangeboren afweersysteem: de intra-epitheliale lymfocyten. Deze cellen komen direct in actie wanneer ziekteverwekkers op één of andere manier het weefsel binnendringen. Ze zijn in feite de knokploeg en zorgen voor het doden en opruimen van binnengedrongen ziekteverwekkers. Zo vormen ze de eerstelijns afweer.
Naast de intra-epitheliale lymfocyten zijn er verschillende andere soorten witte bloedcellen te vinden in het weefsel onder de epitheellaag. Sommige van deze cellen (dendritische cellen) hebben onder andere de taak om de omgeving af te speuren op lichaamsvreemde stoffen zoals ziekteverwekkers, maar ook op voedselbestanddelen en andere stoffen. Samen met andere witte bloedcellen (T cellen) bepalen ze vervolgens wat voor een type afweerreactie er moet plaatsvinden.
Voedselbestanddelen zijn ongevaarlijk, en een afweerreactie zal normaliter worden onderdrukt. Dit wordt orale tolerantie genoemd. Ziekteverwekkers zullen echter via een hele specifieke afweerreactie worden bestreden, wat meestal leidt tot de vorming van antistoffen. Bovendien ontstaat er in beide gevallen ook een geheugen: bij een volgende ontmoeting met dezelfde lichaamsvreemde stof zal er sneller en efficiënter gereageerd worden. Om terug te komen op de vergelijking met de uitgaansgelegenheid: er worden pasjes uitgereikt waarmee je een volgende keer weer zonder problemen naar binnen kunt (voedselbestanddelen), of waarop een toegangsverbod is aangegeven (ziekteverwekkers), en deze passen gelden ook bij een andere ingang!
De belangrijkste taken van de GALT zijn dus het opwekken van efficiënte reactie tegen ziekteverwekkers, en het onderdrukken van de reactie tegen onschadelijke voedselbestanddelen.

Verstoring van de reactie van de GALT tegen ziekteverwekkers kan leiden tot chronische darmontsteking.
Om gezond te blijven moeten afweerreacties tegen ziekteverwekkers goed worden gereguleerd. Een reactie tegen ziekteverwekkers (een zogenaamde ontstekingsreactie) gaat vaak gepaard met ziekteverschijnselen zoals koorts. Wanneer dat te lang duurt, kan schade ontstaan aan gezond weefsel. Daarom is het belangrijk dat een afweerreactie wordt gestopt op het moment dat alle ziekteverwekkers opgeruimd zijn. Dit is de taak van gespecialiseerde regelcellen en signaalstoffen. Je kunt ze vergelijken

met de thermostaat van de verwarming: als de juiste temperatuur bereikt is, zorgt de thermostaat ervoor dat de ketel uitgaat.
Wanneer deze regulatie niet goed verloopt en een afweerreactie tegen ziekteverwekkers te lang aanhoudt ontstaat schade aan het weefsel. In de darm kan dit leiden tot schade aan de epitheellaag. Daarmee is de barrière tussen de darm en het weefsel verzwakt, en kan de inhoud van de darm (waaronder de bacteriën van de darmflora) in het weefsel terecht komen. Hierdoor kan een aanhoudende ontstekingsreactie ontstaan. Die wordt niet tijdig gestopt (er is immers niet voldoende regulatie), en dit leidt tot nog meer epitheelschade. Zo ontstaat een vicieuze cirkel met chronische darmontsteking tot gevolg.
Wereldwijd lijden ongeveer 4 op 1000 mensen aan een vorm van chronische darmontsteking zoals de ziekte van Crohn of Ulceratieve Colitis. In de ziekte van Crohn zijn afwisselend delen van de dunne en de dikke darm ontstoken. In Ulceratieve Colitis is een aaneengesloten stuk van (meestal) de dikke darm aangetast. Patiënten hebben last van hevige buikpijn, diarree, gewichtsverlies, koorts en ernstige vermoeidheid. Beide ziektes gaan gepaard met periodes waarin minder last wordt ondervonden, maar er is eigenlijk geen genezing mogelijk. In een groot aantal gevallen is chirurgisch ingrijpen noodzakelijk waarbij ontstoken delen van de darm worden verwijderd. Door deze ingrepen en de veelvuldige buikklachten is de sociale belasting voor patiënten aanzienlijk.
De exacte oorzaak van het ontsporen van de regulatie in de GALT die bijdraagt aan het ontstaan van chronische darmontstekingen is niet bekend. Wel is duidelijk dat genetische factoren en omgevingsfactoren een rol spelen. In de laatste decennia van de twintigste eeuw is een gestage groei in het aantal patiënten waargenomen, maar momenteel is de groei gestabiliseerd. De oorzaak van de toegenomen incidentie zou te maken kunnen hebben met de toename in welvaart (andere leefstijl, en daarmee verandering in omgevingsfactoren), maar een precieze verklaring is nog steeds niet gevonden.
In het **eerste deel van dit proefschrift** is gekeken naar mogelijkheden om de GALT functie te versterken en daarmee mogelijke therapieën te vinden voor **chronische darmontsteking**. Voor dit onderzoek is gebruik gemaakt van muizenmodellen voor deze aandoening.
Zoals eerder aangegeven kunnen epitheelcellen van de darm enzymen maken waarmee producten van bacteriën al in de darm onschadelijk gemaakt kunnen worden. Een dergelijk enzym is alkalische fosfatase, en in **hoofdstuk 2** is onderzocht of toedienen van alkalische fosfatase bruikbaar is als therapie in chronische darmontsteking. Uit het

onderzoek is gebleken dat toedienen van alkalische fosfatase aan muizen met veel ontsteking van de darm (dus met veel schade aan het epitheel) leidt tot een afname van deze ontsteking. Dat zou betekenen dat patiënten met ernstige darmontsteking baat hebben bij toediening van alkalische fosfatase om de ontsteking te laten verminderen. Echter, toediening van alkalische fosfatase hielp niet als de darmontsteking minder ernstig was. In die gevallen is de productie van alkalische fosfatase door epitheelcellen zelf waarschijnlijk nog ruim voldoende om de aanwezige bacteriële producten af te breken.

Vergroten van het aantal regelcellen is een andere optie om regulatie in de GALT te verbeteren. In chronische darmontsteking (en in iedere andere ontsteking) heb je regelcellen nodig op de plaats waar weefselschade optreedt, om ter plekke de reactie te stoppen. Nu is het zo dat in gebieden met weefselschade een bepaald type eiwitten veel meer wordt aangetroffen, namelijk de zogenaamde stresseiwitten (in het Engels: heat shock proteins, HSP). Wanneer regelcellen HSP herkennen (en daarmee weefselschade) zullen ze trachten de ontsteking te stoppen. Indien er meer HSP-specifieke regelcellen aanwezig zijn, zou dit kunnen leiden tot een afname van de ontsteking. Het aantal HSP-specifieke regelcellen kan gestimuleerd worden door toediening van HSP voordat er een ontsteking plaatsvindt, om zo eventuele ontstekingen te voorkomen. Deze aanpak werkt heel goed in een muizenmodel voor reuma. **Hoofdstuk 3** laat zien dat deze aanpak niet werkt in twee verschillende modellen voor darmontsteking in muizen. Er zal verder onderzoek worden gedaan naar de rol van HSP in darmweefsel, en hoe HSP kunnen bijdragen aan verbetering van de GALT functie.

Verandering van de reactie van de GALT tegen voedselbestanddelen kan leiden tot voedselallergie

Naast de regelcellen die betrokken zijn bij het stoppen van een afweerreactie tegen ziekteverwekkers zijn er in de GALT ook regelcellen en signaalstoffen aanwezig om de afweerreacties tegen voedselbestanddelen te voorkomen. Zoals eerder aangegeven, is dat een lastige taak, aangezien er naast het voedsel zoveel andere stoffen in de darm aanwezig zijn.

Bij een tekort aan (of verandering in) regulatie kan er een afweerreactie tegen voedselbestanddelen en daarmee mogelijk voedselallergie optreden. In dit geval gaan witte bloedcellen van de GALT namelijk signaalstoffen afscheiden die leiden tot de vorming van IgE antistoffen. Deze voedselspecifieke IgE antistoffen plakken vast aan mestcellen. Een volgende keer dat het voedsel wordt gegeten, bindt dit voedsel aan het

IgE op de mestcellen. Mestcellen zullen daardoor mediatoren gaan uitscheiden (waaronder histamine) die een allergische reactie veroorzaken. Deze reactie kan uiteenlopen van tinteling in de mond en diarree tot benauwdheid en bloeddrukdaling en in het ergste geval tot een dodelijke anafylactische shock.
Voedselallergie komt vrij veel voor, namelijk bij 4 op de 100 personen. Hiervan is de grootste groep allergisch voor melk of pinda. Allergie voor melk is er vooral op jongere leeftijd, terwijl pinda-allergie vaak levenslang is. Tot op heden is de enige remedie om klachten te voorkomen het niet eten van het voedsel waarvoor men allergisch is. Het ontstaan van voedselallergie is gedeeltelijk genetisch bepaald (zogenaamde atopie), maar ook leeftijd, dieet en infecties kunnen een rol spelen. Ook voor voedselallergie is in de tweede helft van de twintigste eeuw een gestage toename van het aantal patiënten gevonden. Net als bij chronische darmontsteking wordt dit in belangrijke mate geweten aan verandering in levensstijl, en dus aan een verandering in omgevingsfactoren.
In **het tweede deel van dit proefschrift** is gekeken naar het effect van vetzuren en medicijnen op het ontstaan van **voedselallergie** en naar de rol van bepaalde signaalstoffen hierbij. Verder is onderzocht wat er eigenlijk verandert in de GALT op het moment van het ontstaan van voedselallergie. In dit onderzoek is gebruik gemaakt van een muizenmodel voor pinda-allergie.
Signaalstoffen die de regelcellen ondersteunen dragen bij aan een betere GALT functie. Een belangrijke groep signaalstoffen zijn de prostaglandinen. Prostaglandinen worden gemaakt uit vetzuren die afkomstig zijn uit het voedsel. In deze grote groep komen stoffen voor die de regulatie stimuleren. Deze groep prostaglandinen worden met name gevormd uit omega-3 meervoudig onverzadigde vetzuren. Om dit te bestuderen zijn in **hoofdstuk 7** muizen gevoerd met diëten die verschillend waren in vetzuursamenstelling. Vervolgens zijn de muizen allergisch gemaakt voor pinda. De studie liet geen verschil zien in de hoeveelheid IgE antistoffen tegen pinda tussen de verschillende diëten. Opvallend genoeg leidde een dieet dat rijk is aan omega-3 vetzuren tot een veel hogere afgifte van mediatoren door mestcellen, en dat is niet zo heel gunstig, omdat dit zou kunnen leiden tot benauwdheid, bloeddrukdaling of zelfs een anafylactische shock in voedselallergische mensen. Hoe omega-3 vetzuren dit nu precies veroorzaken, wordt verder uitgezocht.
In het lichaam zorgt het cyclo-oxygenase (COX) enzym voor de omzetting van vetzuren in prostaglandinen. Naast prostaglandinen met regulerende eigenschappen zijn er ook die de afweerreactie stimuleren. Deze prostaglandinen veroorzaken ook het pijngevoel en de roodheid bij een ontsteking. Een aantal pijnstillers, zoals paracetamol

en diclofenac werken via de remming van COX-enzymen. Deze medicijnen zorgen dus voor vermindering van prostaglandinen en onderdrukken zo ziekteverschijnselen zoals koorts. **Hoofdstuk 6** laat zien dat toedienen van diclofenac aan muizen leidt tot een toename van allergische reacties tegen pinda in het muizenmodel. Het is wel zo dat er in muizen een trucje uitgehaald moet worden om ze allergisch te maken: de muizen krijgen pinda gemengd met cholera toxine. Als cholera toxine wordt weglaten worden de muizen niet allergisch. Blootstelling aan diclofenac zonder gebruik te maken van cholera toxine leidt niet tot inductie van pinda allergie. Dat wil zeggen dat slikken van diclofenac niet zomaar leidt tot voedselallergie en alleen een risico zou vormen wanneer andere omstandigheden al aanleiding geven tot vermindering of verandering van de regulering door de GALT. Hierbij valt te denken aan een bacteriële of virale infectie of koorts, maar ook aan schade aan het darmepitheel. Verder onderzoek is nodig om deze omstandigheden en daarmee het risico van diclofenac en andere COX-remmers in kaart te brengen.
Zoals net al is aangegeven worden muizen niet allergisch als ze alleen pinda krijgen. Pinda is immers een onschadelijk voedselbestanddeel, en een afweerreactie wordt door regelcellen onderdrukt. Om allergie te krijgen is er iets nodig dat de GALT functie verandert. Wat in mensen de oorzaak is voor verandering van de GALT functie is niet echt bekend. Zoals hiervoor al aangegeven wordt in het muizenmodel cholera toxine (een bacterieel product) gebruikt om een allergische reactie tegen pinda te krijgen. In **hoofdstuk 4** wordt beschreven welke veranderingen er in de darm optreden in de dagen die volgen na inductie van voedselallergie. Hieruit blijkt dat het soort dendritische cellen (de witte bloedcellen in het weefsel, die de omgeving afspeuren op lichaamsvreemde stoffen) verandert, dat leidt tot activering van de GALT. Een opvallende bevinding was echter ook dat een bepaalde soort intra-epitheliale lymfocyten, namelijk de gammadelta T cellen, verdwijnen. Daarom is er een volgende studie uitgevoerd waarbij tijdens inductie van voedselallergie de functie van gammadelta T cellen is geblokkeerd. In dat geval wordt een toename in allergische reacties tegen pinda gevonden (**hoofdstuk 5**). Met andere woorden, als gammadelta T cellen hun werk niet kunnen doen, dan wordt de allergie erger. Dus gammadelta T cellen zijn (één van de) regelcellen in voedselallergie. Een therapie die leidt tot een toename van het aantal gammadelta T cellen zou daarom kunnen bijdragen aan het voorkomen van voedselallergie. Hiernaar zal verder onderzoek worden gedaan.

Samenvattend kan worden geconstateerd dat omgevingsfactoren uit de darm zoals bacteriële producten, voedselbestanddelen en medicijnen zeker van invloed kunnen zijn

op de GALT functie, zowel in gunstige als ongunstige zin. Het is belangrijk om de risico's goed in kaart te brengen, maar er liggen zeker ook mogelijkheden om de GALT functie te versterken. Verder onderzoek zal zich richten op de onderliggende mechanismen van de waargenomen effecten van verschillende stoffen.

Curriculum Vitae

Marianne Bol-Schoenmakers was born in Etten en Leur on June 23th, 1961. In 1979, she graduated from the Katholieke Scholengemeenschap Etten-Leur. In the same year she started her study "Hoger Natuurwetenschappelijk Onderwijs" (HNWO) at the Dr. Struycken-Instituut in Etten-Leur. During the last year of her study she attended an internship at the department of Molecular Genetics of the Wageningen University. She graduated in 1983, and started working as a technician in the Immunotoxicology research group of the department of Biological Toxicology (now part of the Institute for Risk Assessment Sciences, IRAS) of the Utrecht University under supervision of Prof. Dr. Willem Seinen. In 1989, after becoming a mother, she partly continued working as a technician and participated in the projects of several PhD students. From 2002, she started to work on her own PhD project under supervision of Dr. Raymond Pieters and more recently Dr. Joost Smit, which resulted in this thesis. She will continue working as a researcher in the Immunotoxicology department of IRAS.

List of publications:

Marianne Bol-Schoenmakers, Daniëlle Fiechter, Willem Raaben, Ine Hassing, Rob Bleumink, Daniëlle Kruijswijk, Kelly Maijoor, Monique Tersteeg-Zijderveld, Ruud Brands and Raymond Pieters. Intestinal alkaline phosphatase contributes to the reduction of severe intestinal epithelial damage. *Submitted*

Marianne Bol-Schoenmakers, Marisa Marcondes Rezende, Rob Bleumink, Louis Boon, Sue Man, Ine Hassing, Daniëlle Fiechter, Raymond Pieters, Joost Smit. A functional, regulatory role of intestinal γδ T cells during establishment of allergic sensitization. *Submitted*

Marianne Bol-Schoenmakers, Rob Bleumink, Marisa Marcondes Rezende, Emily Mouser, Ine Hassing, Irene Ludwig, Joost Smit and Raymond Pieters. Diclofenac enhances allergic responses in a mouse peanut allergy model. *Submitted*

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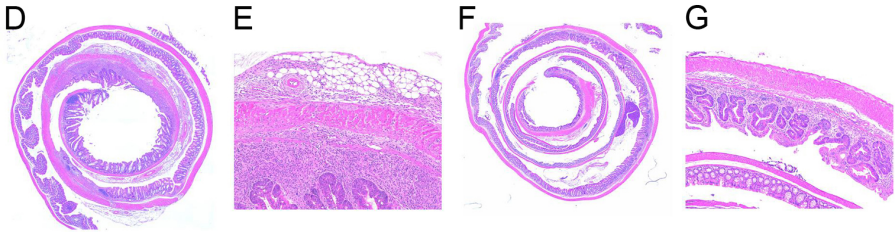
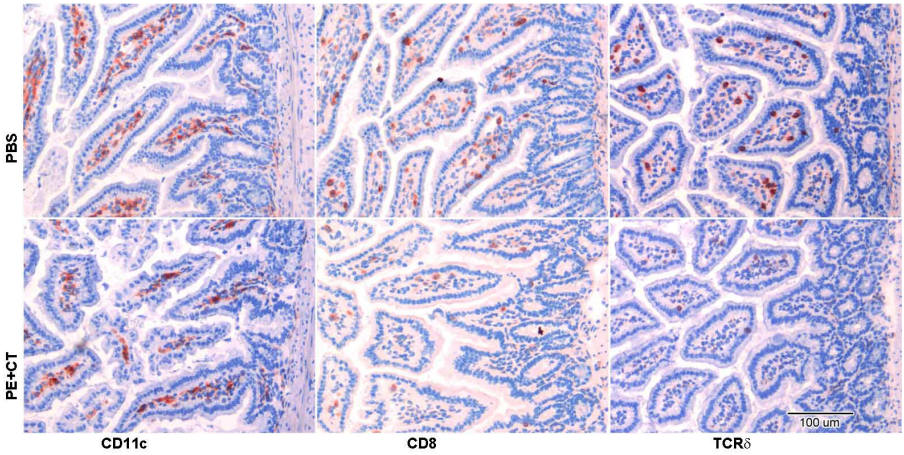
Lieve John: If I could save time in a bottle…… Dankjewel voor alles.

Marianne

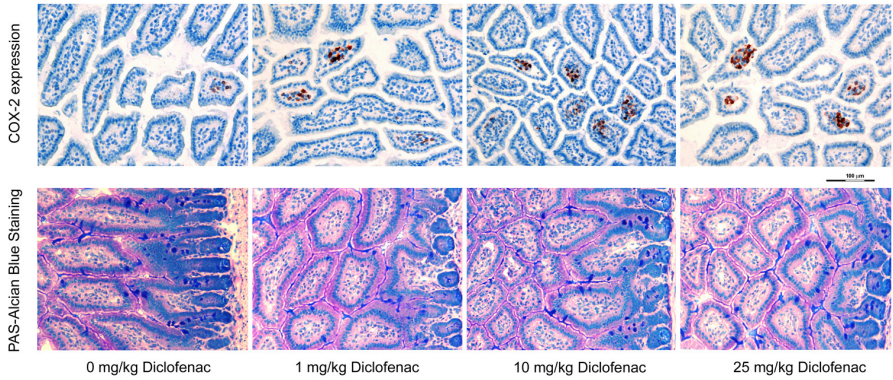
Colour figures



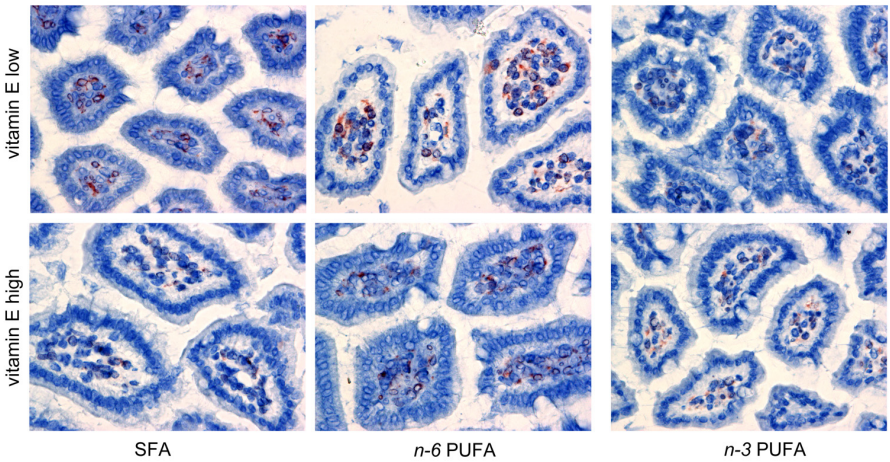
Chapter 2 - page 27
Figure 1: Treatment with iAP after DSS-induced intestinal damage.
D-G: H-E stained paraffin sections from vehicle-treated (**D-E**) or iAP-treated (**F-G**) colon. Original magnitude x40 (**D; F**) and x200 (**E;G***)*.



Chapter 4 - page 57
Figure 1: Immunohistochemical analysis of CD11c, CD8 and TCRδ cells in the intestine.
C57BL/6 mice (n=4) were exposed to PBS or PE+CT as described in materials and methods. On day 3 cryosections of the small intestine were stained in red for CD11c (left panel), CD8 (central panel) or TCRδ (right panel) as described in materials and methods. Representative micrographs of PBS-treated (upper row) or PE+CT treated mice (lower row) are shown. Original magnitude x100



Chapter 6 – page 99
Figure 7: Effect of diclofenac on COX-2 expression in the small intestine
Mice (n=3) were treated orally with 1, 10 or 25 mg/kg diclofenac and small intestine was dissected 2h later. Cryosections of the small intestine were stained in red for COX-2 (upper panel) or with PAS-Alcian Blue (lower panel) as described in materials and methods.



Chapter 7 – page 117
Figure 2: COX-2 expression in small intestine
Mice were fed with SFA-, *n-6* PUFA- or *n-3* PUFA-rich diets supplemented with low or high vitamin E, and sensitized to PE+CT as described in Materials and methods. At the end of the experimental intervention, mice were dissected and cryosections of small intestine were stained for COX-2 (in red) as described in material and methods. Representative sections of one mouse per group are shown (original magnitude *200) upper panel: vitamin E low diet; lower panel: vitamin E high diet. Left panel. SFA diet; middle panel: *n-6* diet; right panel: *n-3* diet