Mucosal Immune Regulation in Early Infancy: Monitoring and Intervention

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Mucosal Immune Regulation in Early Infancy:

Monitoring and Intervention

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Chapter 1

General introduction

INTRODUCTION

The human immune system consists of an innate and an adaptive system, which both sense and react upon antigens. The main function of our immune system is detection and eradication of harmful antigens such as bacteria, viruses and tumor cells. Nonetheless via the same receptors the immune system senses and tolerates self-antigens and harmless antigens like our intestinal flora and food.

During early infancy the immune system is educated to appropriately react to both harmful and harmless antigens. If the immune response is adequate and does not impair the individual this is called homeostasis. Loss of tolerance for example towards food or commensal bacteria leads to specific diseases in which homeostasis is lost, like food allergy or inflammatory bowel disease (IBD).

The innate immune response

The innate immune system differs from the adaptive counterpart by its swift reaction and the fact that this response is neither antigen specific, nor leads to memory. This system has highly preserved genes transcripting for several hundred proteins, mainly receptors. These so-called innate pattern recognition receptors (PRR) such as Toll-like [1,2] and NOD receptors [3,4] are activated after ligation of conserved microbial structures known as pathogen associated molecular patterns.

The innate pattern recognition receptors are found on leukocytes, such as macrophages, monocytes and dendritic cells, but also on epithelial cells. The expression of these receptors is abundant in the mucosa of the gastrointestinal, respiratory and urogenital tract where interaction with microorganisms is a continuously ongoing process.

At least 10 Toll-like receptors have been discovered in humans (TLR1-10). There is a clean segregation of roles between the different Toll-like receptors. Toll-like receptor 2 binds gram-positive and mycobacterial pathogen associated molecular patterns such as lipopeptide, lipotechioic acid, peptidoglycan and soluble tuberculosis factor [5,6]. Toll-like receptor 4 binds lipopolysaccharides from gram-negative bacteria [7] (Figure 1).

Toll-like receptors, have a preserved cytoplasmic domain similar to the intracellular portion of the Interleukin-1 receptor and related molecules and an extracellular portion containing leucine rich repeats [8, 9].

Ligand binding leads to dimerization of the receptor. In contrast to the Toll-like receptor 4 homodimerization, Toll-like receptor 2 signals only as a heterodimer, with either Toll-like receptor 1 or Toll-like receptor 6 [10]. Receptor activation results into the phosphorylation and activation of several intracellular proteins and eventually to the formation of cytokines. Amongst the produced cytokines are the pro-inflammatory Tumor Necrosis Factor (TNF)-α, interleukin(IL)-1 and the chemokine IL-8.

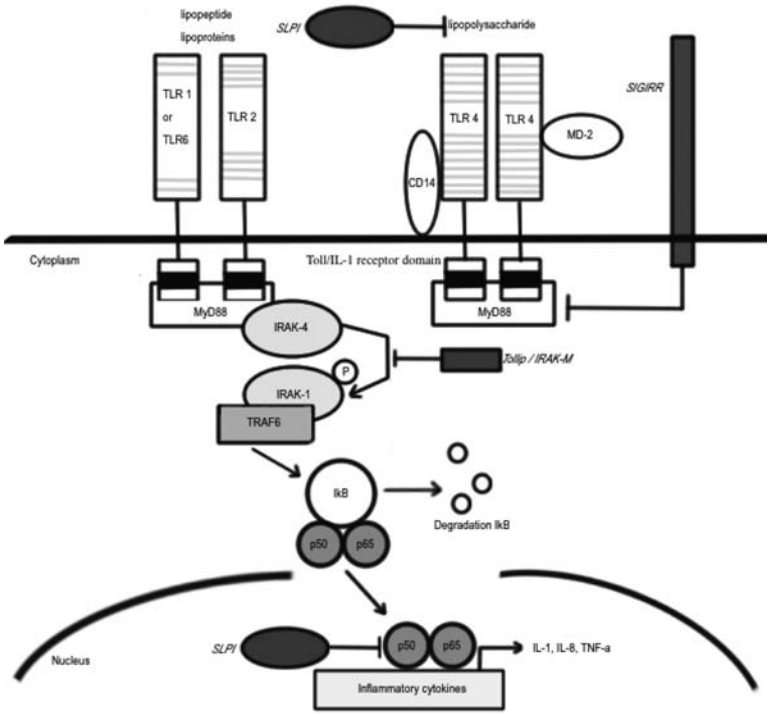

Figure 1 | Overview of TLR signaling and regulators

TLR: Toll Like Receptor, MD-2: Lymphocyte antigen 96, CD14: Cluster of differentiation 14, MyD88: Myeloid differentiation primary response gene (88), IRAK: interleukin-1 receptor-associated kinase, TRAF-6: TNF receptor-associated factor 6, IkB: IkappaB, p50: Transcription factor p50, p65: Transcription factor p65. Regulators: SLPI: secretory leukocyte peptidase inhibitor, SIGIRR: single immunoglobulin IL-1R-related molecule, Tollip: Toll interacting protein, IRAK-M: IL-1 receptor-associated kinase-M. Regulators are depicted in dark grey.

In conclusion, innate receptors are strategically present at sites at risk of encountering microorganisms. The binding of a pathogen-associated molecular pattern to an innate receptor induces an inflammatory response aiming to eradicate the pathogen. As a result, the inflammatory response creates an environment for the erection of an adaptive immune response.

The adaptive immune response

B and T lymphocytes are the main components of the adaptive or antigen specific immune response. Every single lymphocyte expresses a unique receptor, which is able to bind a single antigen. Antigens are picked up and presented by professional antigen presenting cells (dendritic cells and macrophages) via the major histocompatibility complex to naïve lymphocytes. The

antigen presenting cells also express PRR eliciting an innate reaction next to the initiation of an adaptive response.

Naive $CD4^+$ (helper) T-lymphocytes are functional immature. Activation with the appropriate antigen induces proliferation of T-lymphocytes, under the influence of IL-2, enhancing the effect of the immune response. The T-lymphocytes are divided into various subsets based on cytokine profiles in mice [11] and men [12]. Among these subsets are the Thelper 1 (Th1) and Thelper 2 (Th2). Whether naïve $CD4^+$ lymphocytes differentiate into Th1 or Th2 is decided during the first antigen encounter in the peripheral lymphoid tissue. An important factor is the quantity and quality of antigen presentation. Large quantities of peptide or strong affinity with the T-cell receptor will drive the differentiation towards Th1. Next to that the cytokine milieu is pivotal. Naïve $CD4^+$ cells activated in the presence of IL-12 and IFN-γ are dedicated to differentiate into Th1 cells. In contrast, the presence of IL-4, especially in the co-presence of IL-6, directs the cells towards a Th2-phenotype. Th1 cells predominantly produce IL-2 and IFN-γ, while IL-4, -5, and -13 are mainly produced by Th2 cells. Activated Th-cells migrate to the site of infection were the help B-lymphocytes to differentiate into immunoglobulin M (IgM) producing plasma cells (primary B-lymphocyte response). The secondary B-lymphocyte response leads to formation of germinal centers and eventually to plasma cells producing higher-affinity immunoglobulins or memory B-lymphocytes.

To summarize, the adaptive immune response is more potent and specific then the innate response. It can be directed against any antigen, whether it is harmful or harmless, self or non-self. The end product of the adaptive response, the immunoglobulins, specifically target the antigen, leading e.g. to opsonization of the invading pathogen. Alternatively, harmful allergic response to food antigens may occur.

HOMEOSTASIS

Innate tolerance

The mucosal epithelium constitutively expresses pattern recognition receptors and engagement of a pathogenic microorganism mounts an inflammatory immune response to eradicate this pathogen[13]. None of the pathogen-associated molecular patterns is uniquely expressed by pathogens and so the cells also continuously sense the residing commensal microbes. Strikingly, this interaction is not detrimental to the host. This hyporesponsiveness of the innate immune system of the intestinal mucosa is further referred to as “innate tolerance” and will be further discussed in **Chapter 8**.

Mechanisms of innate tolerance

In homeostatic circumstances the commensal flora is in constant contact with the pattern recognition receptors on mucosal epithelial cells without inducing an inflammatory reaction. Intensive research into the mechanisms of the (epithelial) tolerance to intestinal flora has been conducted [14]. In general there are three proposed tolerizing mechanisms:

1. Downregulation of the pattern recognition receptors
2. Inhibition of intracellular signaling
3. The formation of inhibitory proteins

Reduction of the receptor expression on the cell surface can happen either via ubiquitilation and degradation, or via inhibition of receptor-mRNA synthesis. In this way, pattern recognition receptor signaling can be controlled in epithelial and monocytic cells [15-18]. Ubiquitin is a small monomeric polypeptide which binds to the receptor. The binding of (several) ubiquitin molecules labels the receptor for proteasomal degradation. Next to that in our intestinal tract the microbial flora could reduce to expression of pattern recognition receptors or even relocate the receptor from apical to basal [17].

Certain molecules or receptors ensure that continuous exposure to pathogen associated molecular patterns does not lead to a persisting inflammatory response. As such, Single immunoglobulin IL-1R-related molecule (SIGRR) has been implicated in the regulation of pattern recognition receptor responses. Mice deficient for this protein have increased susceptibility to lipolysaccharide induced shock and hapten induced gastrointestinal disease [19,20]. These mice fail to control the inflammatory response and die from a harmless amount of pathogen associated molecular patterns. Other well known regulators are Interleukin-1 receptor-associated kinase-M (IRAK-M) [21] and Toll interacting protein(Tollip) [17,22]. Both these proteins are produced or upregulated upon stimulation of the pattern recognition receptors, and interfere in the activation of the TLR intracellular cascade. The intracellular NOD2-receptor binds a specific part of peptidoglycan and as a result inhibits the TLR2 response [23].

Inhibitory proteins produced by immunological and non-immunological cells can reduce the response of pattern recognition receptor activation. An example is Secretory leukocyte protease inhibitor (SLPI) [24,25] that is able to inhibit intracellular signal transduction at various levels ultimately leading to reduced nuclear factor kappaB (NF-κB) activation and inflammatory gene expression. SLPI knock-out mice showed a higher mortality from endotoxin shock than did wild type mice. NF-κB is rapid-acting transcription factor that controls the transcription of DNA and is involved in cellular responses towards harmful cellular stimuli. Also specific nonvirulent Salmonella strains are able to inhibit the NF-κB pathway indicating that also prokaryotic determinants could be responsible for the unique tolerance of the gastrointestinal mucosa [26]. The desired result is a peaceful coexistence with our bacterial flora, with the ability to remove unwanted pathogenic microorganisms.

Adaptive tolerance

In analogy to the innate immune system, the adaptive immune system also has several mechanisms to tolerate innocuous antigens and maintain homeostasis. During maturation there are three possible outcomes for both sets of lymphocytes: clonal deletion, ignorance /anergy. Autoreactive immature lymphocytes encounter self antigens in the thymus. The signals resulting from the engagement are thought to abort their differentiation and sent the lymphocytes into apoptosis. This process that protects the host from circulating lymphocytes primed to attack host tissue is called clonal deletion. In addition, lymphocytes can be become ignorant or anergic. An ignorant lymphocyte has a receptor that recognizes self, but this antigen is either present in very low concentration or does not lead to cross-linking of the receptors. If the environment of the ignorant B-lymphocytes would change, e.g. in case of inflammation as a result of PRR-activation or increased expression of self-antigen, these cells can become activated and recruited into autoimmune response.

Naturally occurring regulatory T-cells

Unwanted lymphocyte responses can also be actively regulated via a specific subset of T-lymphocytes, the regulatory T-cells. The most studied regulatory T-cells are the naturally occurring $CD4^{+}CD25^{+}$ T-cells. Sakaguchi and co-workers showed that transferring naturally occurring regulatory-cells to thymectomized mice prevented the onset of autoimmune disease [27]. Subsequent studies showed that the transcriptional factor Foxp3 acts as a master for the development of regulatory T-cells, and its constitutive expression is required for its suppressive function [28,29]. Mutations in the Foxp3 gene lead to a severe autoimmune disease called IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked) [30]. The IPEX syndrome combines autoimmune and allergic manifestations, including severe enteropathy, food allergies, atopic dermatitis, hyper-IgE, and eosinophilia [31]. As a consequence of the impaired function or complete absence of regulatory T-cells the other T and / or B-cells to are no longer prevented from attacking the body's own tissues. Affected (male) infants with IPEX exhibit an extreme allergic phenotype, and the absence or dysfunction of naturally occurring regulatory T-cells gives us new insight into immune regulation. However, IPEX is a very rare cause of allergy.

Antigen specific regulatory T-cells

In contrast to the (innate) naturally occurring regulatory T-cells, that are not antigen specific, there are also subsets of antigen-specific regulatory T-cells. These subsets arise from naïve precursors and do not express CD25 [32,33]. Upon antigen presentation and differentiation in the mucosal draining lymph nodes, these cells predominantly produce the "tolerant" cytokines Transforming growth factor β (TGF-β) and interleukin-10 [32,33] and lead to tolerance for the specific antigen.

In humans, Karlsson and coworkers have shown that infants who outgrew cow's milk allergy (tolerant children) had higher frequencies of circulating antigen specific $CD4^+CD25^+$ regulatory T-cells [34]. Depletion of $CD25^+$ cells from peripheral blood mononuclear cells of tolerant children led to a fivefold increase in in vitro proliferation against beta-lactoglobulin. This study suggests that mucosal induction of tolerance against dietary antigens is associated with the development of antigen-specific regulatory T-cells.

INNATE AND ADAPTIVE INTERPLAY

To maintain homeostasis, the innate and adaptive immune responses have to be carefully orchestrated. An unbalanced response to an antigen can lead either to fulminant infections or immunologic diseases. The innate immune system generates an environment in which the required adaptive response can be accomplished.

The role of gut flora in homeostasis

The bulk of the intestinal residing bacteria are commensal and not pathogenic. The total number of commensal bacteria in the intestinal tract is 10^{14} and these bacteria cannot be ignored by the innate immune system [35]. Among individuals there is a high level of variability at bacterial species level [36]. However on a higher level common patterns of microbial communities appear. As part of the symbiosis certain bacteria, like *Bacteroides thetaiotaomicron*, predigest dietary polysaccharides to enable the human host to further utilize them as source of energy [37]. Next to their role in energy efficiency the microbial genome also provide signals for angiogenesis [38] and epithelial cell maturation [39]. Finally the intestinal flora has a direct effect on the maintenance homeostasis through induction of tolerizing cytokines, regulation of the NFkB-pathway [26] and the development of regulatory Tcells [40,41]. Rakoff-Nahoum and coworkers showed that commensal bacteria are recognized by TLRs under normal steady-state conditions and this interaction plays a crucial role in the maintenance of intestinal epithelial homeostasis [42]. In a colitis mouse model germfree conditions or interruption of the TLR-signaling pathway caused increased mortality. In this study it was showed that TLR do not only recognize microbial pathogens, but also commensal bacteria. In case of damage, like the induction of colitis, recognition of the commensal bacteria leads to the production of tissue protective factors [42]. Any interruption in the commensal bacteria – TLR activation cascade has detrimental effects on tissue repair and regeneration.

A key aspect of TLR function in dendritic cells is polarization of effector Th-lymphocytes. In a mouse model of allergic sensitization the amount of LPS present during sensitization determines whether Th1 or Th2 immunity is observed, with a low dosage of LPS inducing a Th2 response [43]. In addition the absence of commensal bacteria or LPS-insensitivity impairs the

ability to acquire oral tolerance to harmless antigens [44,45]. More recently it was shown that SLPI, a regulatory protein of lipopolysccharide signaling, modulates dendritic cell activity and subsequent T-lymphocyte responses in the mucosa-draining lymph nodes [46]. SLPI expression in dendritic cells is proposed to control the intensity of lipopolysccharide signals leading to a tailored adaptive immune response. This control allows for protective lipopolysccharide signals to orchestrate tolerance whereas pathogenic levels of lipopolysaccharide set off an inflammatory immune response [46].

DEFECTIVE INNATE TOLERANCE

Thus, while innate immune signaling induced by pathogenic microorganisms leads to an infectious response, activation of the innate immune system by endogenous bacteria may be part of a protective mechanism to maintain homeostasis. However, improper regulation caused by either absence or over-activation of the pathway may turn a physiological response into a pathological one.

IBD: Unwanted responses towards harmless bacteria

Inflammatory bowel disease, represented by Crohn disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation. IBD is a multifactorial disease in which genetic susceptibility, environmental triggers, and immune dysregulation are thought to play a causative role. The dysregulated immune response is most likely to be directed against the intestinal flora [47]. Inability to maintain homeostasis due to defects in the innate immune response has been hypothesized. A loss of function of the innate immune system leads to failure to respond to commensal bacteria [48]. Subsequently the commensal flora “outgrows” the intestinal tract and may break through the epithelial barrier. Underlying functional immunological cells (T and B-lymphocytes) will sense the bacteria and produce an inflammatory reaction to eradicate the intruder.

The possibility of an unwanted gain of function of the innate immune system has been hypothesized, and is supported by finding of a mutation of the intracellular bacterial sensing gene NOD2 which is associated with susceptibility to CD [49]. Patients homozygotic for this mutation lack the inhibitory effect of NOD-2 and show an overactivation of NF-κB in monocytes and subsequent production of inflammatory cytokines like TNF-α. Failing to restore these defects in innate tolerance results in chronic inflammation and clinical symptoms of IBD.

DEFECTIVE ADAPTIVE TOLERANCE

Allergy: harmful responses towards harmless antigens

In general the response to innocent antigens, such as food proteins, is tolerant as a result of antigen presentation in the presence of pathogen associated molecular patterns (PAMP). Failure to obtain tolerance, or losing tolerance towards innocuous antigens induce immunological reactions may eventually present as allergic disease.

Adaptive immune cells and allergy

Aberrant T-helper cell responses to innocuous antigens can lead to an allergic reaction [50]. In 1988 Del Prete and coworkers showed that interleukin-4 is responsible for IgE switching of B-lymphocytes, indicating the T-lymphocyte as an essential player in the allergic response [51]. IgE directed against harmless antigens, such as cow's milk or pollen, binds to mast cells in the skin and mucosa. Exposition to the specific allergen results in binding of the antigen to IgE and crosslinking of the IgE molecules. The presence of IgE directed against harmless antigens is referred to as sensitization. Sensitization is necessary for an (IgE-mediated) allergic disease, yet sensitization is not the same as disease. The crosslinking of the loaded IgE molecules causes degranulation of mast cells with release of mediators like histamine, bradykinine and prostaglandins. These mediators cause the symptoms associated with allergic diseases.

iNKT-cells in allergy

The classification into the Th1 and Th2 subsets based on cytokine profiles led to the Th1-Th2-paradigm in allergic disease. In the last decade the discovery of regulatory T-cells upgraded this paradigm. The Th1/Th2 hypothesis, in which e.g. allergic disease was explained by increased Th2 activation has become part of a new kind of balance. In this new paradigm regulatory T-cells shape the response or the effector Th1 and /or Th2 cells. Besides the classical Th2-lymphocyte there are novel subsets and functions of immunological cells in allergy, such as invariant Natural Killer T(iNKT)-cells and CRTH2 bearing cells. Both iNKT-cells [52] and $CRTH2^{+}$-lymphocytes [53] are major IL-4 producers upon stimulation.

iNKT-cells are a unique population of T-lymphocytes expressing an invariant T-cell receptor and Natural Killer cell markers [54]. The antigens for iNKT-cells are glycolipids presented by the MHC-class I like molecule CD1d that is present on antigen presenting cells. Upon stimulation, iNKT-cells are able to produce both Th1- and Th2-type cytokines[55]. Mouse models [56,57] as well as human studies suggest a significant role for the iNKT-cells in allergic disease [58].

CRTH2

When a person susceptible for allergic disease encounters an antigen, specific immunoglobulin E will be produced. Mast cells, residing in the mucosa and skin, express a high-affinity receptor

for IgE (FcεRI) that binds IgE molecules irreversibly. When this person re-encounters the antigen it binds to the IgE immediately, causing crosslinking and subsequent degranulation of the mast cells releasing factors like histamine and PGD2. Other PGD2 producing cell types are alveolar macrophages, Th2-lymphocytes and dendritic cells[59, 60]. CRTH2 is the receptor for Prostaglandin D2 (PGD2) [59-61]. In patients with atopic eczema dermatitis syndrome (AEDS) and in aeroallergen-sensitized individuals increased levels of $CRTH2^{+}$-lymphocytes were found in peripheral blood [62,63]. **Chapter 4** will focus on these novel markers of allergy in early childhood.

Hygiene hypothesis

In the last 25 years the prevalence of allergic diseases has doubled in the Western world[64, 65]. This rise corresponded with the implementation of public health measures like indoor plumbing, advanced food processing, childhood vaccination and antibiotic availability. Based upon epidemiological studies and observational data, Strachan formulated the "hygiene hypothesis" stating that a lack of adequate microbial stimulation in early life increases susceptibility to allergic diseases [66].

Cow's milk allergy

Cow's milk is the first food introduced into an infant's diet. Cow's milk allergy (CMA) is the most common food allergy in early childhood [67]. CMA is an example of a defective (adaptive) tolerance towards food antigens. From prospective studies, the estimated incidence of symptomatic CMA is 2% to 5% [68]. Infants with CMA present with symptoms in two or more organ systems, with atopic eczema dermatitis syndrome (AEDS) being the most prevalent[68]. In contrast to other food allergies, which usually manifest later in life, the majority of infants with CMA tolerate cow's milk before the age of 3 years [69]. Environmental factors may play an important role in the development of the mucosal immune system, thereby affecting the development of allergic disease. It has been suggested that CMA is due to immaturity of local and systemic immune responses [70] and is associated with an altered composition of the intestinal flora in Western society [71]. Shifting the composition of the intestinal flora in early infancy could induce an adequate maturation of the immune system and achieving tolerance to cow's milk.

Probiotics to prevent allergy

Eighty years before Strachan [66] introduced the hygiene hypothesis Metchnikoff postulated that the consumption of fermented milk products by Bulgarian peasants contributed to their long and healthy life [72]. Metchinikoff was the first to recommend ingestion of live cultures of beneficial microorganisms, later introduced as probiotics.

Probiotics are defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' [73]. They are normal commensal bacteria of the

human gut and Lactobacilli and Bifidobacteria are the most regular used genera. Rationally, probiotics were studied for their effects on gastrointestinal disorders. More recently focus has shifted as probiotics could influence the host immune system and potentially affect systemic disorders like allergy, IBD or pancreatitis [74-84].

Probiotic rationale

It is suggested that an altered composition of the gut flora is causally related to the increased prevalence of allergy. In consensus with this hypothesis was the finding that two-year-old allergic children were more often colonized with Clostridium difficile and other aerobic bacteria than non-allergic children, whose gut flora contained more Lactobacilli and Eubacteria [71]. Furthermore, formula-fed infants have a complex mixture of anaerobic strains, such as Bacteroides and Clostridium while breast-fed infants were colonized, predominantly with Bifidobacteria and Lactobacilli. It was thought that using probiotics could convert the flora to a “normal healthy” state it might affect the incidence and/or severity of allergic diseases. Finally allergy prevention and intervention trials have shown that the composition of the intestinal flora indeed changed during supplementation with probiotics [80,85,86]. Therefore if probiotic supplementation is able to alter the gut flora composition it seemed that it might as well affect the (developing) mucosal immune system. As an increased knowledge on the interplay between microbial flora, the innate immune system and the adaptive immune systems helps to understand the potential mechanisms of probiotic treatment for immune diseases, it seems that unraveling the factors involved in homeostasis of the (developing) mucosal immune system might lead to new approaches in immune mediated diseases.

Outline of this thesis

General aim: to study the development and regulation of mucosal homeostasis in early infancy in order better understand the development of allergic diseases in early childhood, and to asses effects and mechanisms of probiotic intervention in infancy.

Specific aims

- To study the effect of probiotic supplementation in infants with CMA
- To search for novel immunological markers of for the development of allergic diseases in atopic infants
- To study the effect of probiotic supplementation on the commensal flora in CMA infants
- To evaluate fractional exhaled nitric oxide as a predictor of a positive reaction during Double Blind Placebo Controlled Food Challenge
- To gain further insight into the development of epithelial hyporesponsiveness to commensal flora after birth

– To study the immunological responses to pathogen associated molecular patterns of buccal epithelial cells in IBD

The studies addressing these aims are described in this thesis. **Chapter 2** focuses on the quality of the quotations of a landmark publication on the effect of probiotics in primary prevention of atopic disease. **Chapter 3** describes the results of a multicenter, randomized, placebo-controlled trial into the acquisition of tolerance towards cow's milk through probiotic supplementation. **Chapter 4** focuses on novel immunological markers in atopic infants. In **Chapter 5** the possible predictive value of fractional exhaled nitric oxide during a food challenge test was investigated. **Chapter 6** describes the composition of intestinal flora during the probiotic supplementation in CMA infants. **Chapter 7** describes a clinical study on immunologic activity of buccal epithelium in children with IBD and adults with Crohn disease. **Chapter 8** focuses on the development of epithelial hyporesponsiveness towards commensal bacteria after birth. In **Chapter 9** the results of the studies described in this thesis are summarized and directions for future research are discussed.

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Chapter 2

Quoting a landmark-paper on the beneficial effects of probiotics

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SUMMARY

The quality of the quotations of a landmark publication on the effect of probiotics in primary prevention of atopic disease was poor: one third was incorrect. Misinterpreting manuscripts as a result of misquotations is a peril of biomedical publishing.

Probiotics are defined as 'live microorganisms that when administered in adequate amounts should confer a health benefit on the host' [2]. Intriguingly, the rapid expansion of commercially available products that contain probiotics is in sharp contrast with the lack of scientific evidence for their efficacy and working mechanism. Few well-conducted trials have appeared in high impact journals. Amongst these is a manuscript by Kalliomaki and coworkers [1] "Probiotics in primary prevention of atopic disease: a randomized control trial". To date, this is the most quoted clinical trial on probiotics and atopic disease in peer reviewed journals. Kalliomaki et al [1] reported a double-blind placebo controlled trial on supplementation of *Lactobacillus rhamnosus* GG (LGG) to pregnant women with a family history of atopic disease starting 2-4 weeks before delivery. After delivery, breastfeeding mothers ingested the probiotics, and bottle fed children received the supplementation mixed with water by spoon for 6 months. The intervention resulted in a reduced incidence of atopic eczema at the age of 2 years, but had no effect on allergic sensitization or respiratory allergic disease. We speculate that the high quotation numbers may reflect an apparent urge to establish probiotics as a useful prevention measure. We therefore determined the quality of scientific quotations of this landmark publication.

By November 1st 2008, this paper had been quoted in 663 separate publications. We were able to retrieve and examine 458 English written manuscripts (supplemental text). All quotations were randomly collected in a database, blinding the assessors for authors, year of publication and specific journal. Each quotation was assigned to a specific section of the Kalliomaki paper[1] and assessed for accuracy by two researchers separately. Incorrect quotations were further subcategorized based on 4 model types of errors (*Misquotation of result or over-interpretation of data, secondary citing, no evidence for cited result, specific misquotations of the materials and methods section).* To establish a possible relation between the quality of a quotation and potential interfering factors, we separately obtained the year of publication, journal, 2006 journal impact factor, the number of quotations per publication and the type of publication. Of the total number of 603 quotations 175 (29.0%) were incorrect (Figure 1). Thirty-four percent of the articles had at least one inaccurate quotation. The median impact factor between correct and incorrect quotations did not differ (Correct 3.4 [IQR 3.1] versus Incorrect 2.7 [IQR 3.1]; P=0.74). The annual number of publications citing the paper was stable, and we did not find a change of error frequency in time (Table 1). We anticipated lower error rates in certain paragraphs that would be less subject to interpretation. Surprisingly, quotations concerning the materials and methods section showed the highest number of misquotations, 47.5% of cases. We found lower error rates for the other sections, 28.2% for results and 18.9% for conclusion/hypotheses. We found significantly more misquotations in Basic research manuscripts (37%), followed by Reviews (30.9%), Clinical trials (23.0%) and Miscellaneous (21.1%) (Table 1).

A common error was the generalization that LGG exerts a preventive effect on allergic diseases other than atopic eczema. Notably, the study only reports an effect on eczema, whereas allergic disease or IgE levels remained unaffected. Also statements that LGG supplementation

affected fecal flora composition in this trial were incorrect. This specific error suggests a causative relationship between changes in the intestinal microbial composition and prevention of eczema. For comparison, we performed similar analyses on two additional papers, one on cetirizine treatment as asthma prevention [3]. And the study by Equi et al [4] on azithromycin (antibiotic) treatment in children with cystic fibrosis that was published in the same journal in the same period as the study by Kalliomaki et al [1]. These articles were quoted 78 (60 articles) [3] and 136 (86 articles) [4] times respectively in English written manuscripts that we were able to retrieve. In these two studies we established that 15% and 12.5% of the quotations were incorrect, compared to 29% for the paper by Kalliomaki et al. Respectively 20% and 16% of the articles had at least 1 inaccurate quotation, compared to the 34% of Kalliomaki's paper. When divided into the different subcategories of predefined errors, no significant differences were detected in the two comparator manuscripts. We did not find any misquotations on the Material and Methods of the paper by Equi et al [4] (Figure 1). These findings may indicate that the reason for the high rate of quotation errors concerning the Kalliomaki study is related to the subject, i.e. probiotics.

Studies on the percentage of quotation errors in issues of a journal have been published before. De Lacey et al [5]. found a quotation error rate of 15%, with 12% being major (the potential of dramatically changing the reader's understanding of the original article). More recent work in the field of anatomy [6] and head and neck surgery [7] established similar figures of 19% and 17% for all inaccuracies and 18% and 11% for major errors. These number are similar to our results on the papers by Warner et al [3] and Equi et al [4], but much lower than the misquotations of the paper by Kalliomaki et al [1].

Papers describing misquotations of a single manuscript are rare. Recently, Porrino and coworkers found an error rate of 41% in articles citing a commonly referenced hand surgery study, with 34% classified as major[8]. These findings are comparable to our results on the paper by Kalliomaki et al [1]. They also reported more errors in review articles, which we were unable to detect. A study analyzing the results on HIV and primary care study only found 11% errors in quotations on the primary outcome [9]. Porrino et al [8] suggested that the original article is widely misunderstood, without knowing if this is the by-product of authors referencing from articles other than the original [8]. We cannot exclude that this is also the case for the manuscript by Kalliomaki et al [1].

We conclude that misquotation is a common problem in biomedical literature and that the high percentage of errors on the most quoted probiotic trial calls for more awareness of editors, referees and, above all, authors.

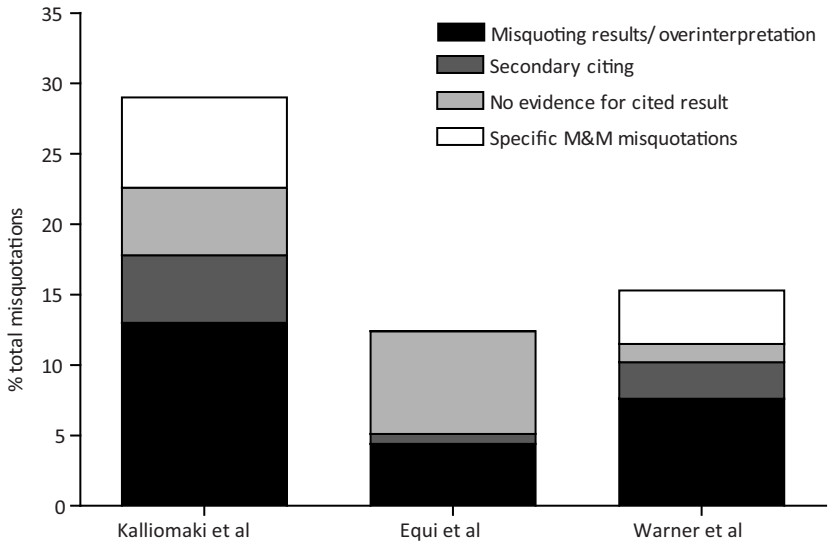

Figure 1 | Total percentage of incorrect quotations of the landmark paper by Kalliomaki et al [1], Equi et al [4] (antibiotic treament in cystic fibrosis) and Warner et al [3] (asthma prevention) further subcategorized into the different model types of error.

Table 1 | Quotation accuracy categorized by year and type of publication

Year	Clinical trial		Review		Basic research		Miscellaneous		Total percentage	
	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
2001	4	1	2	2	0	0	0	1	60%	40%
2002	12	4	43	17	4	1	12	2	75%	25%
2003	13	2	25	12	8	6	3	0	71%	29%
2004	9	4	30	10	15	9	3	1	70%	30%
2005	19	8	30	12	12	3	3	1	73%	27%
2006	29	9	27	15	13	5	4	2	70%	30%
2007	30	11	7	8	12	7	5	1	67%	33%
2008	28	4	15	4	11	13	0	0	72%	28%
Total	144	43	179	80	75	44	30	8	71%	29%

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SUPPLEMENTAL TEXT

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Chapter 3

The acquisition of tolerance towards cow's milk through probiotic supplementation: A randomized controlled trial

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ABSTRACT

Background: Cow's milk allergy (CMA) is the most frequently diagnosed food allergy in infancy. In general, patients have a good prognosis, as the majority acquires tolerance within the first years. Interventions have been proposed to accelerate tolerance and to reduce morbidity. Probiotic supplementation could be effective through modulation of the immune system.

Objective: To determine whether supplementation with a combination of probiotics (*Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12) accelerates tolerance to cow's milk (CM) in CMA infants.

Methods: Double-blind randomized placebo-controlled trial in 119 CMA infants. Infants received CRL431 and Bb-12 supplemented to their standard treatment of extensively hydrolyzed formula for 12 months. Primary outcome was clinical tolerance to CM at 6 and 12 months of treatment. Furthermore, we analyzed T and B lymphocyte subsets ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD20^+$) in peripheral blood at randomization and at 12 months with flow cytometry, and examined the presence of viable probiotic strains in fecal samples.

Results: The cumulative percentage of tolerance to CM at 6 and 12 months was similar in both groups: 56 (77%) respectively in the probiotics group versus 54 (81%) in the placebo group. Infants in the placebo group had higher percentage of $CD3^+$ and $CD3^+CD4^+$ lymphocytes compared to probiotic treated infants. Probiotic intake was confirmed as probiotics were isolated from feces more often in treated infants than in the placebo group.

Conclusion: Supplementation of CRL431 and Bb-12 to extensively hydrolyzed formula does not accelerate CM tolerance in infants with CMA.

INTRODUCTION

CMA is the most common food allergy in early childhood[1]. From prospective studies, the estimated incidence is 2-5% [2]. The majority of infants becomes tolerant before the age of 3 years [3]. However, children with CMA have an increased risk of developing atopic diseases in later life [4]. It has been hypothesized that CMA is due to immaturity of local and systemic immune responses [5] and is associated with an altered composition of the intestinal microbiota in Western society [6]. Indeed, in the last 25 years the prevalence of allergic diseases has doubled in the industrialized parts of the world [7,8]. The rise in allergic disorders appears to correspond with the implementation of public health measures in the prevention of infectious diseases and increased availability of antibiotics. Based upon epidemiological studies and observational data, Strachan formulated the so called hygiene hypothesis stating that reduced microbial stimulation in early life increases susceptibility to allergic diseases [9]. Specifically, alterations in the gut flora composition could be relevant to explain the increased prevalence of allergy. An association has been described between gut colonization with *Clostridium difficile* and allergy in children [10], while *Lactobacilli* and *Eubacteria* were more frequent in non-allergic children [6]. Based on these findings, it has been proposed that specific bacteria, denoted as probiotics, could potentially restore intestinal homeostasis and prevent allergy by interaction with the intestinal immune cells [11,12]. Probiotics have been extensively studied in the treatment of atopic eczema / dermatitis syndrome (AEDS) [13-18] and allergic rhinitis [19]. In CMA, the use of *Lactobacillus GG* reduced the severity of AEDS and diminished intestinal inflammation [20,21]. To date, no studies however, have been performed to assess whether probiotics can induce CM tolerance in established CMA.

The main purpose of our study was to determine whether supplementation of extensively hydrolyzed formula with a combination of 2 probiotics (*Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12) would affect tolerance acquisition to CM.

As it was shown that CMA infants have increased percentages of B-lymphocytes ($CD19^+$) and decreased $CD8^+$ T-lymphocytes in peripheral blood compared to non-CMA infants [22], we also studied changes in B- and T-lymphocyte populations upon tolerance acquisition and in relation to probiotic treatment by means of a secondary endpoint.

METHODS

Study design

The CAMEL project (Cow's milk Allergy Modified by Elimination and Lactobacilli) was a randomized double blind, placebo-controlled study carried out between March 2004 and May 2007. Infants younger than 6 months with a diagnosis of CMA were included [23,24]. 193 infants with suspected CMA, who had shown improvement of food-related symptoms with an extensively

hydrolyzed formula referred from regional clinics and hospitals. Of the 193 infants, 119 met our inclusion criteria (mean aged 4.2 months, range 1.4-6.0 months; 55% boys). At least one parent of each infant gave a written informed consent. The local ethics committee approved the study protocol. The study design is depicted in Figure 1.

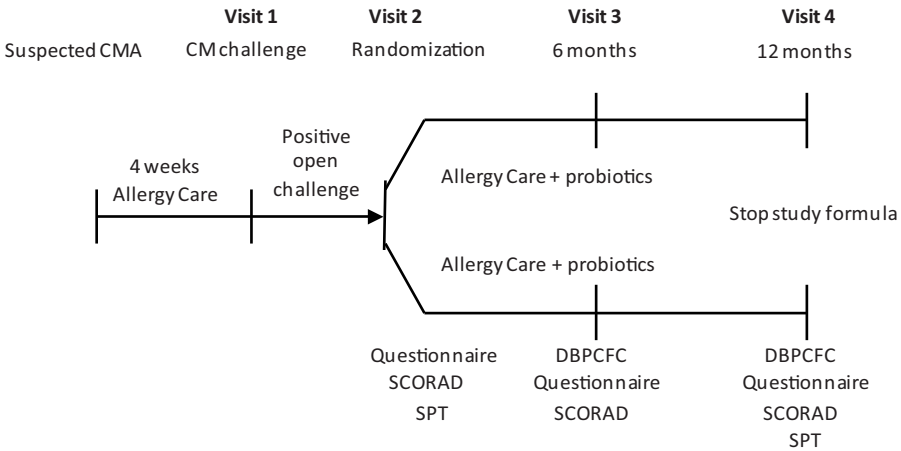

Figure 1 | Study protocol

Diagnosis of cow's milk allergy

Infants referred to the CAMEL-study were prescribed an extensively hydrolyzed casein based formula (Friso 1 Allergy Care®, Royal Friesland Foods, Meppel, The Netherlands) for at least 4 weeks. CMA was diagnosed with a food challenge according to the guidelines of ESPGHAN and ESPACI and criteria by Bock and Sampson [23,24]. At inclusion (visit 1), an open CM challenge was conducted in one of the participating hospitals by the investigators (J.H. and B.E.S.). The test formula was Friso 1 Allergy Care® mixed with additional cow's milk proteins (Protifar®, Nutricia, Zoetermeer, The Netherlands) to achieve the CM protein concentration of standard infant formula. The challenge consisted of administration of 2 ml followed by 6, 20, 60 and 200 ml at 30min intervals. Infants were scored for 9 items divided into 4 main categories (general, skin, gastro-intestinal, respiratory) on a 0-3 scale (0=none, 1=light, 2=moderate, 3=severe). If both investigators independently scored any item 3, or 2 (or more) items 2, the test was considered positive. Two hours after the final dose, infants were discharged and contacted by telephone the next morning to check for symptoms suggestive of a late response, such as vomiting or diarrhoea. Patients returned to the outpatient clinic the next day only when parents reported

skin problems that needed to be evaluated by a physician. If infants did not show any symptoms within the initial 24 hours, parents were advised to reintroduce cow's milk, starting 48 hours after the initial test at an increasing daily dosage. All of these presumed non-allergic children were followed up for at least 1 week. Late responders were rechallenged and included in our studies when positive.

All infants that were positive on challenge were randomized at visit 2. After 6 and 12 months of study formula, a double blind placebo controlled food challenge (DBPCFC) was performed (visit 3 and 4).

All DBPCFC took place in the outpatient clinic of the Erasmus MC – Sophia Children's Hospital on 2 separate days, with a 1-week interval and were carried out by J.H. and B.E.S. The test formula was Friso 1 Allergy Care® mixed with additional cow's milk proteins (Protifar®), the placebo formula was standard Friso 1 Allergy Care®. After completion of the DBPCFC, the code of test formulas was broken.

Assignment

The groups were stratified and block-randomized according to age at inclusion (<20 weeks or ≥20 weeks), birth weight (<2500 grams or ≥2500 grams), and (reported) atopic diseases in first degree relatives (yes or no). A computerized randomization schedule was prepared by the biostatistician.

Probiotic supplements

Infants in the probiotic group received *Lactobacillus casei* CRL431 (Lactobacillus paracasei subspecies paracasei) and *Bifidobacterium lactis* Bb-12 (Bifidobacterium animalis subspecies lactis; 10^7 cfu/g formula for each of the probiotic bacterium used) supplemented to Friso 1 Allergy Care® whereas the control group received Friso 1 Allergy Care® alone. The CFU/g of the formula was measured monthly and remained stable during the study. If infants had become CM tolerant after 6 months, they received either CM-containing follow-up milk (Friso 3®) supplemented with CRL431 and Bb-12 or Friso 3® alone for 6 months.

CRL431 and Bb-12 fulfil the requirements of the Food and Agriculture Organization (FAO)/ World Health Organization expert panel guidelines for probiotics [25]. Earlier clinical studies have shown CRL431 [26,27] and Bb-12 [14,28] to be well-tolerated and safe in young infants.

Blinding

Probiotics and placebo formula were image- and taste-matched and participants and researchers remained blinded to group assignment for the duration of the study. To optimize compliance, participants were supplied with study formula via the study team, and batches were delivered at home.

CLINICAL OUTCOME

The primary endpoint was clinical tolerance to CM at 6 and 12 months after initial CMA diagnosis. J.H. and B.E.S. performed visits at randomization and at 6 and 12 months after start of study formula. Structured interviews on symptoms of allergic disease, adverse events, use of antibiotics, day care attendance, smoking in the house and pet exposure, as well as an inspection of the skin were performed. The SCORing Atopic Dermatitis (SCORAD) index was used to assess the severity of the eczema[29]. Skin tests were performed at randomization and at 12 months of study formula (visit 2 and 4). Fresh foods were applied to the volar side of the patient's forearm (i.e., fresh CM, hen's egg and soy). Skin prick tests (SPTs) were performed with a 1-mm single-peak lancet (ALK, Copenhagen, Denmark). Reactions were read at 15 min, and considered positive if the weal was 3 mm or larger, with NaCl 0.9% as negative control and 10 mg/mL histamine dihydrochloride (ALK) as a positive control.

Fecal samples

Fecal samples were collected from 24 infants (11 on placebo and 13 on probiotics) at visit 3. All samples were frozen to -20°C within 15 min, brought to the hospital, and stored at -80°C until analyzed. Samples were analyzed with a microarray to detect and quantitate the small subunit ribosomal RNA of *Bifidobacterium animalis* and *Lactobacillus casei* & *paracasei*, adapted from Palmer et al [30].

Flow cytometry

Peripheral blood samples were collected by venipuncture at randomization and after 12 months of study formula (visit 2 and 4). Erythrocytes were lysed and cells washed twice with Phosphate Buffered Saline with 1% Newborn Calf Serum (denoted as FACS buffer). Next, samples were incubated with human serum for 10 min, washed with FACS buffer and incubated with anti-CD3-FITC (clone HIT3a), anti-CD4-PE (clone RPA-T4), anti-CD8-PerCP (clone SK-1) and anti-CD20-CyChr (clone 2H7), all from Becton, Dickson and Company, Franklin Lakes, NJ, USA or appropriate isotype control for 30 min on ice. After incubation, cells were washed, resuspended in 1% paraformaldehyde and fluorescence was measured using a FACSCalibur™ (Beckton Dickinson and Company). For each sample 50 000 events were analyzed. Cells that had been incubated with isotype control served as negative controls. The results were analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

SPSS software package (version 12.01 for Windows, Chicago, Illinois, USA) was used for statistical analysis. To examine differences between treatment groups the Pearson χ^2, Fisher exact test, student t-test, ANCOVA, the Mann-Whitney test and repeated measurements ANOVA were

applied. With multivariate stepwise logistic regression analysis we assessed predictors for the persistence of CMA at 6 and 12 months after randomization. The model used data available at randomization: gender, delivery type, age at randomization, family history of atopy, presence of furred pets, SPT to milk and hen's egg, duration of breast feeding, daycare attendance and antibiotic use. Outcomes are reported as odds-ratios [OR]. For all statistical tests, a two-tailed p value of < 0.05 was considered significant.

RESULTS

Baseline characteristics

At visit 1, 119 (61%) of the recruited infants showed urticaria, worsening of AEDS, vomiting, diarrhoea, physician-diagnosed wheezing or convincing behavioral symptoms upon challenge with CM-containing formula. Forty percent of the included infants expressed symptoms in 2 or more organ systems, 32% skin reactions, 18% subjective reactions and 10% gastro-intestinal symptoms. These 119 infants were randomized and enrolled in the intervention study. 74 infants who were negative for the CM-challenge were not included.

Baseline characteristics were comparable between the 2 intervention groups (table 1). There were no differences between the 2 intervention groups in onset and type of CM-related complaints and age of first formula use.

One hundred and eleven infants (93%, 56 placebo, 55 probiotics) underwent DBPCFC after 6 months of study formula. A total of 106 infants (89%, 55 placebo, 51 probiotics) completed the entire study (Figure 2).

Development of CM tolerance

The first DBPCFC at 6 months was carried out in 111 infants. In the probiotics group 31/55 infants (56%) had become tolerant, compared to 30/56 (54%) in the placebo group (difference 2% [95% CI -15.7 to 21.3], not significant, P=0.92). At 6 months 44% of the responsive infants expressed symptoms in two or more organ systems, 36% skin reactions, 4% subjective reactions and 16% gastrointestinal symptoms. Infants with persisting CMA were re-challenged at 12 months. In the probiotics group 11/23 (48%) of the infants were tolerant versus 15/25 (60%) in the placebo group (difference -12% [-40.1 to 15.9], not significant, P=0.58). At 12 months the cumulative tolerance was 81% in the placebo group and 77% in the probiotics group (P = 0.95; OR 1.1 [0.6-1.9]). At 12 months the majority of the responsive infants expressed symptoms in 2 or more organ systems (27%) or skin reactions (41%). Less frequent were subjective reactions (18%), gastrointestinal (9%) and respiratory symptoms (5%), Data on tolerance to CM after 6 and 12 months of study formula are summarized in Figure 2. Using logistic regression analysis, none of the parameters used for block randomization was found to affect the outcomes.

Table 1 | Baseline characteristics of the study population

Characteristics	Placebo n=60	Probiotics n=59
Boys % (n)	60 (36)	51 (30)
Age at inclusion (SD)	4.1 mo (1.5)	4.3 mo (1.2)
Birth weight (SD)	3445 gram (556)	3456 gram (504)
Gestational age (SD)	39.6 wk (1.7)	39.5 wk (1.6)
Caesarean delivery % (n)	17 (10)	20 (12)
Breastfed, ever % (n)	75 (45)	73 (37)
Duration breastfeeding % (n)		
<2 month	45 (27)	49 (25)
≥2 month	30 (18)	23 (12)
Furred pet % (n)	42 (25)	36 (21)
Day care attendance % (n)	53 (32)	46 (27)
Indoor smoking % (n)	10 (6)	12 (7)
Antibiotic before intervention % (n)	22 (13)	31 (18)
Positive SPT milk % (n)	21 (12)	10 (6)
Positive SPT hen's egg % (n)	22 (13)	17 (10)
Positive SPT soy % (n)	3 (2)	2 (1)

Effects of probiotics on SCORAD

Overall the SCORAD improved at 6 and 12 months (Figure 3). The probiotics group (n=51) showed improvement at 6 and 12 months, the placebo group (n=54) only at 6 months. However, after adjusting for the baseline values there were no significant differences in the change from baseline between probiotic or placebo treatment at 6 months (p=0.92) and 12 months (p=0.14).

Since the SCORAD in the overall population was low, we performed a subgroup analysis of infants with moderate to severe eczema (SCORAD ≥ 15) at randomization. The probiotics group (n=18) had a mean SCORAD of 27.6 [95% CI;19.1-36.0] at randomization improving to 14.7 [7.2-22.1] and 8.1 [1.9-14.3] after 6 and 12 months of study formula respectively (P<0.01). The mean SOCRAD for the placebo (n=14) group were 26.8 [21.2-32.4] at randomization and 11.2 [5.0-17.4] and 8.5 [3.7-13.3] at 6 and 12 months, respectively (P<0.001). In this subgroup the SCORAD improved independent of probiotic treatment.

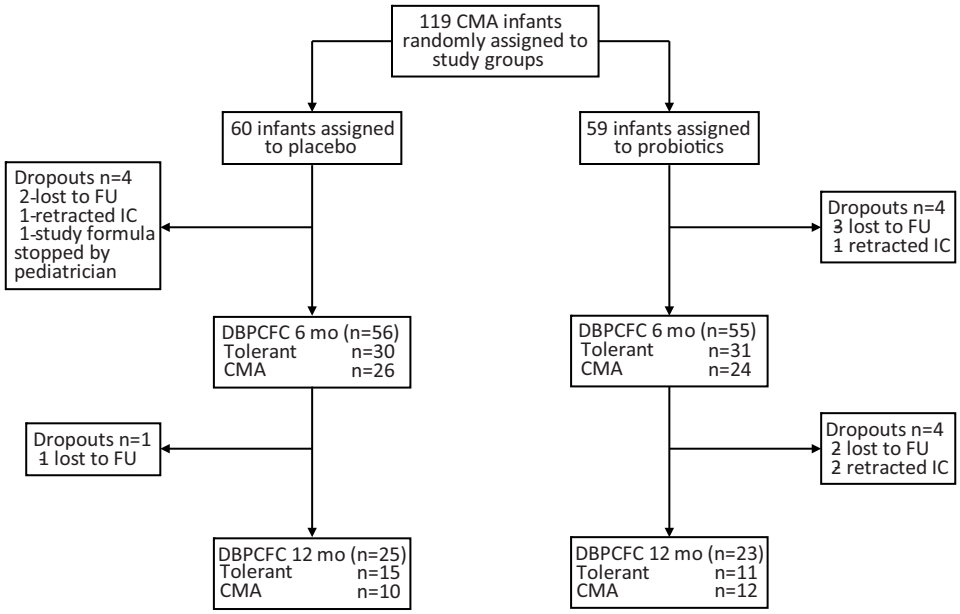

Figure 2 | Flow chart of the study population

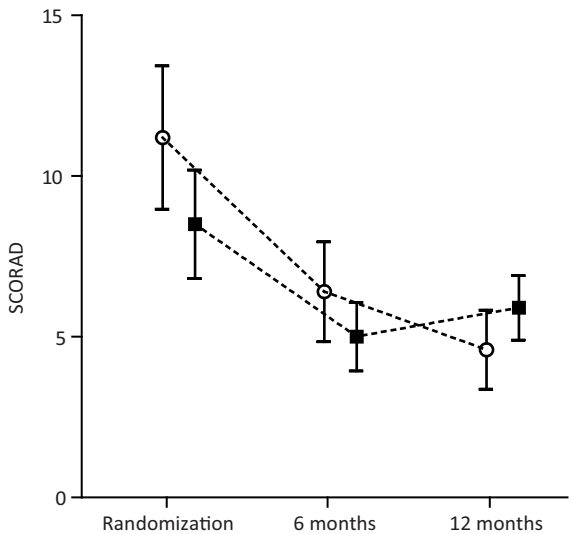

Figure 3 | No additional effect of probiotics on clinical severity of AEDS.

Mean SCORAD (± SEM) at randomization, 6 and 12 months of study formula. Filled squares Placebo (n=54) and open circles Probiotics group (n=51). SCORAD significantly improved from baseline in the probiotics group at 6 and 12 months, in the placebo group only at 6 months.

Effects of probiotics on symptoms and medication use

We found no difference in hospital admissions and reported wheezing between de study groups. The use of medication was comparable for both groups during the study period (table 2). Parents were asked to document if their infants showed any symptoms as a result of the study formula. The study formula, with or without the probiotic supplementation, was well tolerated. We did not note any difference between the groups.

Table 2 | Clinical characteristics of infants in the study population during period of study formula

	Placebo	Probiotics	*P* value
Hospital admission			
inclusion-6 months	4/56 (7%)	7/55 (13%)	0.36
6 months-12months	4/55 (7%)	6/51 (12%)	0.52
Wheezing			
inclusion-6 months	20/56 (36%)	23/55 (42%)	0.56
6 months-12months	14/55 (26%)	19/51 (31%)	0.21
Use of asthma medication			
inclusion-6 months	15/56 (27%)	17/55 (31%)	0.68
6 months-12months	11/55 (20%)	17/51 (33%)	0.13
Use of topical steroids			
inclusion-6 months	18/56 (32%)	15/55 (27%)	0.68
6 months-12months	14/55 (26%)	10/51 (20%)	0.50
Use of antibiotics			
inclusion-6 months	17/56 (30%)	21/55 (28%)	0.43
6 months-12months	18/55 (33%)	25/51 (49%)	0.11
Positive SPT milk 12 months	8/54 (15%)	10/49 (20%)	0.60
Positive SPT hen's egg 12 months	14/54 (26%)	16/49 (33%)	0.52
Positive SPT soy 12 months	6/54 (11%)	3/49 (6%)	0.49

Predictors of CMA persistence

A positive SPT to CM at randomization was a strong predictor of persisting CMA at 6 months (OR 5.0, [95% CI 1.5-16.4], P=0.009). A positive SPT to hen's egg also predicted the persistence of CMA at 12 months (OR 4.0, [1.4-11.4], P=0.01). A positive SPT to milk and hen's egg at 12 months were both predictive for a positive DBPCFC at 12 months (milk P<0.001 and hen's egg P=0.007).

Flow cytometry We found similar $CD3^+$, $CD3^+CD4^+$,$CD3^+CD8^+$ and $CD20^+$ percentages in both treatment groups at randomization (table 3). After 12 months of treatment there was a significant change from baseline of the $CD3^+$ cells in the placebo versus the probiotics group, placebo being 4.5% higher. (P=0.016; ANCOVA, adjusted for baseline).

Additional analysis revealed that the $CD3^+CD4^+$ population mainly contributed to this change of the $CD3^+$ cells. The change from baseline within the $CD3^+CD4^+$ was 3.5% higher in placebo (P=0.038; adjusted for baseline). No difference in change from baseline was found in

the $CD3^+CD8^+$ and the percentage of mature B-cells ($CD20^+$) subpopulation (P=0.85 and P=0.22 respectively, adjusted for baseline).

Further analysis indicated that the $CD3^+$ change from baseline for infants with persistent CMA compared to tolerant infants also was significantly different, being 4.7% higher in the persistent CMA group (Figure 4, P=0.032, adjusted for baseline). However, this difference was independent of the probiotic intervention.

Table 3 | Lymphocyte subset percentages of lymphocytegate at baseline (T= 0) and 12 months

	Probiotics		**Placebo**	
Lymphocyte subpopulation	**T=0**	**T=12**	**T=0**	**T=**
$CD3^+$	66.4 (9.2, n=33)	64.4# (7.9)	66.5 (10.0, n=44)	68.9# (9.6)
$CD3^+CD4^+$	46.9 (8.9, n=33)	41.4†* (6.2)	47.7 (10.7, n=44)	45.2† (10.1)
$CD3^+CD8^+$	16.9 (6.7, n=30)	18.9* (6.6)	16.2 (5.2, n=42)	18.2 (5.9)
$CD20^+$	21.6 (7.2, n=30)	20.8 (9.6)	20.9 (7.7, n=46)	18.0 (8.9)

Data given are percentages of the lymphocytegate of patients with paired data (Mean (SD), n). # p=0.016 for the change from baseline (probiotics vs. placebo). † p=0.038 for the change from baseline (probiotics vs. placebo). * p<0.05 T=0 vs. T=12 months, paired sampled t-test.

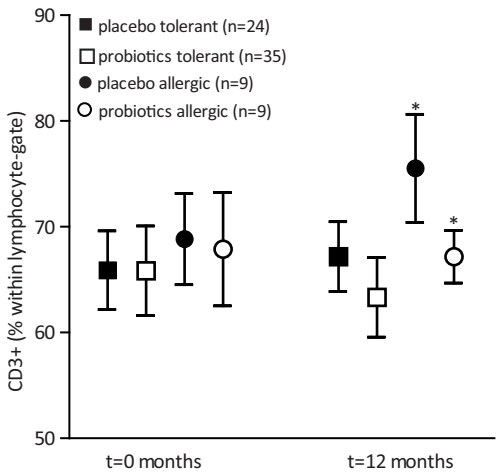

Figure 4 | $CD3^+$ percentage in lymphocyte-gate at 0 and 12 months, categorized into intervention and outcome at 12 months. $CD3^+$ change from baseline for infants with persistent CMA compared to tolerant infants was significantly different, being 4.7% higher in the persistent CMA group (* P=0.032, adjusted for baseline). This difference was independent of the probiotic intervention. Data expressed as means [95% CI].

Fecal sample analysis

After 6 months of study formula, *Bifidobacterium animalis* was detected in 58% and *Lactobacillus casei & paracasei* in 22% of the infants receiving probiotics. In the placebo group the percentages were significantly lower, 5% and 4% respectively (data not shown) .

DISCUSSION

We show that supplementation of a combination of CRL431 and Bb-12 to hydrolyzed formula fails to induce additional or accelerated CM tolerance during 12 months of treatment in CMA infants. Tolerance for CM developed in the majority of infants in both groups already after 6 months. We did find minor effects of probiotic treatment on lymphocyte subpopulations in peripheral blood, and viable probiotic strains were often detectable in fecal samples of treated infants, confirming that the treatment had actually succeeded in modifying the intestinal microflora. To our knowledge we are the first to study the effect of probiotic supplementation on established CMA.

There is convincing data that specific probiotic strains can influence immune function through different pathways including effects on local immune cells and T and B cells. An in vitro study has shown that a strain of *Lactobacillus paracasei* (subspecies Paracasei B21060) inhibited the proliferation of $CD3^+CD4^+$ from both healthy donors and patients with inflammatory bowel disease [31]. Although we were unable to detect a probiotic effect on the primary outcome, the flow cytometric data indicate that probiotics did modulate the immune system. The $CD3^+$, $CD3^+CD4^+$,$CD3^+CD8^+$ and $CD20^+$ lymphocyte percentages before the start of treatment were comparable between both treatment groups and to those of earlier studies of healthy infants in the same age range [32,33]. Twelve months of probiotic supplementation significantly decreased the $CD3^+$ and the $CD3^+CD4^+$ percentages compared to placebo. This probiotic effect was most prominent in CMA-persistent infants.

Treatment of allergies with probiotics focuses on early allergic manifestation such as AEDS. The first studies of probiotic treatment on AEDS [14,20] have reported a positive effect on the severity of AEDS. Recent and larger trials could not confirm a probiotic effect on the severity of AEDS [13,16,34]. The results are difficult to compare, mainly because of the different patient populations.

Advantageous effects of probiotics on AEDS and fecal alpha1-antitrypsin/ TNF-alpha were reported by Majamaa et al [20], whereas Viljanen et al found no effect on AEDS or fecal inflammatory markers in the CMA infants using the same probiotic strain (*Lactobacillus* GG) [21,34]. We observed a significant overall improvement in the severity of AEDS as determined by the SCORAD. The change from baseline was not different between infants on probiotics or placebo. Improvement of AEDS upon using an extensively hydrolyzed formula was earlier

described by Brouwer and coworlers [13]. Hence, our findings indicate that these probiotics did not improveAEDS in infants with established CMA.

Food allergy has been studied in several probiotic trials. Previous studies on probiotic supplementation of infants diagnosed with CMA [20] or suspected of CMA [17,21,34,35] have been reported. Brouwer et al have performed a CM-challenge before intervention with probiotics [13]. However, only 4 out of 50 infants included in their study had a proven CMA. In the suspected CMA studies, probiotic intervention took place before the CM challenge. None of these studies specifically addressed the question whether probiotic supplementation affects the outcome of the CM challenge. These trials focussed on AEDS, fecal inflammatory markers and peripheral blood mononuclear cells (PMBC) cytokine production as outcome measures.

There may be several explanations why probiotics failed in our study. First, the probiotics may have had an insufficient immunomodulatory effect. Indeed, the observed effects were small and unlikely to be clinically relevant. This is corroborated by other prevention studies failing to show clinically relevant effects besides prevention of AEDS [36-39]. Next, the compliance with the study protocol could have been suboptimal. We think that this is not the case, as the study formula was delivered at home on request of the parents. It was reasoned that this would enable us to actively monitor the compliance, and facilitate good compliance. The intake of probiotics was actually confirmed by fecal analyses, that identified the specific *Bifidobacteria* and *Lactobacilli* strains mainly in the probiotic treatment group.

Finally, lack of efficacy may have resulted from insufficient numbers of the probiotics reaching the mucosal immune cells. In this respect, recently, a Dutch consumer organization assessed the concentration of viable *Lactobacilli* and *Bifidobacteria* in 28 commercially available probiotic products [40]. It was established that large differences exist between the tested preparations, and that various products did not contain sufficient viable numbers of the probiotic strains to adequately colonize the intestine. In this trial the concentration of the supplemented probiotics was determined monthly. Furthermore, CRL431 and Bb-12 were used in earlier trials in infants and were shown to colonize the gut [41,42]. The concentrations of used probiotics strains in this trial were estimated at 10^9cfu per day for both CRL431 and Bb-12. We demonstrated that these concentrations were sufficient for colonization of the gut.

We have found a high cumulative percentage of tolerance in our study group [3]. It can be argued that not all the included infants suffered from CMA, due to the fact that we applied an open food challenge and subsequent bias at inclusion. Next, this could also explain the relative low rate of IgE sensitised infants in our studies compared to other published cohorts [43-45].

Based on the complete absence of any effect of the probiotic intervention we deduce that the major conclusion of our study (no efficacy of probiotic intervention in ongoing CMA) may not have been seriously influenced by this issue. Furthermore, our subgroup analysis in infants suffering from IgE^+CMA suggest that the acquisition of tolerance in the probiotics group appears

in fact to be diminished. The subgroups however are too small for adequate statistical analyses and subsequent conclusions.

In our model, positive SPT to milk and hen's egg at randomization were predictive for persistent CMA at 6 and 12 months, respectively. These data confirm the report by Saarinen et al who established that a positive SPT to hen's egg at randomization predicts persisting CMA at age 2 years [46]. One could argue that in these children, CM re-challenge should be postponed.

What are the clinical consequences of our findings? The results do not support the use of probiotics supplementation of extensively hydrolyzed casein formula with these specific probiotic strains for tertiary prevention in infants with CMA. It is unclear whether other probiotic strains might be more successful. The potential modulatory effect of probiotics should be weighed against the excellent prognosis of CMA with high likelihood of spontaneous tolerance within the first year. Consequently, the effects of any intervention next to the use of hydrolyzed formula are likely to be of limited impact. We conclude that probiotic treatment with CRL431 and Bb-12 to extensively hydrolyzed formula does not accelerate tolerance acquisition in infants with CMA, despite the evidence of intestinal colonization and support for immunomodulatory activity.

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Chapter 4

iNKT-cells and CRTH2^{+}leukocytes in early childhood allergic disease

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ABSTRACT

Background: Atopic eczema/dermatitis syndrome (AEDS) and food allergy are early indications of an atopic constitution. The risk of developing atopy may be reduced by breastfeeding or probiotics. Allergic immune responses are maintained by T helper2-lymphocytes (Th2-lymphocytes) that produce a specific array of mediators. Recently, invariant Natural Killer T (iNKT-)cells and leukocyte expression of the Prostaglandin (PG)D_2 receptor (CRTH2) have been implicated in pathogenesis of allergy.

Objective: To examine if cell marker expression by blood leukocytes and in particular the iNKT-cell receptors and/or CRTH2 is altered by the duration of breastfeeding or supplementation of probiotics in a cohort of cow's milk allergic (CMA) infants. Furthermore to assess if the expression of leukocyte markers is associated with AEDS and the development of an allergic phenotype

Methods: Patients took part in a randomized controlled trial on the effect of L*actobacillus paracasei* CRL431 and *Bifidobacterium lactis* Bb-12 on the evolution of established CMA in infants. Blood samples were obtained at randomization and after 12 months of study formula, and were analyzed using flowcytometry.

Results: At randomization, infants with AEDS had lower numbers iNKT-cells than infants without AEDS. Probiotics had no effect on iNKT-cells and $CRTH2^+$ lymphocytes. T(h)-lymphocytes were lower after at least 3 months of breast feeding and after probiotic supplementation. At 12 months, hen's egg-sensitized infants showed lower iNKT-cells and higher $CRTH2^+$ lymphocytes.

Conclusion: We found low iNKT-cells and high $CRTH2^+$-lymphocytes in sensitized infants. Breast feeding and probiotics were associated with reduced T(h)-cells.

INTRODUCTION

Atopic eczema/dermatitis syndrome (AEDS) and food allergy are often the first indications of an atopic constitution, later followed by allergic rhinitis and asthma [1]. It is important to identify those patients in whom allergic symptoms will persist, and in whom targeted prevention may be possible. Possible measures to prevent development of atopic disease include breastfeeding and probiotics [2,3]. Unfortunately, due to the lack of validated immunological markers it has been difficult to identify patients who are at risk for persistent disease and thus may benefit from such interventions. iNKT-cells are a specific subpopulation of T-lymphocytes expressing an invariant T-cell receptor and Natural Killer cell markers [4]. Upon activation by glycolipids presented by CD1d on antigen presenting cells, iNKTcells can produce both Th1- and Th2-type cytokines [5]. Earlier studies have suggested a role for these cells in the inception of allergic disease [6,7]. A second subset of effector T-cells in allergy express CRTH2, a membrane receptor for prostaglandin D2. In AEDS and in aeroallergen-sensitized adults, increased levels of $CRTH2^+$Th-lymphocytes were found in peripheral blood [8,9]. As the majority of $CRTH2^+$-lymphocytes exhibit a Th2 profile, altered numbers of $CTRH2^+$Th-lymphocytes may be associated with the allergic response in infants [10]. We assessed whether in infancy the numbers of these cells in peripheral blood were associated with the presence, persistence or development of allergic disease, the use of probiotics and the duration of breastfeeding. As early sensitization to hen's egg has a predictive value in development for atopic disorders we also examined the relationship between sensitization to hen's egg and iNKT-cells / $CRTH2^+$Th-lymphocytes [11, 12].

MATERIALS AND METHODS

Patient population A cohort of 119 CMA infants up to the age of 6 months was enrolled in the Cow's milk Allergy Modified by Elimination and Lactobacilli (CAMEL) project [13]. Inclusion was stratified for being breastfed (n=82) or receiving infant formula (n=37). After 6 and 12 months of intervention, a double blind placebo controlled food challenge was performed [14].

Interventions Infants under the age of 6 months with CMA were randomized in a probiotics and control group, receiving either *Lactobacillus paracasei* CRL431 and *Bifidobacterium lactis* Bb-12 (both 10^7 cfu/g formula) supplemented to an extensively hydrolyzed formula (Friso 1 Allergy Care®) or hydrolyzed formula only. When infants had acquired tolerance to CM after 6 months they received either CM-containing follow-up milk (Friso 3®) supplemented with *Lactobacillus paracasei* CRL431 and *Bifidobacterium lactis* Bb-12 or follow-up milk alone for the next 6 months. After 12 months the study formula was stopped.

Clinical parameters The SCORing Atopic Dermatitis (SCORAD) index was used to assess the severity of eczema [15]. Skin prick tests (SPTs) were performed at randomization and at 12

months of study formula. Fresh foods were applied to the volar side of the patient's forearm (i.e., fresh CM, hen's egg and soy) and pricked with a 1-mm single-peak lancet (ALK, Copenhagen, Denmark). The response was assessed at 15 min, and considered positive if the weal was 3 mm or larger, with NaCl 0.9% as negative control and 10 mg/mL histamine dihydrochloride (ALK) as a positive control.

Flow cytometry and IgE Erythrocytes were lysed and cells washed twice with Phosphate Buffered Saline with 1% Newborn Calf Serum (denoted as FACS buffer). Next, samples were incubated with human serum for 10 min, washed with FACS buffer and incubated with anti-CD3-FITC (clone HIT3a), anti-CD4-PE (clone RPA-T4), anti-CD8-PerCP (clone SK-1), anti-CD25-FITC (clone M-A251,all from Becton, Dickson and Company, Franklin Lakes, NJ, USA), anti-CRTH2-PE (clone BM-16, Miltenyi Biotec, Bergisch Gladbach, Germany), anti TCR-Vα24-FITC (clone C15) and anti-TCR-Vβ11-PE (clone C21, both from Beckman Coulter, Inc., Fullerton, CA, USA) or appropriate isotype control for 30 min on ice. After incubation, cells were washed, resuspended in 1% paraformaldehyde and fluorescence measured (FACSCalibur™ (Beckton Dickinson and Company). For each sample 50 000 events were analyzed, except for the staining of iNKT-cells for which 150 000 events were analyzed. The results were analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA). Total and serum allergen-specific IgE levels against cow's milk, egg white, peanut, dog, cat and house dust mite were measured by means of immunoassay (Phadiatop Infant, Pharmacia CAP System, fx5, Pharmacia Diagnostics) at randomization (n=80) and after 12 months (n=68, 38 paired samples). The cut-off level was >0.35kU/l according to manufacturer's protocol.

Statistical analysis SPSS software package (version 12.01 for Windows, Chicago, Illinois, USA) was used for statistical analysis. To examine differences between the groups at indicated time points, non-parametric tests were applied. For all statistical tests, a two-tailed p value of <0.05 was considered significant. All data were expressed as medians (interquartile range).

RESULTS

One hundred and nineteen infants were randomized in the probiotics group (n=59) or in the control group (n=60); 106 infants completed the study after 12 months. Blood samples were obtained from101 infants at randomization (mean aged 4.2 months, range 2.1-6.0, 51% boys) and from 91 infants after 12 months of study formula, with paired samples available from 83 infants.

Overall, we observed a decrease in the percentages of $CD4^+$ T-cells and iNKT-cells, an increase in the percentages of $CD8^+$ T-cells and no change in $CRTH2^+$-T-cells between randomization and the end of the intervention (Table 1).

Table 1 | Peripheral blood leukocyte subsets

CDsubset	Cell population	t=0	t=12 months	P-value
$CD3^+$	Total T-cells	65.8 (12.2)	67.4 (13.1)	0.62
$CD3^+CD4^+$*	T-helper	48.0 (13.6)	43.9 (13.4)	**0.001**
$CD3^+CD8^+$*	T-cytotoxic	15.9 (7.1)	17.7 (9.7)	**0.002**
$CD3^+V\alpha24^+V\beta11^+$*	Invariant NKT-cells	0.04 (0.04)	0.02 (0.02)	**<0.001**
$CD4^+CRTH2^+$	$CTRH2^+$Th-cells	0.11 (0.09)	0.16 (0.22)	0.19

* CD subset determined within CD3 lymphocyte-gate. Values represent the percentages of the cells expressing the indicated markers, presented as medians (IQR). P-value is of Wilcoxon signed rank test.

We observed a lower percentage of $CD3^+$T-cells and $CD3^+CD4^+$ T-cells in peripheral blood in infants who received at least 3 months of breastfeeding (Figure 1a-b). No correlation was found between the duration of breastfeeding and numbers of iNKT cells or $CRTH2^+$Th-lymphocytes (Figure 1c-d). As we reported before probiotic supplementation for one year significantly decreased the T-lymphocyte and T-helper percentages compared with placebo [13]. However, probiotic supplementation did not affect the percentages of iNKT- and CRTH2-lymphocytes (table 2).

Table 2 | Effects of probiotic intervention on peripheral blood leukocyte subsets

Cell population	Intervention	t=0	t=12 months	P-value
Total T-cells	Placebo	67.3 (12.4)	69.6 (13.2)	**0.02**
	Probiotics	64.2 (11.4)	67.1 (12.3)	
T-helper	Placebo	47.7 (16.1)	46.2 (15.6)	**0.04**
	Probiotics	48.1 (10.6)	41.2 (9.8)	
T-cytotoxic	Placebo	15.6 (6.7)	17.3 (9.5)	0.85
	Probiotics	16.7 (8.2)	18.8 (10.0)	
Invariant NKT-cells	Placebo	0.04 (0.04)	0.02 (0.02)	0.10
	Probiotics	0.03 (0.03)	0.03 (0.03)	
$CTRH2^+$Th-cells	Placebo	0.11 (0.10)	0.20 (0.26)	0.13
	Probiotics	0.12 (0.08)	0.10 (0.16)	

Values represent the percentages of the cells expressing the indicated markers, presented as medians (IQR). P-value indicates for the change from baseline (probiotics vs placebo, analysis of covariance, adjusted for baseline).

At randomization, the percentage iNKT-cells was significantly lower in AEDS infants (n=31) compared to those without AEDS (n=70): 0.03% (0.02%) vs 0.04% (0.03%); P: 0.006 (Figure 2). The number of $CRTH2^+$ lymphocytes was similar for AEDS (n=21): 0,13% (0,2%) vs non-AEDS (n=54) 0,11% (0,11%); P: 0.141.

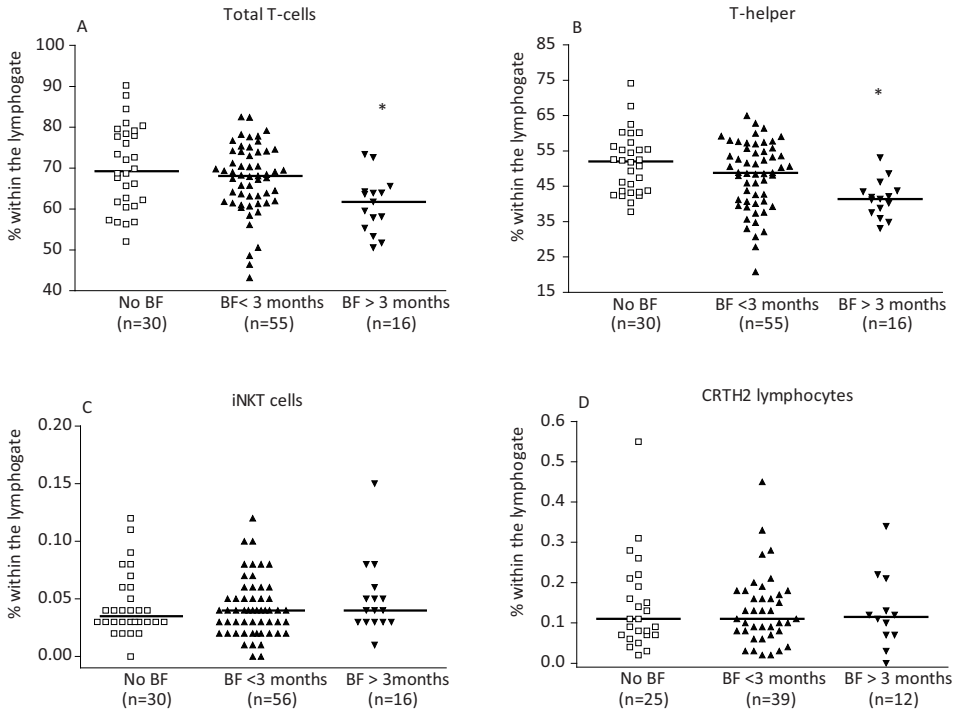

Figure 1A-D | Total T-cells and Thelper-cells, iNKT and CRTH2^{+} lymphocytes percentages within the lymphocyte-gate at randomization, categorized for the duration of breastfeeding (BF).

Data expressed as medians. *P<0.05 compared to no breastfeeding.

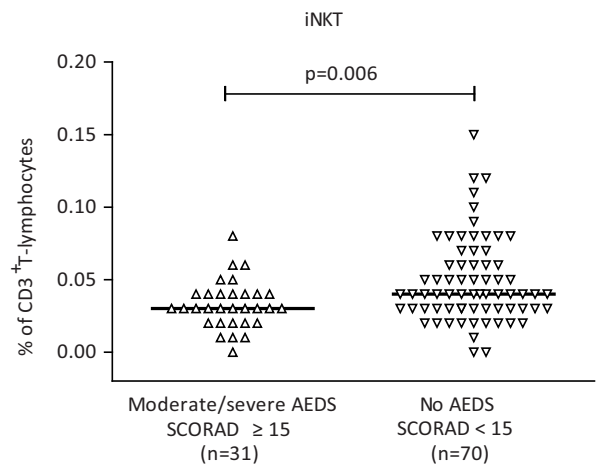

Figure 2 | iNKT percentages at randomization of infants with or without AEDS.

Horizontal bars = medians.

At 12 months, infants sensitized to hen's egg at that age (n=28) had lower percentages of iNKT-cells compared to non-sensitized children (n=66) 0.015% (0.03%) vs 0.03% (0,02%), P: 0.016 (Figure 3a), and CRTH2$^+$Th-lymphocytes were increased (0,2% (0,2%) vs 0,1% (0,2%) P: 0.04; Figure 3b).

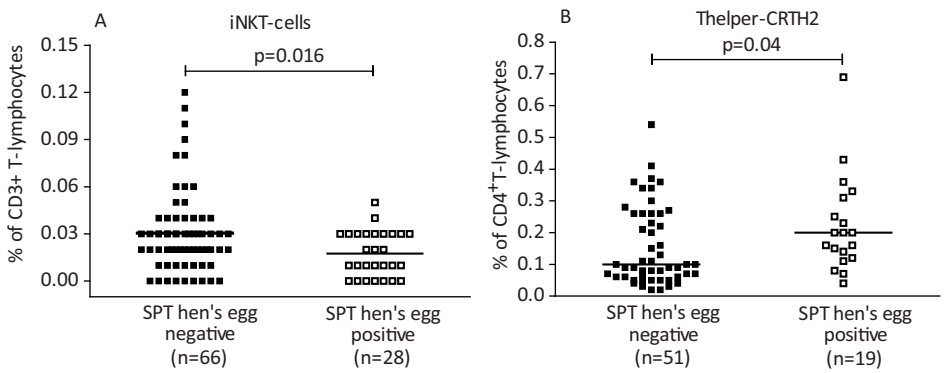

Figure 3A/B | iNKT and T-helper-CRTH2$^+$ percentages at t=12 months of infants with and without positive SPT to hen's egg at 12 months. Horizontal bars = medians.

DISCUSSION

We evaluated the expression of lymphocyte markers in a cohort of CMA infants, focusing on iNKT-cells and CRTH2$^+$ leukocytes. We found lower percentages of circulating iNKT-cells in infants with AEDS at baseline, and in hen's egg sensitized infants after 12 months. Furthermore, CRTH2$^+$-leukocytes were higher in children sensitized to hen's egg after 12 months.

Reduced iNKT-cells in were observed previously in peripheral blood of adult individuals with AEDS [16] and atopy [17]. Moreover, pediatric patients with cow's milk or hen's egg allergy showed lower iNKT-cells peripheral blood [18]. It remains to be established whether the detection of low iNKT-cell percentages are associated with altered iNKT-cell function. A recent study showed that iNKT-cells from children with food allergy preferentially mounted a Th2-lymphocyte response [18] to milk-derived sphingomyelin. This suggested that functional changes may indeed be present as well. As iNKT-cells respond to glycolipids [5], and not to protein antigens, these cells might well act in synergy with protein specific CD4+ T-cells in the inflammatory response.

Among human Th-lymphocytes, CRTH2 is preferentially expressed on Th2-lymphocytes. Upon ligation with PGD2, CRTH2 activation elicits a broad spectrum of responses including chemotaxis in lymphocytes, eosinophils, basophils [19] and monocytes. In particular, PGD2

mediated CRTH2 signals enhanceTh2-lymphocyte polarization via inhibition of the D prostanoid receptor (DP1) on Th1-lymphocytes [20]. In mouse models of airway hyperreactivity it has been shown that aerosol administration of PGD2 leads to an increased Th2 type inflammatory response in the airways(21). Recently, studies in patients revealed that changes in percentages of peripheral blood CRTH2$^+$-lymphocytes are associated with allergic disease and AEDS in particular (9) [22]. Hence, we hypothesized that increased CRTH2$^+$ cells would be used as early markers of atopic disease. Our findings did not confirm this, as we did not find an association between the percentage of peripheral blood CRTH2$^+$ leukocytes and AEDS at randomization or at 12 months. As previous studies showed that early sensitization to hen's egg has a high positive predictive value for atopic disorders [11,12], we compared CRTH2$^+$ cells between infants with and without sensitization to hen's egg at 12 months and observed higher percentages of peripheral blood CTRH2$^+$Th-lymphocytes in children that were sensitized. Hence, in contrast with iNKT cells, CTRH2$^+$Th-lymphocytes seem a marker for atopy in this specific subgroup at high risk for persisting atopy.

In a previous publication we reported on the lower percentages of T-lymphocytes and T-helper lymphocytes after probiotic supplementation [13]. A recent study in infants suffering from AEDS has shown that administration of a probiotic mixture containing *L. acidophilus* DDS-1 and *B. lactis* UABLA-12 for 8 weeks also lowered T-helper lymphocytes [23]. However, none of these changes was associated with any clinical improvement of CMA, AEDS or development of sensitization(13). Moreover in the current study we show that the probiotic intervention had no effect on iNKT cells or CTRH2$^+$Th-lymphocytes.

Exclusive breastfeeding up to the age of 4 months might protect against allergic disease [3,24]. We observed that breastfeeding >3 months prior to the start of the study formula was associated with lower T-lymphocytes and Th-lymphocytes in general, but no effect was found for iNKT cells or CTRH2$^+$Th-lymphocytes. Furthermore, in our intervention study the frequency of iNKT-cells and the frequency of CRTH2$^+$ lymphocytes in peripheral blood did not change with the development of tolerance. This is in contrast to the diminished iNKT-cells in CMA infants described by Jyonouchi et al [18]. A possible explanation for this discrepancy may be the high cumulative percentage of tolerance acquisition in our study group [13] with perhaps insufficient power resulting to detect differences in lymphocyte subpopulations.

In summary, our results confirmed that infants with early atopic disease have reduced frequencies of iNKT-cells. Neither probiotic supplements nor the development of tolerance to cow's milk did affect the expression of the lymphocyte surface marker CRTH2$^+$ in infants with CMA. Interestingly, we found an increased percentage of CRTH2$^+$leukocytes and reduced iNKT cells in a subgroup of infants with increased risk for development of atopy.

We speculate that CRTH2$^+$leukocytes are potentially interesting markers for monitoring the development of atopy. Additional studies are necessary to elucidate if monitoring of these

specific parameters in infants and children may be helpful in predicting the development of allergic diseases.

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Chapter 5

Fractional Exhaled Nitric Oxide in infants during cow's milk food challenge

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ABSTRACT

Background: Cow's milk allergy is the most common food allergy in early childhood. The golden standard for the diagnosis of cow's milk allergy is a food challenge after a period of elimination. Increased levels of exhaled nitric oxide (FE_{NO}) have been shown after bronchial allergen provocation.

Objective: We evaluated whether FE_{NO} may also be a predictor of a positive reaction during cow's milk challenge in infants.

Methods: Forty-four infants (mean age [range]: 4.2 [3.7-4.6] months) suspected of cow's milk allergy underwent an open food challenge with cow's milk formula administered in ascending quantities, starting with 2ml and then 6, 20, 60 and 200ml until a clinical reaction occurred. Off-line FE_{NO} samples were obtained during tidal breathing by means of a facemask covering infants' nose and mouth. FE_{NO} was measured twice before the challenge (baseline), immediately before each new dose of milk and after a positive reaction or after the last dose of milk.

Results: Eleven children showed immediate positive clinical responses to cow's milk, whereas 13 infants presented only a late-type reaction. FE_{NO} values before or after a positive reaction (either immediate or late) were not different from FE_{NO} values at baseline. Baseline FE_{NO} in infants with a positive reaction did not differ from FE_{NO} in infants without a reaction at any time point.

Conclusion: We conclude that FE_{NO} values are not predictive and not related to the occurrence of a positive reaction during a cow's milk challenge in infants, suggesting that a positive reaction may not result from eosinophilic activation.

INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy in early childhood [1]. From prospective studies, the incidence is estimated to be 2-5% [2]. It has been hypothesized that CMA is due to immaturity of local and systemic immune responses combined with an increased permeability of the gut [3]. This could lead to presentation of large milk peptides to the immune system, triggering an allergic immune reaction in susceptible infants [4]. Although the majority of infants with CMA become tolerant in the first years of life, many continue to have CMA throughout childhood [5].

To date, the golden standard for the diagnosis of CMA is a food challenge after a period of elimination [6]. The food challenge includes subjective assessment, is time consuming and carries a small risk of anaphylactic shock. The allergic response to a food challenge can occur in different organ systems. The skin, upper and lower respiratory system and the gastrointestinal tract might be involved in case of a positive reaction to food challenge. How and in which organ an individual reacts is therefore dependent on systemic and local circumstances. Sub-clinical inflammation could be present in several organs and may well remain undetected during a food challenge. An objective marker of a positive response would therefore be desirable. Fractional exhaled nitric oxide (FE_{NO}) is a marker of bronchial inflammation in atopic asthma [7] and correlates with IgE levels in children with asthma and allergic rhinitis [8]. It has been shown that FE_{NO} values increase after bronchial allergen provocation in adults [9-11], whereas in children no change in FE_{NO} has been reported after a nasal allergen challenge [12]. Furthermore, in mouse models it has also been shown that induced gastrointestinal allergy enhances allergic airway responses also to unrelated allergens, indicating the systemic nature of an allergic reaction [13].

Since no data is available on infants, we explored the relation between food allergy and airway inflammation in atopic infants. Specifically, we sought to evaluate if changes in FE_{NO} during a cow's milk challenge test would allow us to differentiate between tolerant and allergic infants.

METHODS

The CAMEL-study (Cow's Milk Allergy with Elimination and Lactobacilli) explores the effects of non-pathogenic bacteria of the gut (probiotics) on the development of the immune system in infants with CMA. Parents were informed and asked to participate as soon as an infant suspected of CMA was seen by the healthy baby clinics or by the general practitioner. Then infants were referred to the Sophia Children's Hospital in order to perform an open cow milk challenge test at the start of the study according to the guidelines of ESPGHAN and ESPACI and criteria of Bock and Sampson [2,14]. Verum formula was Allergycare® (Royal Friesland Foods, Leeuwarden) and Protifar® (Nutricia, Zoetermeer) in an 11:3 mixture, resulting in 1.8 gram of cow milk protein per

100 ml. During the food challenge the formula was administered in ascending quantities, starting with 2ml and then 6, 20, 60 and 200 ml. The time between two consecutive doses ranged between 30 and 60 min. Infants were scored for 9 items divided into four main categories (general, skin, gastro-intestinal, respiratory) in a 0-3 system (0=none, 1=light, 2=moderate, 3=severe). A test was considered positive if one item scored 3 or if 2 or more items scored 2 (table 1).

One to 2 hours after the final dose patients were discharged and contacted by telephone the next morning to assess whether a late-type reaction had occurred, such as vomiting and diarrhoea. Patients attended the outpatient clinic the next day only if parents reported skin problems that needed to be evaluated by a physician. Due to the design of the CAMEL-study, total and cow's milk specific IgE and skin prick test (Pharmacia, Uppsala, Sweden) were only performed in infants with a positive challenge.

FE_{NO} measurements were performed off-line, during tidal breathing and without the use of sedation, with infants in supine position, as previously described in detail [15]. Briefly, we collected mixed exhaled air during quiet breathing via a silicon facemask covering infant's nose and mouth. A measurement was considered successful if at least 5 breaths could be sampled during quiet tidal breathing with the facemask tightly fitted to nose and mouth. Before each measurement, ambient NO was recorded. FE_{NO} was measured twice within 10 min before the challenge and the mean of the two measurements was taken as baseline. Then FE_{NO} was measured immediately before every new dose of cow's milk. The last FE_{NO} measurement was performed 1 hour after the highest dose, or after a positive reaction. Infants were included in the analysis only if they successfully performed at least 75% of the attempted FE_{NO} measurements including baseline and final FE_{NO} measurement.

The medical ethical committee of the Sophia Children's Hospital approved the study. Parents gave written informed consent.

Statistical Analysis

FE_{NO} values were log-transformed and then analyzed by means of parametric tests. FE_{NO} reproducibility was assessed according to Bland and Altman [16] and was quantified by the intraclass correlation coefficient (ICC). The Cox proportional hazards regression model, with FE_{NO} changes as a time dependent variable, was used in order to investigate whether these changes were related to a positive clinical reaction to cow's milk. Additionally, baseline FE_{NO} values of infants with a positive reaction were compared to those of infants without reaction at any time point by means of the t-test. Regression analysis was used to evaluate the influence of ambient NO on baseline FE_{NO}. FE_{NO} values are reported in parts per billion (ppb).

For all statistical tests, a two-tailed p value < 0.05 was considered significant.

Table 1 | Scoring system for response to cow's milk food challenge. Infants were scored for 9 items on a 0-3 scale each. A test was considered positive if one item scored 3 or if 2 or more items scored 2

Score	Subjective symptoms	Anaphylaxis	Urticaria	Rash	Itch	SCORAD increase	Gastrointestinal: Vomiting, diarrhoea	Sneezing	Wheezing
0	Absent	no change	none	absent	absent	0-10%	absent	absent	absent
1	Minimal nausea or pain, no change in activity	n/a	<3	minimal, small areas of faint erythema (<20% surface)	minimal, occasional scratching moderate	10-20%	minimal, 1 episode	minimal, sneezes rarely	minimal, expiratory wheezing to auscultation
2	moderate, frequent nausea or pain, decreased activity	n/a	>3 and <10	moderate, areas of erythema and macula (>20% en <50% surface)	scratching continuously for >2 minutes at a time	20-30%	moderate, 2-3 episodes (or 1 episode of vomiting and 1 episode of diarrhoea)	moderate, sneezes <10; intermittent rubbing of nose and/or eyes	moderate, dyspnoea; inspiratory en expiratory wheezing
3	severe, notably distressed, continuous crying	Anaphylactic shock	>10 (generalized)	severe, generalized erythema (>50%) or >25% of the surface erythematosquamous lesions / vesicles	continuous scratching with excoriations	>30%	severe, 3 episodes (or 2 episode of vomiting and 2 episode of diarrhoea)	severe, continuous rubbing of nose / eyes; periorbital swelling	severe, dyspnoea, use of accessory muscles

RESULTS

Fifty-eight children underwent the open challenge with cow's milk between September 2004 and March 2005. Forty-four infants (83%, 32 boys, mean age [range]=4.2 [3.7-4.6] months) successfully performed FE_{NO} measurements during the open food challenge (Figure 1). The two baseline FE_{NO} measurements were successfully performed in 39 infants and showed good reproducibility (intraclass correlation coefficient=0.88, mean difference [SD]: -0.29 [4.6] ppb) (Figure 2). Hence, the geometric mean of the 2 baseline values was calculated and used as the individual baseline for the analysis. Immediate reactions occurred in 11 infants (25%), whereas in 13 infants (29%) a late-type reaction was recorded (table 2).

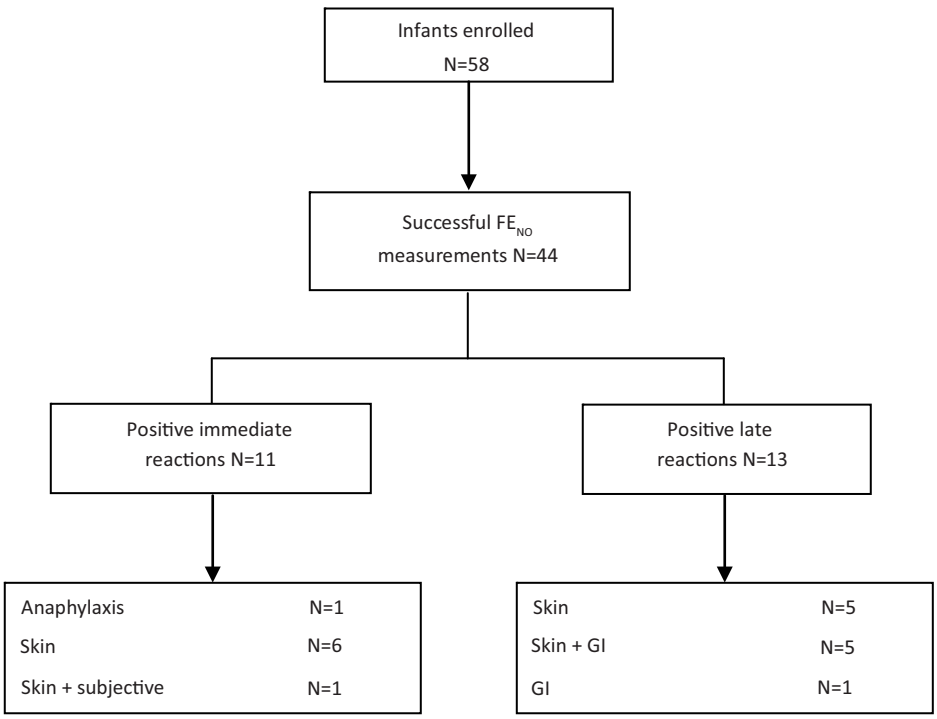

Figure 1 | Flow chart of the study population. GI : gastrointestinal symptoms (vomiting and diarrhoea); subjective symptoms included: nausea or pain, decreased activity and continuous crying.

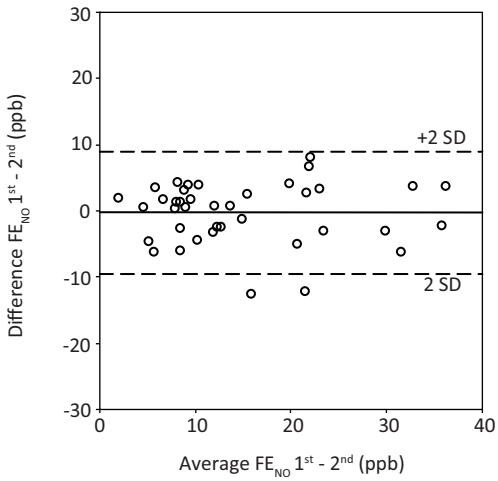

Figure 2 | Bland and Altman plot showing good agreement of the two baseline FE_{NO} measurements in 39 infants (mean difference [SD] of the two mesurements = -0.29 [4.6] ppb)

Table 2 | Geometric mean baseline FE_{NO} in infants undergoing cow's milk challenge test. No difference was observed in geometric mean baseline FE_{NO} depending on the clinical reaction to cow's milk challenge.

Cow's milk challenge	No reaction (n=20)	Early reaction (n=11)	Late reaction (n=13)
Geometric mean baseline FE_{NO} [95%CI]	13.4 [10 – 17.8]	12.1 [8.2 – 17.9]	10.8 [7.5 – 15.5]

Subjective symptoms, whether during the challenge or reported by parents, are difficult to interpret and debatable. Therefore we performed a separate analysis after excluding all patients who reported only subjective symptoms (such as nausea or pain, decreased activity and continuous crying) during and after the challenge. Such exclusion did not modify our results.

FE_{NO} values before and after a positive reaction did not differ from FE_{NO} at baseline. FE_{NO} was not different in infants with a positive immediate clinical reaction compared to infants without reaction at any time point (Figure 3). The results did not change when infants with a late-type reaction were considered in the analysis. Independently of the type of reaction, none of the infants presented respiratory symptoms during the challenge. Individual FE_{NO} values of infants with a positive immediate reaction at different time points are reported in Figure 4. A single infant developed a severe, systemic immediate response (anaphylaxis). In this case we

found a rise in FE_{NO} from 5.4 ppb at baseline to 9.9 ppb after the last dose of milk was introduced. However, a similar increase in FE_{NO} was also observed in other infants with immediate positive reaction (Figure 4). Infants with a positive challenge (n=24) underwent a skin prick test using fresh cow's milk and venous IgE assessment. Total IgE levels ranged from 0.1 to 270 kU/L, mean 35.8 kU/L. Seven infants (24%) had a positive skin prick test and IgE directed against cow's milk. No correlation was found between IgE (total or cow's milk specific) and FE_{NO} (Spearman's coefficient = -0.24; p=0.2) No relation was found between FE_{NO} and ambient NO.

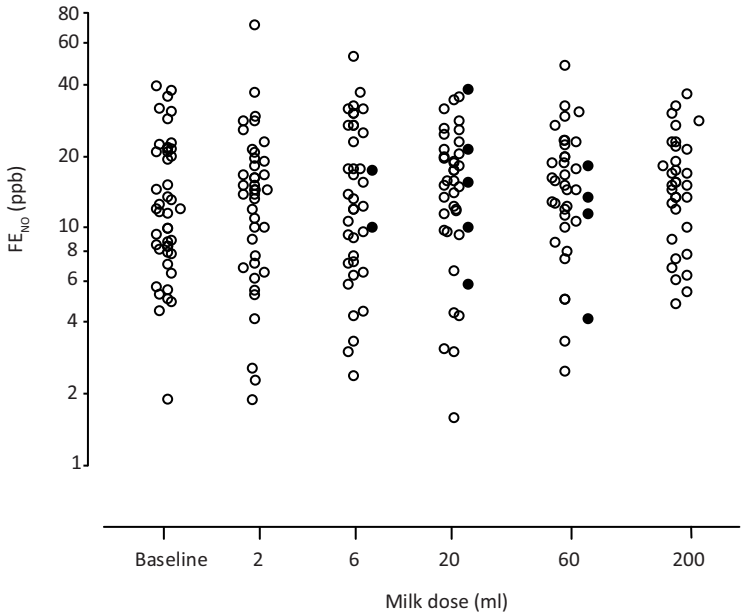

Figure 3 | Individual FE_{NO} values of 44 infants suspected of CMA.

Open circles: children without immediate clinical response; solid circles: children with immediate clinical response (n=11) to the subsequent cow's milk dose. There was no difference between FE_{NO} of children with or without positive immediate reaction.

DISCUSSION

We evaluated whether changes in FE_{NO} levels were predictive of a positive response in infants undergoing cow's milk challenge. This study was set up based on previous data that link FE_{NO} production to (the severity of) airway inflammation, mucosal recruitment and activation of

eosinophils in particular. To our knowledge, this is the first study that investigated the possible role of FE_{NO} during a food challenge in infants. The results do not support the hypothesis that FE_{NO} can be used as a predictor or marker of a positive clinical reaction to cow's milk in an open food challenge. However, our results have to be interpreted with caution, as none of the infants presented respiratory symptoms during the challenge. Therefore, high FE_{NO} levels at baseline or relevant FE_{NO} changes in infants with respiratory symptoms after a food challenge could not be excluded.

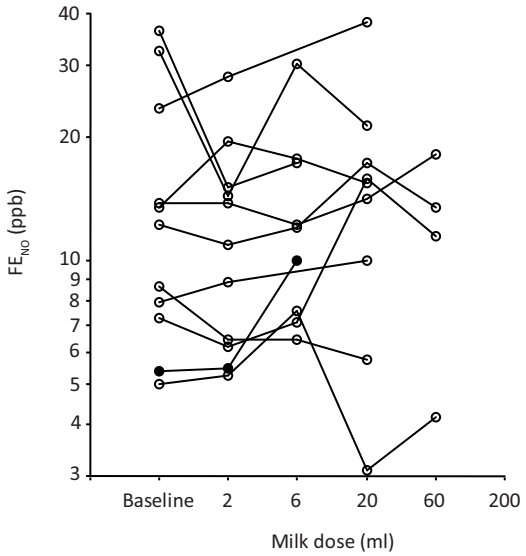

Figure 4 | Individual FE_{NO} values of infants with immediate positive reaction (N=11) at different time points, until a reaction occurred. Solid circles represent individual FE_{NO} values of the infant who experienced anaphylactic shock.

The lack of association between FE_{NO} and the positive reaction to cow's milk in our study might be due to the absence of eosinophilic infiltration in the lower airway mucosa. Indeed, none of the 11 infants with immediate positive reactions showed respiratory symptoms during allergen challenge. Baseline FE_{NO} did not differ between infants with or without immediate or late response to cow's milk, suggesting no pre-existing eosinophilic airway inflammation. It could be argued that FE_{NO} is not a suitable marker in case of absence of airway symptoms. However, we don't think this is valid as eosinophilic airway inflammation and airway symptoms correlate weakly or not at all [17].

In the last decade several studies evaluated the effect of allergen challenge on FE_{NO} in asthmatics. Kharitonov et al demonstrated that the late asthmatic response to bronchial allergen provocation in adults is associated with elevated FE_{NO}, while the early response showed no significant increase in FE_{NO} [9]. Olin et al found that atopic subjects have elevated levels of FE_{NO} only if they had recently been exposed to the relevant allergen [18]. Atopic subjects not exposed to a relevant allergen or without symptoms of asthma or rhinitis showed normal $FE_{NO.}$ The relevant allergens of this study however did not include food allergens. Pedroletti et al demonstrated that a single nasal allergen challenge with cat dander did not induce bronchial inflammation and increase in FE_{NO} in a pediatric population [12]. Although the challenge caused a local reaction in the nose, the authors speculated that a single nasal allergen challenge might have been insufficient to induce bronchial inflammation. Our study is in line with the findings of Kharitonov and Pedroletti, since we did not find any change in FE_{NO} related to the early reaction to cow's milk challenge. We could not evaluate a late FE_{NO} response to the allergen, since infants were discharged within 2 hours after the challenge was completed. Apparently, FE_{NO} was not predictive of a late response on the day of the challenge. Nonetheless, the usefulness of FE_{NO} measurements to assess a late-type reaction needs to be further studied, as in our study FE_{NO} assessment did not take place at the time of the late responses.

Most infants with a positive challenge presented a late-type reaction only with a combination of gastrointestinal and subjective symptoms. It has been hypothesized that delayed gastrointestinal symptoms due to CMA are related to a variant Th-2-type immune response, mainly characterized by the production of IgG_4 rather than an IgE mediated immune response [19]. Since FE_{NO} is a marker of eosinophilic inflammation, which is unlikely in non-IgE mediated intolerance, an IgG-mediated response might explain why no change in FE_{NO} values was observed in infants with a late-type positive reaction to cow's milk.

A single infant developed a severe systemic reaction (anaphylaxis), and this child showed a consistent increase in FE_{NO} values preceding the response. This rise might have been useful in characterizing the systemic reaction at individual level, but the magnitude of the change was such that it could escape detection due to within-subject short-term variability. Indeed, an increase in FE_{NO} was also observed in other infants with immediate reaction, indicating that the extent and the direction of change of FE_{NO} values was not related to (the severity of) the clinical reaction.

Could our results be due to selection bias? All doctors collaborating in the CAMEL-study were trained to recognize and refer children with a high probability of CMA based on history and/or physical examination. Hence, in our study group a high percentage of the cow's milk challenges were positive. Any selection bias would therefore have led to an overestimation of the association. As we found no association at all, it seems unlikely that the selection of the population might have biased the results.

The method used for the measurement of FE_{NO} in non-sedated infants might have introduced variability, since no control for the expiratory flow neither for the breathing frequency was performed, and it could be argued that this would reduce the possibility of detecting FE_{NO} changes. This seems unlikely, however, as our method showed good reproducibility within infants and could differentiate between infants with different airway diseases [15,20].

In summary, our data indicate that no correlation exists between changes in FE_{NO} and the outcome of a food challenge in allergic children, suggesting that a positive reaction may not result from eosinophilic activation. We conclude that FE_{NO} measurements cannot be used to predict or characterize a positive reaction to cow's milk in infants.

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Chapter 6

Fecal microbial composition correlates to the acquisition of tolerance towards cow's milk

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ABSTRACT

Background: Allergy is one of the most prevalent chronic diseases in the industrialized world. The intestinal flora of allergic versus non-allergic individuals differs. In infants with "bad" gut microbiota the supplementation of "good" commensal bacteria, probiotics, could alter the flora composition and subsequently prevent or treat allergic diseases.

Objective: To examine if probiotic supplementation for 12 months in a cohort of cow's milk allergic infants alters the intestinal flora composition and whether the acquisition of tolerance to cow's milk was associated with changes in intestinal flora.

Methods: Patients took part in a randomized controlled trial on the effect of L*actobacillus paracasei* CRL431 and *Bifidobacterium lactis* Bb-12 on the evolution of established cow's milk allergy in infants. Fecal samples were obtained at start, 6 and 12 months of study formula. All fecal samples were analyzed using quantitative polymerase chain reaction. In a subgroup, intestinal flora was analyzed with a micro-array to detect and semi-quantitate the small subunit ribosomal RNA.

Results: Total numbers of bacteria and bifidobacteria did not differ between placebo and probiotics before and during the trial. Within the probiotics group the supplemented probiotic bacteria were significantly more detected. Acquisition of tolerance was associated with increased levels of several species including bacteroidetes.

Conclusion: Probiotic administration for 12 months in a cohort of cow's milk allergic infants did not affect the numbers of specific microbial strains. We detected significant variations in the composition of the intestinal microbiota between tolerance-acquiring and persistent cow's milk allergic infants that were independent of probiotics supplementation.

INTRODUCTION

Allergy is an aberrant immunological response towards harmless environmental allergens in genetically susceptible individuals. The rising prevalence of allergic diseases in the last 3 decades prompted intensive research into the pathophysiology of this complex disease. Early infancy is thought to be an important period for fine-tuning of the immune system. As such, inadequate stimulation of immune cells in infants has been linked to allergic diseases in later life [1]. In line with this hypothesis, a lower number of bifidobacteria has been found in the intestinal flora of allergic children in comparison to non-allergic individuals [2]. Based on this, it has been put forward that supplementation of specific bacteria such as probiotics could be beneficial in the prevention or treatment of allergic diseases. Probiotic bacteria are defined as "living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition [3]". Current probiotic research does not only investigate whether a specific strain benefits specific patient populations but is also aimed at the probiotic working mechanism during and after supplementation. The principle hypothesis on the working mechanism of probiotics is restoration of the mucosal homeostasis. Homeostasis can be achieved by (1) alteration or balancing of the intestinal flora, (2) restoration or maintenance of the intestinal barrier function and (3) regulation of immunological responses. Several allergy prevention- and intervention trials have shown that the composition of the intestinal flora changes during supplementation [4-7]. To date, no studies however, have been performed to assess the effect of probiotics on the intestinal flora in established cow's milk allergy (CMA). The main purpose of this study was to determine if probiotic supplementation for 12 months in a cohort of cow's milk allergic infants alters the intestinal flora composition and whether these changes reflect the acquisition of tolerance towards cow's milk (CM).

MATERIALS AND METHODS

The CAMEL project (Cow's milk Allergy Modified by Elimination and Lactobacilli) was a randomized double blind, placebo-controlled study carried out between March 2004 and May 2007 and was described earlier [8]. In summary 119 infants younger than 6 months with a diagnosis of CMA were included. Infants in the probiotic group received Lactobacillus casei CRL431 (Lactobacillus paracasei subspecies paracasei) and Bifidobacterium lactis Bb-12 (Bifidobacterium animalis subspecies lactis) 10^7 cfu/g formula for each of the probiotic bacterium used) supplemented to Friso 1 Allergy Care® whereas the control group received Friso 1 Allergy Care® alone. The cfu/g of the formula was measured monthly and remained stable during the study. After 6 and 12 months of study formula, a double blind placebo controlled food challenge (DBPCFC) was performed. If infants had become CM tolerant after 6 months, they received either CM-

containing follow-up milk (Friso 3®) supplemented with CRL431 and Bb-12 or Friso 3® without probiotic supplementation for the next 6 months. The local ethics committee approved the study protocol.

Fecal samples

Fecal samples were collected at inclusion and at 6 and 12 months. All samples were frozen and stored at -80°C and analyzed using quantitative polymerase chain reaction (qPCR) for total bacteria and bifidobacteria. The qPCR of the mentioned genera was performed according to the methods as derived from Kimura et al [9] and Fenicia et al [10]. In case of sufficient fecal material fecal samples were also analyzed by small subunit rRNA microarray to determine and semi-quantify most major intestinal microorganisms. The microarray with 394 probes was performed according to the method as described by Schuren et al [11].

Statistical analysis

SPSS software package (version 12.01 for Windows, Chicago, Illinois, USA) was used for statistical analysis of qPCR results. To examine differences between the groups at indicated time points, non-parametric tests were applied. TM4 software was used for analysis of microarray data[12]. For all statistical tests, a two-tailed p value of <0.05 was considered significant.

RESULTS

At inclusion, fecal samples of 65 infants were collected, 34 infants of the placebo group and 31 of the probiotic intervention group. Baseline characteristics did not differ between the study groups (Table 1). At 6 and 12 months we found no significant difference in the total number of bacteria between the probiotics and placebo groups ($p=0.41$). Also, no significant differences were found between the total number of bifidobacteria present in the fecal samples that were obtained from the probiotic and placebo group ($p=0.56$) (Figure 1A and B). We considered whether there were specific confounders at baseline (gender, type of delivery, breastfeeding, antibiotic use, or the presence of eczema). None of these characteristics influenced the outcome. Since probiotic supplementation for 12 months did not alter the number of total bacteria or bifidobacteria in CMA-infants we used a microarray to detect subtle shifts within classes of specific bacteria. In infants receiving probiotics, the administered probiotic strains were abundantly present After 6 months of study formula, Bifidobacterium animalis was detected in 58% and Lactobacillus casei & paracasei in 22% of the infants receiving probiotics. In the placebo group the percentages were significantly lower, 5% and 4% respectively ($p<0.05$ versus controls). Next, we investigated if alterations within the composition of the microbiota that were independent of probiotic intervention would be related to the acquisition of tolerance towards cow's milk. We found that

fecal samples of infants who acquired tolerance at 6 months contained significantly increased levels of *Bacteroidetes, Prevotella*, *Enterobacteriaceae, Mollicutes, Leuconostoc (/Streptococcus), (Firmicutes), Streptococcus (/Enterococcus),* and *Ruminococcus species* (Figure 2). Analysis of the microarray data at 12 months generated comparable results.

Tabel 1 | Baseline characteristics of the placebo and probiotics group.

Characteristics	Placebo (n=34)	Probiotics (n=31)
Boys % (n)	56 (19)	55 (17)
Birth weight (SD)	3520 (422)	3470 (502)
Gestational age (SD)	40.0 (1.5)	39.5 (1.3)
Caesarean delivery % (n)	9 (3)	19 (6)
Breastfed, % (n)	74 (25)	65 (20)

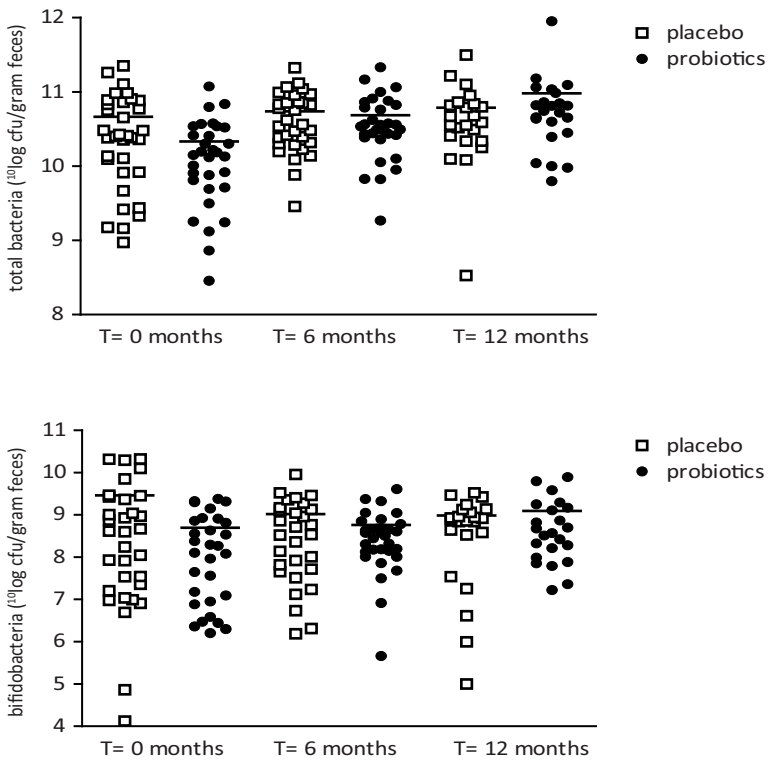

Figure 1 | qPCR data at baseline, 6 and 12 months for total bacteria (A) and bifidobacteria (B) separated in placebo and probiotic administration. Bacterial numbers are depicted as ^{10}log colony forming units per gram of feces. No differences were detected between placebo and probiotic administration.

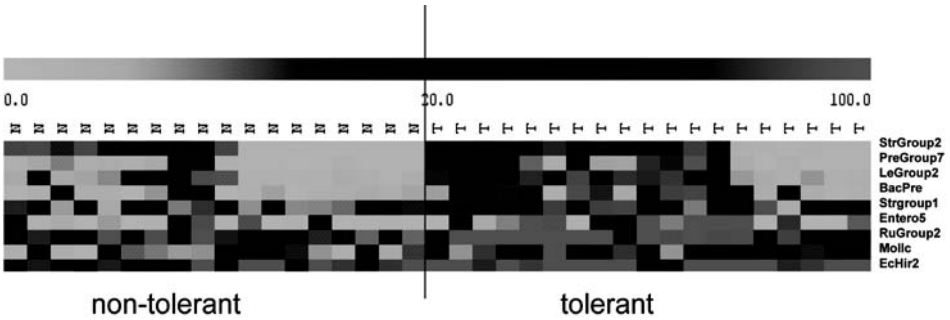

Figure 2 | Microarray analysis for 37 fecal samples obtained at 6 months. Eighteen samples were from infants still suffering from CMA (marked N), 19 samples from infants that had acquired tolerance for CM.

Only markers that were significantly different between the two groups are shown. The upper colour scale indicates a semi-quantitative value for the micro-array analysis. The hybridisation values are scaled from 0-100; of which 100 corresponds to the strongest hybridisation signal strength, indicating most dominant presence of the species or group. StrGroup2= Streptococcus species; PreGroup7= Prevotella (Bacteroides) species; Le group2= Leuconostoc (Streptococcus) species ; Bac Pre= Bacteroides/Prevotella species; StrGroup1= Streptococcus (Enterococcus) species ; Entero5= Enterobacteriaceae; RuGroup2= Ruminococcus species; Mollc= Mollicutes; EcHir2= (Firmicutes). Names mentioned in brackets refer to hits found when 1 mismatch in the sequence is allowed, other names are perfect match hits.

DISCUSSION

In this study we show that probiotic administration for 12 months in a cohort of CMA infants did not affect the numbers of specific microbial strains, but did affect the composition of specific supplemented members within the genus of *Bifidobacteria* and *Lactobacilli*. This indicates that these specific probiotic bacteria, when administered in adequate amounts, will survive the gastrointestinal tract and are detectable in fecal sampling, as was shown for other probiotic strains [6,13]. However many trials have also shown that these levels of probiotic bacteria quickly disappear after discontinuation of the treatment [14,15]. Previously, we established that probiotic intervention with CRL431 and Bb-12 did not result in enhanced acquisition of tolerance towards CM[8]. This suggests that the survival of our specific probiotic strains in the gastrointestinal tract was ineffective, and perhaps insufficient to modulate the immunesystem and lead to clinical effects. Whether variations in probiotic bacteria, dosage or composition will have beneficial effects, and if any effect will persist after cessation of administration is not clear, and remained to be studied.

We therefore investigated if alterations within the composition of the microbiota that were independent of probiotic intervention would be related to the acquisition of tolerance

towards cow's milk. We found that fecal samples of infants who acquired tolerance at 6 and 12 months contained increased levels of *Bacteroidetes, Prevotella*, *Enterobacteriaceae, Mollicutes, Leuconostoc, Streptococcus,* and *Ruminococcus species*. These data suggests that shifts within certain phyla are associated with the acquisition of tolerance towards CM. As reported earlier by the groups of Vael et al [16].and Suzuki et al [17], we also detected raised levels of *Bacteroides* correlating with tolerance acquisition. The increased *Firmicutes* in allergic infants are in line with the report of Pakarinen, who found more *Firmicutes* in the house dust atopic Russian infants [18]. To our knowledge we are the first to link the acquisition of CM-tolerance to fecal microarray data. Yet we are cautious in interpreting our data. The microarray technique used is under continuous development and the number of patients included was relatively small. Studies using larger cohorts are needed to validate our results.

In conclusion, our results suggest that the evaluation of the effect of probiotic supplementation and the incidence of allergic diseases should not be based on total bacteria counts but rather on the differentiations within certain groups of bacteria. Further studies have to elucidate the exact nature of the differences observed in this study.

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Chapter 7

Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn's disease

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ABSTRACT

Objectives: Inflammatory Bowel Diseases (IBD) represent an aberrant immune response by the mucosal immune system to luminal bacteria. Since the oral mucosa harbors the first epithelial cells that interact with microorganisms, we assessed the immunological activity of buccal epithelium in children with IBD and adults with Crohn's disease.

Methods: Buccal epithelial cells were obtained from 17 children and 14 adults with Crohn's disease, 18 children with ulcerative colitis, and 40 controls. Cells were cultured with and without microbial stimulation. Chemokine levels were determined in culture-supernatants by cytometric bead array and ELISA. CXCL-8 production was studied by immunohistochemical analysis of these cells. CXCL-8 production by lipopolysaccharide stimulated monocyte-derived dendritic cells from these patients was determined.

Results: Compared to controls, pediatric ulcerative colitis patients, and adult Crohn's disease patients, only in children with Crohn's disease buccal epithelial cells exhibited enhanced production of CXCL-8, CXCL-9, and CXCL-10. *In vitro* stimulation with lipopolysaccharide or zymosan resulted in a further increase of chemokine-levels only in cells from pediatric Crohn's disease patients. CXCL-8 production by stimulated monocyte-derived dendritic cells from children with Crohn's disease was equal to that of children with ulcerative colitis.

Conclusions: Buccal epithelium of children with Crohn's disease is immunologically active, even in the absence of oral lesions. The enhanced chemokine production is associated with pediatric Crohn's disease and seems restricted to cells derived from the epithelial barrier. Assessment of chemokine production by buccal epithelial cells may become a new, rapid, non-invasive test for screening and classification of IBD in children.

INTRODUCTION

Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis represent diseases of chronic intestinal inflammation, also called inflammatory bowel diseases (IBD). Genetic susceptibility, environmental triggers and immune dysregulation have been described as the main factors involved in the establishment and development of IBD [1]. The aberrant response of the mucosal immune system associated with IBD is thought to be directed towards microorganisms that are present within the intestinal lumen [2,3]. This hypothesis is supported by the evidence of a mutation of the bacterial sensing gene NOD2 being strongly associated with susceptibility to CD [4,5].

Intestinal epithelial cells can play a role in initiating and regulating mucosal innate and acquired immune responses through the secretion of cytokines and chemokines. In IBD patients, epithelial cells derived from colonic specimens are able to produce significant amounts of chemokines including CXCL-1 (Groα), -2 (Groβ), -5 (ENA-78), -8 (IL-8), and CCL-2 (MCP-1), -3 (MIP-1-α), -4 (MIP-1-β), -7 (MCP-3), -8 (MCP-2)[6-14]. These studies indicate that intestinal epithelial cells are an important source of chemokines that play a role in the recruitment of neutrophils and T lymphocytes to the epithelial layer, which may initiate and/or promote intestinal inflammation in IBD.

CD can be localized throughout the entire digestive tract. In up to 40% of children with CD, biopsies from the upper gastrointestinal tract may reveal granulomas, even in mucosa that appears normal on endoscopy [15,16]. Accordingly, we speculate that even in the absence of oral lesions buccal epithelial cells from children with CD may display pro-inflammatory immune responses.

We studied the chemokine production by buccal epithelial cells in pediatric IBD patients, in adult CD, as well as in healthy controls. CXCL-8 (i.e. interleukin-8) production was assessed as it represents the most commonly produced chemokine by the epithelial cells. The production of related chemokines, such as CXCL-9 (i.e. monokine induced by interferon-gamma [Mig]) and CXCL-10 (interferon-inducible protein-10 [IP-10]) was also determined. In addition CCL-2 (monocyte chemoattractant protein-1 [MCP-1]) and CCL-5 (regulated upon activation, normal T cell expressed, and secreted [Rantes]) were determined as several studies have indicated enhanced production of these molecules in colonic biopsies from IBD patients. Next to the evaluation of spontaneous production of these chemokines we also established an *in vitro* assay to determine whether these molecules could be induced by microbial stimuli. Finally, we determined the response of monocyte-derived dendritic cells (moDCs) to lipopolysaccharide (LPS). MoDCs were included as they represent a non-epithelial cell-type that is involved in mucosal microbial-host interactions [2,17,18].

MATERIALS AND METHODS

Patient characteristics

All children with (suspected) IBD that were admitted to the clinic or outpatient clinic of the department of Pediatric Gastroenterology at the Sophia Childrens Hospital from September 2003 to September 2004 were included. The included adult CD patients visited the clinic or outpatient clinic of the department of Gastroenterology at the ErasmusMC in September 2003. The study was approved by the Medical Ethical Committee of the Erasmus MC, and the Central Committee on Research Involving Human Subjects.

In children with UC, disease activity was assessed by the modified Truelove and Witts score [19]. This score is based on clinical symptoms (number of stools a day and amount of bloodloss in the stools), laboratory parameters (hemoglobin levels and erythrocyte sedimentation rate [ESR]), and physical examination (axillary temperature and pulse rate). On a scale of 6-18 points, a score of 6 indicates inactive disease, 7-10 mild disease, 11-14 moderate disease, and 15-18 severe disease. In children with CD, disease activity was expressed by means of the Paediatric Crohn's Disease Activity Index (PCDAI) [20]. The PCDAI is a disease activity index based on symptoms, laboratory parameters (haematocrit, ESR, and albumin), and physical examination, including changes in linear growth. On a scale of 0-100 points, a score <15 indicates inactive disease, 15-30 mild disease, and >30 moderate to severe disease.

Buccal epithelium

Buccal epithelial cells were collected by gently rubbing a Cytobrush® Plus (Medscand Medical AB, Sweden) over the inside of the cheeks. Cells were washed twice in RPMI 1640 (Invitrogen, Merelbeke, Belgium). In a 96-wells flat bottom plate 3,5 x 10^4 cells per well were incubated in 200 µl medium RPMI 1640 supplemented with 10% fetal calf serum (FCS; Integro, Leuvenheim, the Netherlands), hepes 15 mM, L-glutamine 2 mM, penicilline 100 U/ml, streptomycin 100 µg/ml, amphotericin B 500 µg/ml and mercaptopurine 50 µM. When more than 10,5 x 10^4 cells were obtained lipopolysaccharide (from E. Coli serotype 005:B5, Sigma-Aldrich, Zwijndrecht, the Netherlands) and/or zymosan A (from Saccharomyces Cerevisiae, Sigma-Aldrich) were added at different concentrations (as indicated), starting from February 2004. Buccal epithelial cells do not proliferate *in vitro* and have limited viability. In a pilot experiment CXCL-8 was produced already within the first hours of incubation, and reached a plateau at 8 to 12 hours. This is in agreement with another publication concerning the production of CXCL-8 by buccal epithelial cells upon microbial stimulation[6]. Therefore, we decided to collect (and store at minus 80°C) the supernatants of the cell-cultures at 24 h of incubation. The remaining cells were washed thrice with phosphate-buffered saline (PBS) and suspended in 50 µl PBS and 50 µl 10% human serum albumin (HSA). Cytospins were prepared by centrifugation (50g, 7 min) of the cell suspension. Cytospins were air-dried on silicagel overnight and either used directly or stored at minus 20°C.

Monocyte-derived dendritic cells (MoDCs)

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation of anti-coagulated human blood from IBD patients using Lymphoprep™ (Nycomed, Oslo, Norway). $CD14^+$ cells were isolated by applying MACS CD14 MicroBeads and the Magnetic cell separator MidiMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified cells were typically >95% $CD14^+$ as determined by flow cytometry. To obtain MoDCs [21], $CD14^+$ cells (5×10^5 cells/well) were cultured in RPMI 1640 with L-glutamine (Invitrogen), 10% FCS (Integro), gentamycin 56 mg/ml, recombinant human GM-CSF (molgramostim) at 500 U/ml (Novartis, Arnhem, the Netherlands) and recombinant human IL-4 at 250 U/ml (Pepro Tech, Rocky Hill, USA). At day 6 LPS was added to a subset of wells, at indicated concentrations. Supernatants were collected at day 7.

Measurement of chemokines in the cell culture supernatants

CXCL-8 levels in the cell culture supernatants were determined by ELISA according the manufacturers protocol (BD Biosciences, San Diego, USA). CXCL-9 and -10, and CCL-2 and -5 were detected by application of the standard protocol of the Cytometric Bead Array (CBA) using the human chemokine-I kit specific for these chemokines (BD Biosciences).

Immunohistochemical staining of buccal epithelial cells for CXCL-8

For immunohistochemical detection of CXCL-8 the peroxidase-labeled avidin-biotin method was used. Buccal epithelial cells on the cytospins were fixed by immersion into fresh acetone containing 0.02% (vol/vol) H_2O_2. Slides were then air-dried for 10 min. Histochemical revelation of endogenous peroxidase activity was performed with 4-chloride-1-naphtol (4-Cl-1-napthol). A solution of 80 mg 4-Cl-1-napthol in 1 ml of ethanol 100% was added to 200 ml Tris-HCL buffer together with 6.2 µl 30% H_2O_2. This solution was filtered. Slides were immersed into this solution for 15 min at RT. Slides were washed with phosphate-buffered saline (PBS) 1x1 min and with PBS/Tween20 0.05% 1x10 min. Subsequently cells were incubated with the primary mouse antihuman CXCL-8 antibody (BD Biosciences) overnight at 4°C in a humidified atmosphere, or with the irrelevant isotype-match antibody (IgG2b; Dako, Glostrup, Denmark). The next morning, cells were incubated with the secondary biotinylated rabbit anti-mouse antibody (Dako) for 30 min at RT. Between incubation steps slides were washed twice with PBS/Tween20 0.05%. Subsequently cells were incubated with avidin-biotin compex labeled horseradish peroxidase-conjugated (ABC complex HRP; Dako) for 1 h at RT. Slides were washed twice in PBS. The peroxidase activity was revealed by incubation with 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich) for 10 min at RT, leading to a bright red precipitate. Again slides were washed twice with PBS. Finally cells were counterstained using Mayer's haematoxylin (Merck) and embedded in glycerol gelatin. The same method was used for detection of CD45 (using a primary mouse anti-human CD45 antibody; BD Biosciences).

Statistical analysis In order to obtain approximate normal distributions patient groups were compared using the t-test after logarithmic transformation. Outcomes less than the lower limit of detection were set at this limit, and the resulting value was analyzed as a left-censored observation using STATA software (proc CNREG). Paired data (LPS 0,01 versus LPS 0,1 mcg/ml) were compared using the Wilcoxon signed-rank test. Correlation coefficients given are Spearman's (r_s). P = 0,05 (two-sided) was considered the limit of significance.

RESULTS

Patient characteristics and yield of buccal epithelial cells September 2004 thirty-five children with IBD were included, as well as 14 adult CD patients and 40 controls (children and adults). Patient demographics and disease characteristics are shown in table 1. In IBD patients as well as in adult CD patients disease activity ranged from mild to severe. Six children with CD and 6 children with UC were newly diagnosed with IBD and had not received anti-inflammatory medication yet. None of the adult CD patients was newly diagnosed (they were all tertiary admitted). Treatment strategies of the IBD patients, children as well as adults, were very different. Five adolescents stopped their medication without consulting the pediatric gastro-enterologist. Other patients used 5-ASA, prednisone, azathioprine, methotrexate, anti-tumor necrosis factor, or a combination of these. Most of the controls (children and adults) were healthy. Others were admitted under the suspicion of IBD, but turned out not to have IBD.

Table 1 | Patient characteristics and patient demographics

	Pediatric UC	**Pediatric CD**	**Adult CD**	**Controls**
Number	18	17	14	40
Male / female	10 / 8	10 / 7	4 / 10	18 / 22
Age at diagnosis (in years)	1.5-15 (median 11)	1.2-16 (median 10)	>18 years	n.a.
Age at study entry (in years)	4-17 (median 13)	1.7–17 (median 12)	29-63 (median 42)	1-50 (median 17)
Newly diagnosed at study entry	6 / 18	6 / 17	0 / 14	n.a.
PCDAI for children	n.a.	7,5-55 (median 24)	n.a.	n.a.
CDAI for adults	n.a.	n.a.	47-325 (median 164)	n.a.
Modified Truelove-Witts score	7–16 (median 10)	n.a.	n.a.	n.a.

n.a. = not applicable

The median yield of buccal epithelial cells for the pediatric CD patient was 30 x 10^4 cells per patient (ranging from 3.5 to 81 x 10^4 cells per patient). The median yield of buccal epithelial cells for the pediatric UC patient was comparable (24 x 10^4 cells per patient, ranging from 3.5 to 85.6 x 10^4 cells per patient). The yield of buccal epithelial cells for the adult CD patients and the controls was also within the same range.

CXCL-8 production by buccal epithelial cells is exclusively enhanced in children with Crohn's disease Figure 1 shows the levels of CXCL-8 in the culture supernatants of buccal epithelial cells. In children with CD, CXCL-8 production was significantly higher than in children with UC (p=0.001), or controls (p<0.001). Four out of 6 children with CD were newly diagnosed, had a moderate to severe disease activity and were not receiving medication yet. These four patients presented with a high CXCL-8 production (of more than 300 pg/ml). CXCL-8 production by buccal epithelial cells derived from adult CD patients was comparable to that of controls. In children with CD the CXCL-8 production by buccal epithelial cells was correlated with the erythrocyte sedimentation rate (r_s=0.61; p=0.016) as is shown in Figure 2. In these children, CXCL-8 production did not correlate with the C-reactive protein (r_s=-0.13; ns), the hemoglobin (r_s=-0.37; ns), the thrombocytes (r_s=-0.06; ns), the leukocytes (r_s=-0.10; ns), or the albumin (r_s=-0.42; ns) in the blood (data not shown). Finally, the CXCL-8 production was not related to clinical disease activity expressed as PCDAI (r_s=0.33; ns)(data not shown).

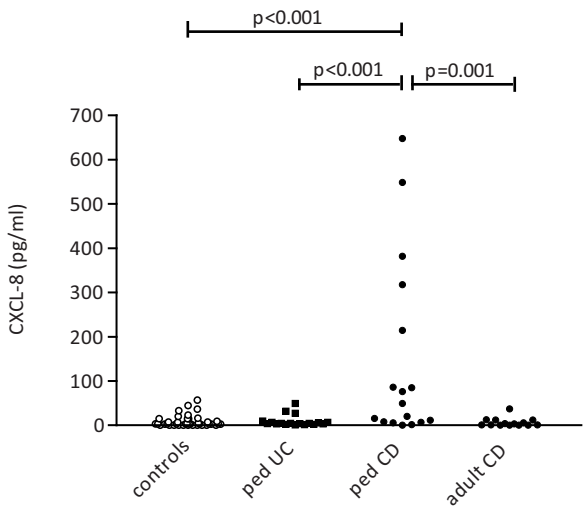

Figure 1 | CXCL-8 is exclusively enhanced in pediatric CD patients. CXCL-8 levels in the culture-supernatants of buccal epithelial cells of controls (adults and children), children with ulcerative colitis (ped UC), children with Crohn's disease (ped CD), and adults with Crohn's disease (adult CD). Samples were taken at 24 h of incubation. In children with CD, CXCL-8 production was significantly enhanced compared to children with UC (p<0.001) and controls (p<0.001). Adults with CD did not show enhanced CXCL-8 production.

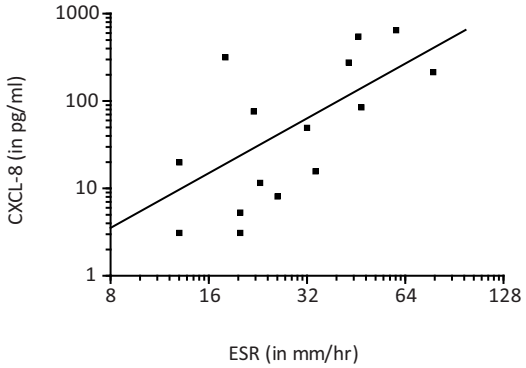

Figure 2 | In children with CD, CXCL-8 production by buccal epithelium correlates with ESR. CXCL-8 levels in the culture-supernatant of buccal epithelial cells derived from children with CD correlate with the erythrocyte sedimentation rate in the blood of these patients (r_s=0.61; p=0.016). The line represents least-squares regression line after logarithmic transformation of both axes.

In order to conclusively identify the epithelial cell as the source for the CXCL-8, we performed immunohistochemical analysis of these cells. Figure 3 shows representative examples of CXCL-8 production by buccal epithelial cells. CXCL-8 was particularly detected at the periphery of the cells, and was located in granules. As shown, buccal epithelial cells were not contaminated with cells positive for the haematopoietic marker CD45 (i.e. monocytic cells or macrophages).

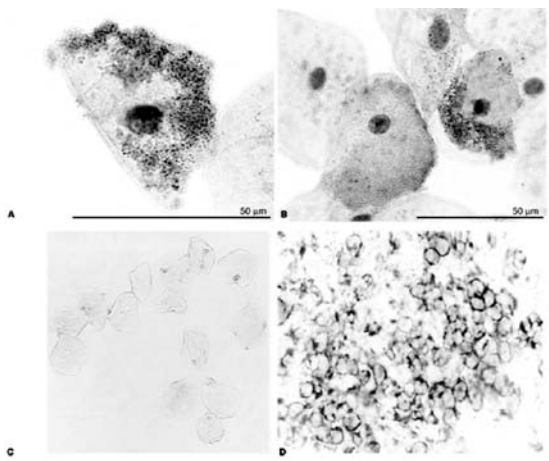

Figure 3 | Representative examples of CXCL-8 production by buccal epithelial cells are shown. Immunohistochemical analysis for CXCL-8 was performed on cytospins of buccal epithelial cells. In the examples above (Figure 3A and 3B) buccal epithelial cells of a child with CD were identified as the source for CXCL-8. The orange-red pigment indicates positive CXCL-8 staining. In contrast to bone marrow cells, positive for CD45 (i.e. the red staining, Figure 3D), buccal epithelial cells were all negative for the haematopoietic marker CD45 (Figure 3C) which excludes contamination with monocytic cells or macrophages.

Enhanced production of other chemokines by buccal epithelial cells from pediatric CD patients

Next to CXCL-8, the levels of CXCL-9, CXCL-10, CCL-2 and CCL-5 were determined in the culture-supernatant of the buccal epithelial cells at 24 hours of incubation by using cytometric bead array. Figure 4 shows that in children with CD, CXCL-9 production was significantly higher compared to children with UC (p=0,008). In children with CD, CXCL-10 production was also higher compared to children with UC (p=0,024). The production of CCL-2 and CCL-5 were equally low in both patient groups.

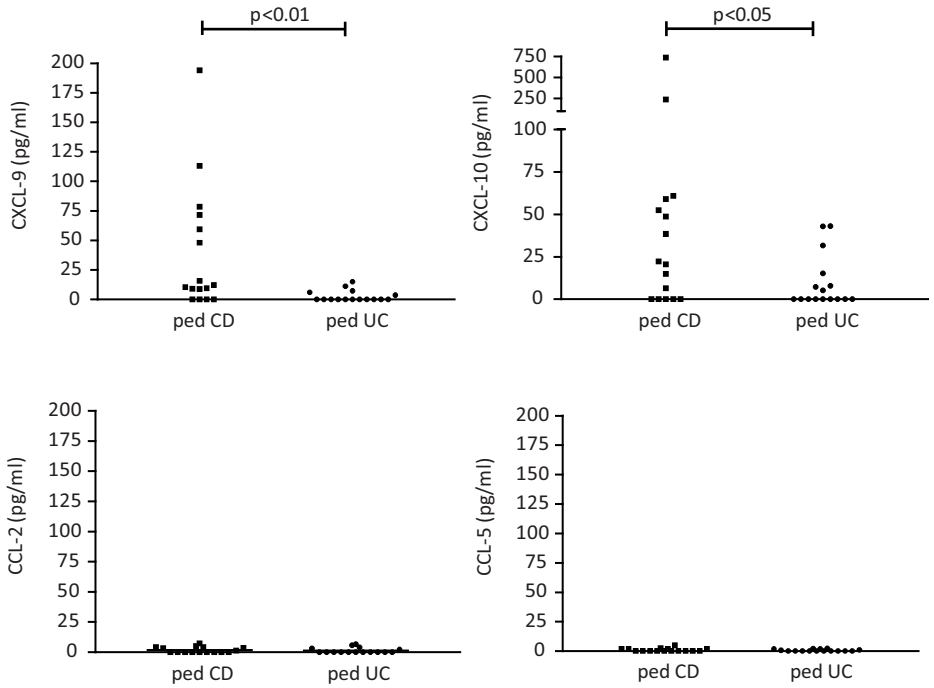

Figure 4 | Levels of CXCL-9, CXCL-10, CCL-2 and CCL-5 in the culture-supernatants of buccal epithelial cells of children with Crohn's disease (ped CD) and ulcerative colitis (ped UC). Samples were taken at 24 h of incubation. CXCL-9 and CXCL-10 production was significantly enhanced in CD patients compared to UC patients (p<0.01 and <0.05 respectively). CCL-2 and CCL-5 levels were very low in both CD and UC patients.

Stimulation of buccal epithelial cells with LPS or zymosan

In order to determine whether the buccal epithelium of pediatric CD patients produced more CXCL-8 as a result of a lower threshold for microbial stimulation we next performed an *in vitro* stimulation assay. In this assay, buccal epithelial cells were cultured in the presence of LPS or

zymosan at different concentrations. In 4 out of 10 children with CD the production of CXCL-8, CXCL-9 and/or CXCL-10 increased more than 50 pg/ml. Figure 5 shows a representative example of enhanced chemokine production upon microbial stimulation. No induction (either by LPS or zymosan) of chemokine production (CXCL-8, CXCL-9, CXCL-10, CCL-2 and/or CCL-5) was found in any of the buccal epithelial cells that were derived either from children with UC (n=10), or in cells from healthy controls (n=20).

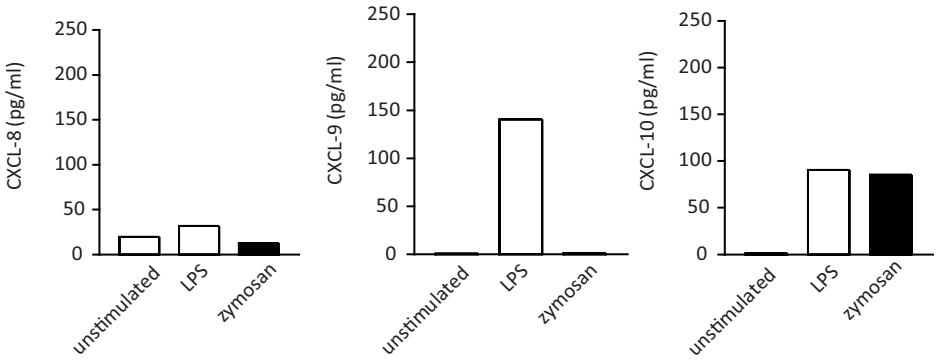

Figure 5 | Representavive example of inducible chemokine production by buccal epithelial cells from pediatric CD patients. Cells were stimulated with lipopolysaccharide (LPS) at 0.1 mcg/ml or zymosan at 100 mcg/ml. At 24h of incubation chemokine production was determined in the culture-supernatants.

The response of monocyte-derived dendritic cells to lipopolysaccharide

Next we determined whether the enhanced chemokine production by buccal epithelium demonstrated in pediatric CD could be extended to other immunocompetent non-epithelial cells. As such, CXCL-8 production by moDCs in response to LPS was measured in pediatric IBD patients. MoDCs were derived from children with CD and UC and stimulated with different concentrations of LPS as described. As is illustrated in Figure 6, CXCL-8 production in response to LPS 0.1 mcg/ml generally was higher than in response to LPS 0.01 mcg/ml ($p=0.004$ for CD; $p=0.04$ for UC). In response to LPS 0.01 mcg/ml moDCs of children with CD produced the same amounts of CXCL-8 as those of children with UC ($p=0.64$; ns). In response to LPS 0.1mcg/ml moDCs of children with CD also produced comparable amounts of CXCL-8 compared to that of children with UC ($p=0.89$; ns). In conclusion, in response to LPS, moDCs of children with CD did produce comparable amounts of CXCL-8 as did moDCs of children with UC.

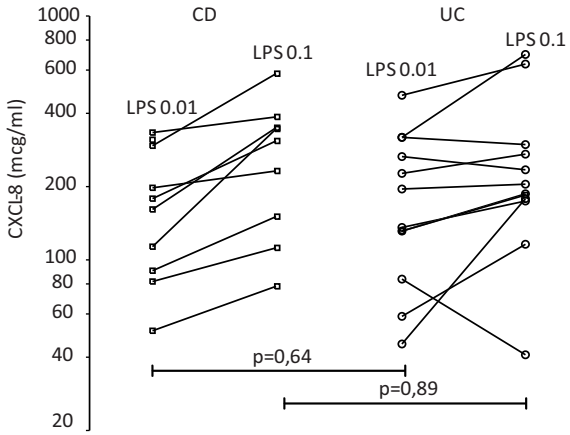

Figure 6 | CXCL-8 production by stimulated monocyte-derived dendritic cells in children with CD is comparable to that of children with UC.

Levels of CXCL-8 in the culture-supernatant of monocyte-derived DCs (moDCs) of children with CD (n=10) and UC (n=13) are compared. CXCL-8 production in response to LPS 0.1 mcg/ml generally was higher than in response to LPS 0.01 mcg/ml (p=0.004 for CD; p=0.04 for UC). In response to LPS 0.01 mcg/ml moDCs of children with CD produced the same amounts of CXCL-8 as those of children with UC (p=0.64; ns). In response to LPS 0.1mcg/ml moDCs of children with CD also produced comparable amounts of CXCL-8 compared to that of children with UC (p=0.89; ns).

DISCUSSION

One hypothesis on the etiology of IBD is that these diseases represent an aberrant immune response by the mucosal immune system, to either pathogenic or resident luminal bacteria. According to this hypothesis, resident bacteria can persistently stimulate the mucosal and systemic immune system, thereby perpetuating the inflammatory response. Intestinal epithelial cells (IECs) and dendritic cells are among the first cells that are capable of sensing microbial signals through the expression of pattern recognition molecules such as the Toll-like receptors. Through the presentation of antigens and the production of chemokines and cytokines, IECs and DCs are involved in the initiation and regulation of the acquired immune response. Several studies have identified the IECs as a major source of chemokines that play an important role in chemotaxis, adhesion, activation and degranulation of migratory immune cells. In response to microorganisms or pro-inflammatory cytokines, IECs are capable of producing CXCL-1, -3 (GROγ), -5, -8, -9, -10, and -11 (I-TAC) as well as CCL2, -3, -4, and -5 [6,22-27]. In freshly obtained specimens from IBD patients, epithelial cells of the lower GI tract showed an increased expression of CXCL-1, -5, and –8, as well as CCL-2, -3, -4, -7 and -8 [6-14]. These studies used in situ hybridisation

with radiolabelled probes of the chemokine-genes, immunohistochemical analyses, and/or specific protocols for the isolation of intestinal epithelial cells. Importantly, other studies that made use of the same techniques failed to demonstrate such chemokine production by colonic epithelial cells *in vivo* [28-31]. Although buccal epithelial cells seem to be an obvious subject for experimentation as these cells are so readily available without the need for endoscopy or biopsies, to our knowledge this is the first functional study on these cells in IBD patients. In fact, chemokine production or expression by buccal epithelial cells in these patients has never been studied before.

Here we demonstrate that in children with CD, buccal epithelial cells produce significantly higher levels of CXCL-8, CXCL-9 and CXCL-10, in comparison to children with UC, to controls or to adults with CD. Interestingly, not all newly diagnosed children with CD presented with an enhanced chemokine production by buccal epithelial cells. Also, no relation was found between the disease activity (pCDAI) or a specific drug that was used and chemokine production. These findings may suggest that the enhanced chemokine production is specifically associated with a subset of patients with a specific (genetic) ethio-pathogenesis.

In children with CD, the production of CXCL-8 by buccal epithelial cells was correlated with the ESR in the blood. No such correlation was found in children with UC despite the fact that the range of ESR in these children was comparable to that of the children with CD. The correlation between ESR and CXCL-8 production in CD patients may represent a phenomenon that is associated only with pediatric CD, and not with pediatric UC. As an enhanced ESR is found in active pediatric CD as well as in active pediatric UC, CXCL-8 production by buccal epithelial cells seems the better test to discriminate between the two diseases.

Upon stimulation with LPS or zymosan, only buccal epithelial cells derived from pediatric CD patients show an inducible production of chemokines. Based on our preliminary data (11 out of 17 children with CD had an enhanced release of CXCL-8, CXCL-9 and/or CXCL-10 spontaneously, and 4 out of 10 children with CD had an increased release upon microbial stimulation), we estimate that over 72% of all children with CD will exhibit an enhanced chemokine production, spontaneous or upon microbial stimulation. These striking differences in response patterns by buccal epithelial cells can be explained by various mechanisms. Alterations in the local (oral) flora of pediatric CD patients may be associated with an enhanced chemokine production. The fact that chemokine production by microbial stimuli could only be induced in pediatric CD patients points to an enhanced ability of these cells to become stimulated. As we could not show the same differences in moDCs, this mechanism seems to be limited to epithelial cells of pediatric CD patients. The enhanced chemokine production by buccal epithelial cells from pediatric CD patients may be associated with mutations in the NOD2 molecule, such as described [32]. A recent paper by Watanabe et al [33] elucidated how signaling through mutated NOD2/CARD15 molecules may lead to disease by causing an excessive T_H1 response. The authors present a model whereby NOD2 senses muramyl dipeptide (a breakdown product of peptidoglycan) within

the cell which leads to a blockade of TLR2 signaling upon activation by peptidoglycan at the cell surface. This may in fact represent a physiological mechanism through which the inflammatory response to gut flora is limited. Mutant NOD2 (in Crohn's disease) will not sense MDP and will be associated with unopposed TLR2 signaling, which leads to enhanced IL-12 production, one of the essential mediators of intestinal inflammation in IBD. Another approach may be that the expression of molecules such as TLR2 and 4 is specifically enhanced in the epithelial cells of pediatric CD patients as is suggested by various authors [34-36]. Finally, the results may also be explained by alterations in the expression of molecules such a TOLLIP. Recently it was reported that these types of molecules might contribute to a state of hypo-responsiveness of epithelial cells to microbial stimuli [37-39].

Finally, we found a striking difference in epithelial chemokine-response patterns by comparing pediatric to adult IBD patients. None of the adult CD patients in this study were diagnosed in childhood. The question whether pediatric CD patients will also exhibit this enhanced chemokine production into adulthood remains to be elucidated. Interestingly, recent reports suggest that, in comparison to adults, pediatric CD may represent a distinct disease that may be associated with an enhanced incidence of NOD2/CARD 15 mutations [40,41].

A high production of CXCL-8 or CXCL-9 by the buccal epithelium, either spontaneously or upon microbial stimulation, increases the suspicion of CD in non-diagnosed children. In children with indeterminate colitis an enhanced production of these chemokines may contribute to further discrimination. Determining the chemokine production by these cells can be of great value in making the correct diagnosis, and in deciding on a specific treatment modality. Enhanced chemokine production by buccal epithelial cells may well provide us with the first soluble marker that is exclusively linked to pediatric CD, and may therefore become a new, rapid, and non-invasive test in children with (suspected) IBD.

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Chapter 8

Human primary buccal epithelium acquires microbial hyporesponsiveness at birth, a process involving increased secretory leukocyte protease inhibitor (SLPI) expression

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ABSTRACT

Background: The oral cavity is rapidly colonized upon birth followed by continuous exposure of the mucosal epithelium to harmless microbial stimuli. Previously, we have reported that in healthy individuals, buccal epithelial cells are unresponsive to microbial stimulation.

Objective: The aim of this study was to determine whether interaction with microbial stimuli determines that primary buccal epithelial cells from healthy individuals become hyporesponsive to TLR stimulation.

Methods: Buccal epithelial cells collected directly after birth and in later stages of life were investigated. Spontaneous chemokine release and enhanced release after microbial stimulation were used as a read-out for cellular activation. Regulatory signaling pathways were studied using primary buccal epithelial cells and the colonic epithelial cell line Caco-2.

Results: Primary neonatal buccal epithelial cells spontaneously produced CXCL-8 and were highly responsive to microbial stimuli. Within the first weeks of life these epithelial cells readily attained a state of hyporesponsiveness that was associated with acquisition of sustained levels of IκBα while activated neonatal buccal epithelial cells displayed degradation of IκB. Modelling of microbially induced hyporesponsiveness using Caco-2 cells showed that microbial encounter elicited expression of the NF-κB inhibitor secretory leukocyte protease inhibitor (SLPI). Similarly, hyporesponsive adult epithelium exhibited SLPI mRNA and nuclear expression of SLPI whereas activated neonatal buccal cells did not express nuclear SLPI.

Conclusion: We show that human primary buccal epithelium acquires microbial hypo/responsiveness soon after birth, accompanied by decreased degradation of IκB and accumulation of the regulatory protein SLPI in the nucleus.

INTRODUCTION

Mucosal epithelial cells are the first cells that interact with microbial flora. These cells are equipped with pattern recognition receptors (PRR), such as Toll-like (TLR) [1,2] and NOD receptors [3,4] that incite cellular activation after ligation of defined bacterial structures. In healthy individuals the interaction with commensal intestinal flora does not lead to an inflammatory response. However, upon encounter of harmful bacteria PRR convey the proinflammatory immune responses that are required to eradicate these pathogens [5]. The mechanisms that account for this tailored hyporesponsiveness of epithelial cells are a topic of extensive research [6]. Using murine models it has recently been shown that epithelial cells of neonatal mice become hyporesponsive to TLR stimuli immediately after birth [7,8]. The acquisition of this hyporesponsiveness was dependent on a transient cellular activation induced by contact with exogenous TLR ligands [7]. Transient epithelial stimulation was found to induce a negative regulatory mechanism through depleting IL-1 receptor associated kinase-1 (IRAK-1) which rendered the cells unresponsive to subsequent stimulation [7,8]. Acquisition of microbial hyporesponsiveness is seen in several innate cell types and can be regulated via multiple mechanisms. Functional experiments with epithelial- and monocytic cells have shown that PRR signaling can be controlled by reducing the surface expression of the receptors through ubiquitination and degradation or inhibition of mRNA synthesis [9-12]. In addition, a network of intracellular negative regulators can limit PRR mediated over-activation. Amongst these IL1 receptor-associated kinase M (IRAK-M) [13], Nucleotide-binding Oligomerization Domain containing 2 (NOD2)[14], Tollip [12,15], A20 [16], Single Immunoglobulin Interleukin-1 Receptor-related protein (SIGIRR) [17,18] and SLPI [19] have been found to inhibit intracellular signal transduction at various stages during the signaling cascade; ultimately leading to reduced NF-κB activation and inflammatory gene expression. In particular, SLPI is a pleiotropic inhibitor which can inhibit the NF-κB pathway directly in the nucleus by binding an NF-κB consensus sequence in the promoter region of the CXCL-8 gene.

Currently, it is unclear how human primary epithelial cells acquire microbial hypo/responsiveness. This is of particular interest as in patients with Crohn's disease and Ulcerative Colitis aberrant epithelial responses to commensal microbial flora contribute to chronic inflammation of the gastrointestinal tract [20-23]. Recently, we have observed that buccal epithelial cells from pediatric Crohn's Disease patients spontaneously released increased amounts of CXCL-8, CXCL-9, and CXCL-10 when compared to epithelium from healthy controls and children with Ulcerative Colitis [24]. Stimulation with bacterial products resulted in a further increase of chemokine production by buccal epithelial cells from Pediatric Crohn's disease patients whereas the epithelium of healthy controls and Ulcerative Colitis patients remained hyporesponsive to TLR stimulation. These data reveal that primary buccal epithelial cells are a valuable tool to study acquisition of hyporesponsiveness in human primary epithelium and may help identify dysregulation of this mechanism in Crohn's disease.

The aim of this study was to address how primary buccal epithelial cells from healthy individuals become hyporesponsive to TLR stimulation. It is likely that the transition from the sterile *in utero* environment to continuous contact with microbial products after birth is a pivotal event for shaping epithelial responses. Therefore, we investigated the chemokine release and microbial responsiveness of buccal epithelial cells directly after birth and later, and examined the role of SLPI in the development of hyporesponsiveness.

MATERIALS AND METHODS

Reagents

Phosphate-buffered saline (PBS) was used to collect buccal epithelial cells and wash the cells. Buccal epithelial cells were cultured in RPMI 1640 medium (Gibco/Invitrogen Ltd, Carlsbad, California, United States of America) supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), HEPES 15mM, L-glutamine 2mM, Sodium-penicillin-G 100U/ml, streptomycin 100µg/ml, amphotericin B 500µg/ml and β-mercaptoethanol 50µM (Gibco/Invitrogen). For in vitro experiments the Caco-2 cell line (Human, Caucasian, colon, adenocarcinoma ATCC number HTB-37) was obtained and cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Integro) 15mM HEPES, 2mM L-glutamine, sodium-penicillin G (10,000IU/ml) and streptomycin (10,000UG/ml; Gibco/Invitrogen). For stimulation assays DMEM supplemented with 1% non-essential amino acids (Gibco/Invitrogen) and 0.1% penicillin / streptomycin (10,000IU/ml/10,000UG/ml; Gibco/Invitrogen) was used.

Cells were stimulated with Lipopolysaccharide (LPS, 1µg/ml Escherichia Coli serotype 005:B5, Sigma-Aldrich, Zwijndrecht, The Netherlands), Peptidoglycan (PG, 10µg/ml, Bacillus subtilis, Sigma-Aldrich), Pam3Cys (10µg/ml, EMC Microcollections, Tuebingen, Germany) and IL-1β (1µg/ml = high dose or 0.1µg/ml = low dose, Sigma-Aldrich). Phorbol myristate acetate (PMA, 0.05µg/ml, Sigma-Aldrich). Amniotic fluid was obtained with informed consent from a single donor at 26 weeks gestational age.

Buccal epithelium cell collection

Buccal epithelial cells were collected from both at term neonates and controls (ages indicated in figure legends). For neonates the cells were obtained within 10 minutes after birth. The buccal epithelial cells were collected by gently rubbing the inside of the cheeks with a Cytobrush® Plus (CooperSurgical Inc. Trumbull, CT). Buccal epithelial cell suspensions were washed twice in PBS and centrifuged for 5 minutes at 485g. Buccal epithelial cell numbers, viability and the presence of blood contamination were determined by counting with trypan blue in a Bürker counting

chamber. Buccal epithelial cells contaminated with blood were excluded from further analyses. Written informed consent was obtained from each donor or when applicable from the parents.

LPS and PG stimulation of buccal epithelial cells

For culture, 3.5 x 10^4 buccal epithelial cells from neonates or controls were seeded per well of a 96-wells flat bottom plate (Corning B.V. Life Sciences, Amsterdam, The Netherlands) and incubated in 200µl of medium per well. Buccal epithelial cells were left untreated or were stimulated with either LPS (1µg/ml) or PG (10µg/ml) for 24 hours. After 24 hours supernatants of the cell-cultures were collected for ELISA. The remaining buccal epithelial cells were divided into two aliquots which were used for cytospin analysis or intracellular protein analysis respectively. To obtain cytospins, cells were washed three times with PBS and resuspended in 50µl PBS and 50µl 10% human serum albumin. Cytospins were prepared by spinning the cells at 50g, for 7 minutes onto microscope slides, after which the slides were air-dried for 18 hours on silicagel. The slides were then either used directly for immunohistochemical staining or stored at -20°C.

Amniotic fluid stimulation of buccal epithelial cells

Buccal epithelial cells were either stimulated with 200 µl of amniotic fluid for 6 hours or left untreated. After 6 hours cells were washed twice. The buccal epithelial cells were subsequently incubated for 24 hours in 200µl medium without stimulus or containing Pam3Cys (10µg/ml). After 24 hours supernatants of the cell-cultures were collected for ELISA.

Whole cell, nuclear fraction and cytosolar fraction lysates of buccal epithelial cells

Buccal epithelial cells were harvested and lysed directly after stimulation assays using the protocol from BD Biosciences. Briefly, the cells were spun for 5 minutes at 450g and washed twice in ice cold PBS, after which the cell pellet was dissolved in the extraction buffer and incubated on ice for 30 minutes. Whole cell extraction buffer consisted of 20mM Hepes, 20% glycerol, 1% Nonidet P40, 1mM $MgCl_2$, 1mM Phenylmethylsulfonylfluoride, 0.5mM EDTA, 0.1mM Dithiothreitol and 15µl protease inhibitor cocktail (Aprotinin, Pepstatin and Leupeptin). Subsequently the mixture was spun for 20 minutes at 20,000g, after which the supernatant was collected into a clean tube. Cellular lysates were stored at -80°C. To extract cytosolar and nuclear fractions the cell suspension was centrifuged at 420g, at 4°C for 5 minutes, and the supernatant was discarded. Next, the mixture was incubated on ice for 15 minutes in lysis buffer, consisting of 100mM Hepes, 15mM $MgCl_2$, 100mM KCl, 0.1mM Dithiothreitol, 1mM Phenylmethylsulfonylfluoride and 15µl protease inhibitor cocktail (Aprotinin, Pepstatin and Leupeptin). Subsequently, the sample was centrifuged at 420g, at 4°C for 5 minutes and the supernatant was discarded. The pellet was resuspended in lysis buffer and the further dissociated by rapid strokes of a 27 gauge needle and syringe, after which the mixture was centrifuged for 20 minutes at 4°C, at 11,000g. The

supernatant containing the cytosolar fraction was collected and stored at -80°C. The remaining pellet was taken up in extraction buffer, consisting of 20mM Hepes, 1.5mM $MgCl_2$, 0.42mM NaCl, 0.2mM EDTA, 25% glycerol, 0.1mM Dithiothreitol, 1mM Phenylmethylsulfonylfluoride and 15µl protease inhibitor cocktail (Aprotinin, Pepstatin and Leupeptin). As described in the cytosolic protein extraction procedure, a 27 gauge needle and syringe was used to disrupt the cell nuclei after which the mixture was incubated on ice for 30 minutes. The disrupted nuclei were then centrifuged at 21,000g for 5 minutes, after which the supernatant containing the nuclear protein fraction was collected and stored at -80°C.

Desensitization of Caco-2 cells

Caco-2 cells were used at passages ranging from 30-46 and cultured in 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cells were seeded at a density of $1x10^5$ cells/ml in a 24 well plate (Corning B.V. Life Sciences) and cultured 3-5 days, changing the culture medium every 2nd day until confluency was reached.

Caco-2 cells ($1x10^5$ cells/ml) were stimulated with Pam3Cys (10µg/ml) for 24 hours or left untreated after which supernatant was collected for ELISA. For restimulation, the cells were washed and subsequently were stimulated with Pam3Cys (10µg/ml) or IL-1β (1µg/ml) or left untreated and incubated for another 24 hours after which the supernatant was collected for ELISA.

Examining NF-κB activation in desensitized Caco-2 cells

Caco-2 cells ($1x10^5$ cells/ml) were stimulated with Pam3Cys (10µg/ml) for 24 hours or left untreated. Supernatant was collected for ELISA and some cells were collected for analysis of intracellular protein. The remainder of the cells was restimulated with Pam3Cys for 5, 15, 30 or 45 minutes and subsequently collected, after which cells were harvested and their whole cell lysate was analyzed.

ELISA

Quantification of CXCL-8 (BD Biosciences, Mountain View, CA) was performed by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol. For quantification of human SLPI, monoclonal anti-human SLPI capture antibody, (R&D biosystems, Abingdon, UK MAB1274, clone 20409) was coated on a 96-wells flat bottom plate (Corning B.V. Life Sciences) over night. The wells were then washed three times with PBS/Tween 0.05% and subsequently blocked with PBS/FCS 10% for one hour and washed again three times with PBS/ Tween 0.05%. The plate was subsequently incubated with 50ml of supernatant for 2 hours and washed five times with PBS/Tween 0.05%. Polyclonal goat anti-human SLPI (R&D biosystems, BAF1274) was used as detection antibody and the plate was incubated with 50ml of the detection antibody for 1 hour. This incubation was followed by 5 wash steps with PBS/ Tween 0.05%, after which

the plate was incubated for 1 hour with 50ml Peroxidase-conjugated streptavidin in PBS/FCS 10% and washed another 5 times. Next, wells were incubated for 20 minutes with 50ml tetramethylbenzidine. Finally, the reaction was stopped with 50ml of 1M H_3PO_4 and the plate was read at 450/570 using a nanometer.

Immunoblotting

Protein from whole cell lysates was separated by SDS-PAGE and transferred to a Millipore Immobilon-P Transfer Membrane (a PVDF membrane; Polyvinylidene fluoride) membrane. Western blots were conducted for I kappa B alpha (IκBa) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), SLPI (R&D biosystems) and β-actin (R&D biosystems) and developed with the appropriate horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (GE Healthcare Europe GmbH, Diegem Belgium). IκBα and β-actin levels in whole cell lysates were determined by densitometry using a Syngene resolution imaging system to obtain an image of the blot and the GeneTools program for densitometry analysis. IκBα levels determined by densitometry were corrected for β-actin and noted as aribitary unitis (AU).

Immunohistochemistry

Immunohistochemical detection of CXCL-8 was performed as previously described by Damen et al [13]. In short, the peroxidase-labeled avidin-biotin method was used. Cytospins of buccal epithelials cells were incubated overnight at 4°C with the primary mouse antihuman CXCL-8 antibody (BD Biosciences) or with the irrelevant isotype-matched antibody (IgG2b; Dako, Glostrup, Denmark) in a humidified atmosphere. The next day, cells were incubated with the secondary biotinylated rabbit anti-mouse antibody (Dako) for 30 minutes at room temperature. Subsequently, cells were incubated with the avidin and biotin complex labeled horseradish peroxidase-conjugated (Dako) for 1 hour at room temperature. For detection of CXCL-8, a peroxidase-labeled DAB (3,3-diaminobenzidine) method was used (Vector Laboratories, Peterborough, United Kingdom).

RT-PCR analysis

Total RNA from buccal epithelial cells was isolated using the Nucleospin RNA XS kit (Machery-Nagel, Düren, Germany) and following the manufacturer's protocol. RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5mM) and oligo dT primers (20nM). The reverse transcription reaction was performed in a total volume of 25ml containing 0.2mM of each dNTP (Amersham Pharmacia BioTech Piscataway, NJ), 200U Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT;, M3683 Promega, Madison, WI), and 25U RNAsin (Promega). The reverse transcription reaction was performed at 37°C for 45 minutes, 42°C for 15 minutes, and 94°C for 5 minutes. Real-time quantitative PCR was performed using an ABI PrismR 7900 Sequence Detection System (Applied Biosystems, CA) based on specific primers and

general fluorescence detection with SYBR green. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for sample loading and to allow normalization between samples. The expression levels relative to GAPDH were calculated using the following equation: relative expression level = $2^{-\Delta Ct}$, where ΔCt = Ct target – Ct housekeeping. Specific primers were designed across different exons resulting in the following primers:
GAPDH: Fw: 5'-GTCGGAGTCAACGGATT-3', Rv: 5'-AAGCTTCCCGTTCTCAG-3'
SLPI: Fw: 5'-TCCAGGGAAGAAGAGATGT-3', Rv: 5'-TGCCCATGCAACACTT-3'

Statistical analysis
Was performed using Independent sample T-test, Paired sample T-test and one-way and two way ANOVA as indicated in figure legends. P values <0.05 were considered statistically significant.

RESULTS

Neonatal buccal epithelial cells spontaneously produce CXCL-8 and are highly responsive to microbial stimuli

We have collected buccal epithelial cells from 26 newborns within 10 minutes after birth and compared their activation status with epithelial cells from 26 controls aged 2-6 months. Neonatal buccal epithelial cells spontaneously released substantial amounts of CXCL-8 when compared to epithelial cells from controls (Figure 1A). Buccal epithelial cells actively responded to bacterial stimulation as demonstrated by the additional increase in chemokine release upon incubation with LPS or PG (Figure 1A). This responsiveness was restricted to neonatal buccal epithelial cells since further investigation showed that buccal epithelial cells from adult individuals (age 29-55) were also unresponsive to bacterial stimulation (data not shown).

To investigate whether epithelial activation found in neonatal buccal epithelial cells could be a remnant of stimulation of these cells by the amniotic fluid [25] we assessed if amniotic fluid could induce CXCL-8 production in buccal epithelial cells from adult subjects (age 29-55). Neither incubation of adult buccal epithelial cells with amniotic fluid for 6 hours, followed by 24 hour incubation without stimuli nor 6 hour incubation with amniotic fluid to prime sensitivity to a subsequent stimulation with Pam3Cys evoked CXCL-8 production (Figure 1B).

Vaginal birth is likely to be associated with the epithelium coming into contact with a higher number and a larger variety of microbial stimuli than birth by Caesarian section. Therefore, the effect of the method of delivery on epithelial responsiveness was assessed. Spontaneous CXCL-8 release by buccal epithelial cells from neonates delivered through vaginal birth was higher than that from neonates delivered by caesarean section (Figure 1C). Immunohistochemical staining was performed to confirm that CXCL-8 was derived from epithelial cells. Cytoplasmic staining indicated cytokine production by these cells (Figure 1D).

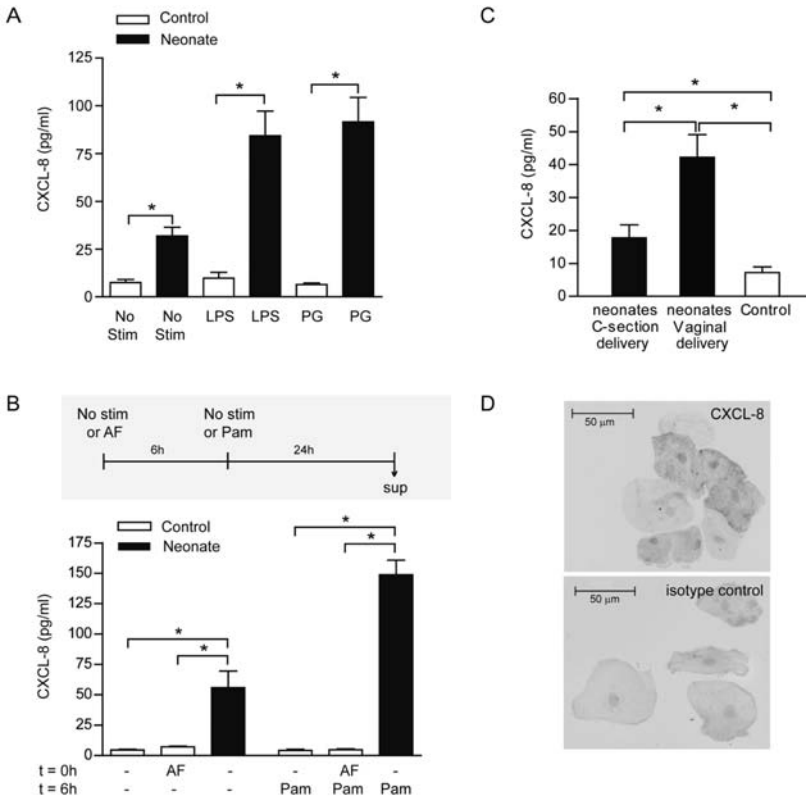


Figure 1 | Neonatal buccal epithelial cells spontaneously produce CXCL-8 and are highly responsive to microbial stimuli

A) Buccal epithelial cells from 26 neonates and 26 controls aged 2-6 months were collected. Cells were cultured for 24 hours in medium only or in the presence of LPS (1µg/ml) or PG (10µg/ml), and CXCL-8 production was analyzed by ELISA in the supernatant. Data are expressed as means with SEM. (*) indicates significant $p<0.05$ assessed by unpaired sample *t*-test with Welch correction.

B) Buccal epithelial cells from 11 controls (age 29-55) were collected and incubated for 6 hours with either medium only (No stim) or in the presence of amniotic fluid (AF). Thereafter, the cells were washed and stimulated with Pam3Cys (10µg/ml) or received no stimulus for 24 hours. As a control buccal epithelial cells from three neonates were incubated for 24 hours without stimulus or with Pam3Cys. CXCL-8 release in the supernatant was analyzed by ELISA. Data are expressed as means with SEM. (*) indicates significant difference $p<0.05$ assessed by one-way ANOVA, with Dunnett's multiple comparison as post test.

C) Buccal epithelial cells were collected from neonates born by Caesarian section (n=12) and born via vaginal birth (n=14). Buccal epithelial cells from 26 subjects (age 2-6 months) served as control. Cells were cultured for 24 hours without stimulus after which CXCL-8 production was analyzed by ELISA in the supernatant. Data are expressed as means with SEM. (*) indicates significant $p<0.05$ assessed by one-way ANOVA.

D) CXCL-8 stain on cytospins of neonatal buccal epithelial cells. Brown staining indicates CXCL-8, staining with the isotype control shown in right panel. Staining is representative for 3 different donors.

Neonatal buccal epithelial cells acquire hyporesponsiveness within weeks after birth

Buccal epithelial cells from neonates born by Caesarean section (n=3) were collected directly after birth as well as on day 1, 4, 11 and 21 after birth. The cells were stimulated with either Pam3Cys or LPS. Within 1 to 4 days after birth neonatal buccal epithelial cells secreted half of the amount of CXCL-8 than on the day of birth (Figure 2). On day 21 after birth CXCL-8 in response to microbial stimuli became undetectable, comparable to the reponse in buccal epithelial cells from infants and adults. (Figure 2).

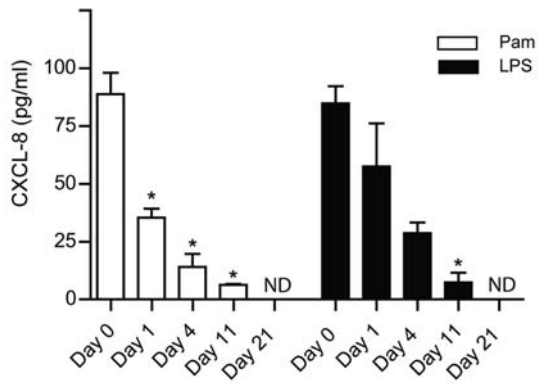


Figure 2 | Neonatal buccal epithelial cells acquire hyporesponsiveness within weeks after birth.

Buccal epithelial cells from 3 neonates delivered by Caesarian section were collected directly after birth, as well as on day 1, 4, 11 and 21 after birth. Cells were stimulated with Pam3Cys (10µg/ml) or LPS (1µg/ml) for 24 hours. CXCL-8 production was measured in the supernatant. ND denotes non-detectable. Data expressed as means with SEM. (*) indicates significant $p<0.05$, assessed by repeated measures ANOVA for both LPS and Pam3Cys, with Dunnett's multiple comparison as post test.

Priming of naive Caco-2 cells with Pam3Cys results in hyporesponsiveness that is associated with the expression of SLPI

As primary epithelium is short-lived we wished to identify a putative mechanism for acquisition of epithelial hyporesponsiveness using the epithelial cell line Caco-2. Caco-2 cells incubated for 24 hours with Pam3Cys released substantial amounts of CXCL-8 (Figure 3A). This release was largely inhibited when the cells were pre-treated with the same concentration of Pam3Cys for 24 hours (Figure. 3A). The hyporesponsiveness of Caco-2 cells treated with Pam3Cys was not due to total unresponsiveness of these cells, as an initial incubation with Pam3Cys did not suppress CXCL-8 production induced by a subsequent stimulation of the cells with high dose IL-1β (Figure

3B). Pre-treatment with low dose IL-1β and subsequent stimulation with low dose IL-1β resulted in a comparable downregulation of CXCL-8 production (Supplemental Figure 1). To identify whether hyporesponsiveness of Caco-2 cells was accompanied by reduced NF-κB activity, IκB levels were measured in whole cell lysates. Caco-2 cells pre-incubated with Pam3Cys for 24 hours and subsequently stimulated with Pam3Cys for 0, 5, 15, 30 and 45 minutes showed no reduction in IκB levels (Figure 3C). In comparison cells which did not receive a pre-treatment with Pam3Cys showed a time dependent IκB degradation (Figure 3C). This time dependent IκB degradation is characteristic for efficient activation of the NF-κB pathway (Figure 3C). In our culture system no Tollip or SIGRR protein could be found in the lysates of Caco-2 cells by Western Blot analysis (data not shown). Previously, it has been shown that the BBe (brush border expressing) subclone of Caco-2 cells constitutively expresses SLPI mRNA[26]. SLPI can act as a potent inhibitor of TLR stimulation by interfering at multiple levels of the NF-κB signaling pathway. We cultured Caco-2 cells with or without stimuli and analyzed changes in SLPI expression by quantitative PCR. Indeed Caco-2 cells expressed SLPI mRNA and expression was increased upon stimulation with PMA (Figure 3D). To examine whether hyporesponsive Caco-2 cells contain SLPI, SLPI protein was determined in cellular lysate. Caco-2 cells were incubated with Pam3Cys or left unstimulated for 24 hours. The presence of SLPI protein was assessed by performing an SLPI specific ELISA on the cell lysate. Hyporesponsive Caco-2 cells were shown to contain higher levels of SLPI protein compared to the responsive control Caco-2 cells (Figure 3E). These data indicated that hyporesponsive Caco-2 epithelial cells had higher levels of IκB and increased levels of SLPI protein.

Neonatal buccal epithelial cell sensitivity to microbial stimulation is associated with decreased SLPI expression

In agreement with the Caco-2 data, lysates of responsive neonatal buccal epithelial cells had lower levels of IκBα than adult hyporesponsive buccal epithelial cells (Figure 4A). To determine whether this involved SLPI production, the amount of SLPI protein in whole cell lysate of neonatal buccal epithelial cells was compared to that of hyporesponsive buccal epithelial cells from adults. Buccal epithelial cells from adults expressed SLPI protein (Figure 4B). Responsive neonatal buccal epithelial cells, however, contained non-detectable levels of SLPI protein without stimulation. Moreover, SLPI was not induced in the neonatal cells by stimulating with PG for 24 hours (Figure 4B). Quantification of the amount of intracellular SLPI in buccal epithelium from adult donors (n=6) showed that SLPI was consistently detectable in whole cell lysates (Figure 4C). In the buccal cavity, SLPI is present as a secretory product of the salivary gland [27-30]. This raises the question whether buccal epithelial cells take up SLPI from their environment or actively produce the protein. Therefore, we determined the presence of SLPI mRNA in adult buccal cells. Hyporesponsive buccal epithelial cells were indeed found to express SLPI mRNA (Figure 4D). As SLPI has been shown to exert its immunosuppressive activities at different cellular sites[24], we

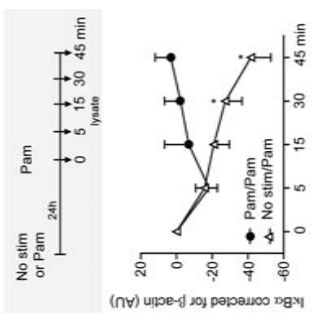


Figure 3 | Priming of naïve Caco-2 cells with Pam3Cys results in hyporesponsiveness that is associated with the expression of SLPI.
A) Caco-2 cells were cultured for 24 hours in the presence of Pam3Cys (10µg/ml) or without a stimulus, after which the supernatant was collected and the cells were washed. The cells were then cultured for another 24 hours in the presence of Pam3Cys (10µg/ml) or without a stimlus after which the supernatant was collected. CXCL-8 production was then analyzed in the supernatant collected at t=24 hours and at t=48 hours. Data are expressed as means with SEM. (*) indicates p<0.05, assessed by unpaired sample *t*-test with Welch correction.
B) Caco-2 cells were cultured for 24 hours in the presence of Pam3Cys (10µg/ml) or without stimulus, after which the supernatant was collected and the cells were washed. The cells were then cultured for another 24 hours in the presence of high dose IL-1β (1µg/ml) after which the supernatant was collected. CXCL-8 production was then analyzed in the supernatant collected at t=24 hours and at t=48 hours. Data are representative of three independent experiments (n=4) and expressed as means with SEM. NS indicates not significant and was assessed by unpaired sample *t*-test.
C) Caco-2 cells were cultured for 24 hours in the presence of Pam3Cys (10µg/ml) or without stimulus. A proportion of the cells was collected after 24 hours of culture and the remaining cells were then stimulated with Pam3Cys for 5, 15, 30 and 45 minutes. IκBα protein bands were analyzed by Western Blot in whole cell lysates. Densitometry of the bands was used to determine IκBα levels relative to β-actin and these were expressed as arbitary units (AU). At time point 0 minutes AU were set at 0. (*) indicates p<0.05 assessed using a two way ANOVA.
D) Caco-2 cells were cultured for 2 hours in the presence of PMA (0.05µg/ml) or without stimulus, after which the cells were harvested and lysed for detection of SLPI mRNA by Real Time PCR.
E) Caco-2 cells were cultured for 24 hours in the presence of Pam3Cys (10µg/ml) or without stimulus, after which cells were harvested and lysed for the detection of SLPI using ELISA. Data are representative of four independent experiments and expressed as means with SEM (n=8). (*) indicates p<0.05 assessed by paired sample *t*-test.

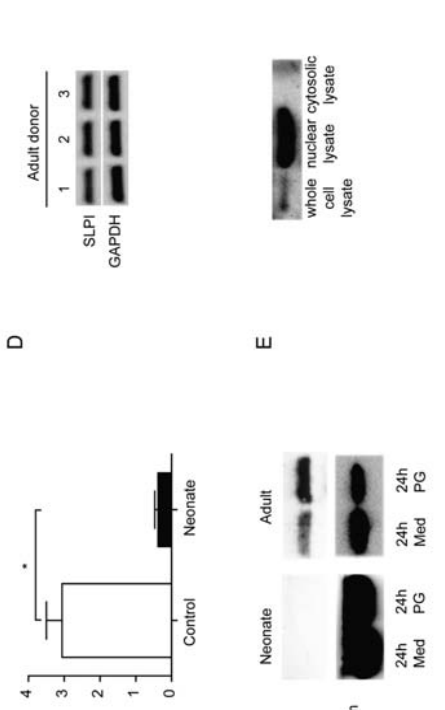


Figure 4 | Neonatal buccal epithelial cell sensitivity to microbial stimulation is associated with decreased SLPI expression.

A) Buccal epithelial cells from controls (age 29-55) and neonates were collected, lysed and analyzed by Western Blot for IκBα and β-actin proteins. Densitometry of IκBα protein bands was then used to determine IκBα levels. Levels were corrected for β-actin and noted as arbitary units (AU). (*) indicates p<0.05 assessed by an unpaired sample *t*-test with Welch correction.

B) Buccal epithelial cells from controls (age 29-55) and neonates were collected and incubated for 24 hours with PG (10μg/ml) or culture medium. After 24 hours cells were lysed and a Western Blot was performed to detect SLPI. Data are representative of 3 experiments using 3 different donors.

C) Buccal epithelial cells from six controls (ages 29-55) were incubated with Pam3Cys (10μg/ml) or culture medium for 24 hours and SLPI expression was assessed by ELISA in whole cell lysate.

D) Buccal epithelial cells from 3 controls (ages 29-55) were analyzed for SLPI mRNA expression by quantitative Syber Green PCR. PCR products were purified and visualized by gelelectrophoresis.

E) Buccal epithelial cells from controls (age 29-55) were collected and incubated without stimulus for 24 hours. Afterwards cells were lysed, nuclear and cytosolic fractions isolated and Western Blot was performed on the fractions to detect SLPI protein.

F) Buccal epithelial cells from controls (age 29-55) were collected and incubated without stimulus for 24 hours. Afterwards cells were lysed, nuclear and cytosolic fractions isolated and ELISA was performed on the fractions to detect SLPI protein. N.d. denotes non-detectable.

further analyzed the cellular location of SLPI protein in buccal epithelial cells from adults. SLPI was found abundantly in the nuclear fraction (Figure 4E, F) of adult buccal epithelial cells. As expected, SLPI was also located within the cytosolic fraction of adult epithelial cells (Figure 4F). However, in certain individuals the concentration of cytosolic SLPI was lower than that of nuclear SLPI (Figure 4E, F). This may explain the finding that cytosolic SLPI was not detected by Western Blot analysis, since the detection limit of this assay was 25ng as assessed with recombinant SLPI.

DISCUSSION

We have shown that primary buccal epithelial cells secrete CXCL-8 at birth and that secretion of this chemokine is enhanced in response to microbial triggering. In the first weeks of life buccal epithelial cells actively acquired hyporesponsiveness to microbial stimulation. Hyporesponsiveness of epithelial cells not only corresponded with a decrease in IκB degradation and consequently a decrease in NFκB activation, but also with an upregulation of SLPI mRNA and protein.

At birth a transition occurs from the sterile *in utero* environment in amniotic fluid to an environment rich in microbial products. Regarding the epithelium this transition is associated with a loss of contact with amniotic fluid and a gain of contact with increasing amounts of microbial products. As such, chemokine release by the epithelium directly after birth could be a remnant of activation by components in the amniotic fluid which were encountered *in utero*. Indeed, prenatal CXCL-8 production by intestinal epithelial cells has been observed [25]. Alternatively, the epithelium is inactive at birth and only starts releasing chemokines after microbial encounter during birth. Our data show that amniotic fluid does not mediate microbial responsiveness of adult epithelium and support the concept that first microbial contact at birth initiates a temporary activation of the epithelium.

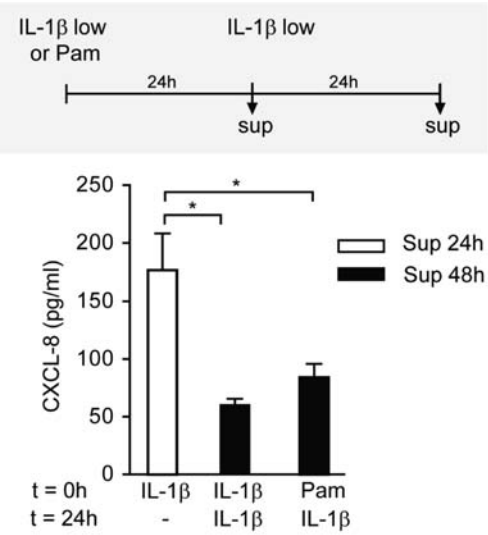
Using human primary buccal epithelial cells we now show that the transition from the sterile *in utero* environment to continuous contact with microbial products after birth is a pivotal event in shaping human epithelial responses. Within minutes after birth buccal epithelial cells from vaginally born neonates were found to release more CXCL-8 than buccal epithelial cells from neonates delivered via primary caesarean section, indicating that differences in microbial presence are detected by the epithelial cells. This epithelial responsiveness to bacterial stimulation is not restricted to the buccal cavity as several groups have shown responsiveness of neonatal intestinal epithelium to PAMPs [7,31,32]. The accessibility of the buccal epithelium allowed us to follow epithelial cell function in the first weeks of life within individual subjects. Despite the initial production of high levels of CXCL-8, buccal epithelial cells of neonates after caesarean section rapidly acquired microbial hyporesponsiveness within the first three weeks of life. In analogy, it was previously shown in a murine model that the first contact with endotoxin after birth elicited

chemokine production by intestinal epithelium [7]. However, 24 hours after birth the exposure to exogenous LPS had rendered the murine intestinal epithelial cells unresponsive towards endotoxin. In our study, the kinetics were different: spontaneous chemokine production was seen at day 0 which had disappeared after 21 days in culture in the presence of a microbial stimulus. Our data indicate that a responsive period precedes the tolerant state of buccal epithelial cells in healthy individuals. To obtain insight in the mechanisms that underlie acquisition of epithelial hyporesponsiveness we used an *in vitro* system. Active induction of hyporesponsiveness to microbial stimulation has been described in multiple intestinal cell lines amongst which Caco-2 cells [33]. Indeed, Caco-2 cells treated for 24 hours with Pam3Cys did not secrete CXCL-8 in response to Pam3Cys re-stimulation. Hyporesponsiveness of the Caco-2 cells was associated with reduced NF-κB activation. However, the cells which had become hyporesponsive to Pam3Cys were still able to respond to high dose IL-1β stimulation to the same extent as responsive cells, indicating that the cells were not defective in NF-κB signaling. Using this model system we searched for molecular pathways in the hyporesponsiveness of epithelial cells. Microbial-associated molecular patterns are known to have the capacity to induce regulatory molecules. As such IRAK-M [13], NOD2 [14], Tollip [12,15], A20 [16], SIGIRR [17,18] and SLPI [19] have been found to inhibit intracellular signal transduction at various stages during the signaling cascade, leading to reduced NF-κB activation and inflammatory gene expression. In our experiments we identified that microbial hyporesponsiveness in Caco-2 cells was associated with upregulated SLPI mRNA expression and SLPI protein. SLPI can directly interact with LPS before the latter molecule interacts with responder cells [34]. Intracellular SLPI can inhibit Toll-like receptor 2 and 4 signaling by direct prevention of the degradation of the inhibitory factor IκBα[35]. In addition, Taggart et al demonstrated in human monocytes that SLPI located in the nucleus directly binds to an NF-κB consensus sequence in the promoter region of the CXCL-8 and TNF-α genes [19]. SLPI competes with the NF-κB p65 subunit for binding to this consensus sequence thus blocking transcription [19]. Strikingly we were able to demonstrate SLPI protein in both the cytosol and nucleus of hyporesponsive primary buccal epithelial cells. The nuclear localization of SLPI protein in hyporesponsive buccal epithelial cells may reflect active suppression of gene expression by SLPI [19]. Currently, it is unclear whether the SLPI protein found in the cytosolic and nuclear fractions derives from mRNA produced by the buccal epithelial cell or from extracellular SLPI which is taken up from the buccal cavity. Saliva of newborns is known to contain large amounts of SLPI [27], however, the fact that no intracellular SLPI can be found in neonatal buccal epithelial cells even though the protein is abundantly present in the extracellular milieu argues against uptake of extracellular SLPI by buccal epithelium.

In previous work we demonstrated that buccal epithelial cells of pediatric Crohn's disease patients without oral lesions are responsive to microbial stimuli while healthy controls were hyporesponsive [24]. This raised the question whether a particular mechanism accounts for the loss of hyporesponsiveness in the epithelium of pediatric Crohn's disease patients. Recently, in

murine experiments SLPI was implicated in recovery from colonic inflammation[36]. Together, these data warrant further study to dissect whether alterations in SLPI-mediated inhibition of NF-κB are involved chronic inflammation in IBD patients.

In conclusion, neonatal buccal epithelial cells are responsive to microbial stimuli directly after birth. In the course of a few weeks these buccal epithelial cells become hyporesponsive to microbial stimuli and exhibit high levels of IκB. Furthermore, these hyporesponsive buccal epithelial cells express mRNA and high protein levels for the regulatory protein SLPI. SLPI may contribute to human epithelial cells acquiring a hyporeponsive state. Whether reduced levels or diminished function of SLPI could be involved in the pathogenesis of chronic intestinal inflammation remains to be elucidated.



Supplemental Figure 1 | Hyporesponsiveness of naive Caco-2 cells can be achieved by low dose IL-1β stimulation as well as Pam3Cys.
Caco-2 cells were cultured for 24 hours in the presence of 0.1 ng/ml IL-1β or 10 μg/ml Pam3Cys, after which the supernatant was collected and the cells were washed. The cells were then cultured for another 24 hours in the presence of 0.1ng/ml IL-1β or without a stimulus after which the supernatant was collected. CXCL-8 production was then analyzed in the supernatant collected at t=24 hours and at t=48 hours. Data are expressed as mean with SEM. (*) indicated $p<0.05$ assessed by one way ANOVA.

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Chapter 9

General discussion

THIS THESIS

The aim of this thesis was to study mucosal immunological homeostasis in early infancy in relation to the development of allergic diseases in early childhood, and to asses effects and mechanisms of probiotic intervention in infants with cow's milk allergy. In this chapter we will discuss the main results of the described studies.

Homeostasis is a multilayered regulatory immune response

Mucosal homeostasis is the result from the interaction between the mucosa and exogenous factors such as dietary and microbial antigens. Induction and maintenance of mucosal homeostasis is a highly regulated immune response that involves different cell types (epithelial cells, antigen-presenting cells, and lymphocytes). If homeostasis is lost, as a result of single or multiple defects, this may lead to disease, including allergy and chronic intestinal inflammation.

In general the mucosal immune system has developed two homeostatic strategies. First, immune exclusion by minimizing bacterial-epithelial cell contact via mucins, antimicrobial peptides and secretory antibodies. This immune response controls epithelial colonization of microorganisms and restrains the infiltration of potentially harmful agents. Penetration of pathogenic microorganism will elicit a pro-inflammatory response to eradicate the invader.

Secondly, immunosuppression to prevent local and peripheral hypersensitivity against innocuous antigens. Induction of oral tolerance to food proteins is controlled by interactions between both innate and acquired regulatory immune responses. However recent research has underlined the redundancy in mechanisms that contribute to this local tolerogenic environment [1]. Consequently the mechanisms resulting in a loss of tolerance remain for the most part unknown.

Homeostasis: nature or nurture?

As part of the microbe-host symbiosis the microbial genome provides signals for angiogenesis [2] and epithelial cell maturation [3] in the host. In addition, the intestinal flora has a direct effect on the maintenance homeostasis through induction of tolerance-inducing cytokines, regulation of the NFkB-pathway [4] and the development of regulatory T-lymphocytes [5]. The mucosal immune system detains the commensal bacterial flora to the lumen of the gut, without excessive and unwanted inflammation. In contrast, the mucosal immune system has to be able to adequately clear pathogenic microorganisms.

From the host perspective the induction and maintenance of mucosal immunity depends on sampling commensal flora, particularly by intestinal dendritic cells in the dome of intestinal lymphoid follicles or Peyer's patches [6], that sample the intestinal lumen by extending their dendrites between the tight junctions of the epithelial cell layer [7]. Although these dendritic cells migrate from the Peyer's patches and lamina propria to the mesenteric lymph nodes,

Macpherson demonstrated that the dendritic cells do not penetrate further into the body[8]. In this way mesenteric lymph nodes operate as an immune firewall that limits systemic penetration of commensal bacteria. Within the mesenteric lymph node B-lymphocytes differentiate into IgA producing plasma cells. The IgA plasma cells secrete dimeric IgA that is transcytosed across the epithelial cell layer and binds to intestinal bacteria, restricting bacterial association with the epithelium [9] and preventing bacterial penetration of host tissue [10].

In addition to the interaction between the commensal flora, dendritic cells, B-lymphocytes and various subsets of T-lymphocytes, the epithelial cell layer can also induce and maintain homeostasis. Lotz and coworkers showed in a murine model that the first encounter to endotoxin postpartum elicits chemokine production by intestinal epithelium [11]. However 1 day after birth the intestinal epithelial cells of the mouse became unresponsive towards endotoxin [11]. Other researchers have esthablished that neonatal intestinal epithelium reacts to microbial stimulation [12,13]. We have demonstrated in **chapter 8** that in the first weeks of human life buccal epithelial cells actively acquire hyporesponsiveness to microbial stimulation which is associated with altered NF-κB signaling and expression of Secretory Leukocyte Protease Inhibitor (SLPI) mRNA and protein. The permanent contact with microbial products directly after birth may therefore be pivotal for regulating epithelial homeostatic responses.

Food allergy

The mucosal epithelial barrier and immunoregulatory network are poorly developed in early life. The development of immune homeostasis depends on a window of opportunity in early infancy during which innate and adaptive immunity are educated to appropriately react to both harmful and harmless antigens. From animal models we have learned that the postnatal development of homeostasis depends on the establishment of a balanced commensal gut flora as well as adequate timing and dosing of the introduction of exogenous food antigens [14].

Oral tolerance is a strong adaptive immune function as significant amounts of intact food proteins are absorbed by the intestine after ingestion. A disturbance in the interplay between innate and adaptive immunity can result in food allergy: an allergic response or the loss of tolerance towards innocuous food antigens.

The position paper by the European Academy of Allergy and Clinical Immunology in 2004 stated that for food allergy the golden standard remains the double blind placebo controlled food challenge [15]. No single laboratory tests is diagnostic although skin prick testing (SPT), the atopy patch test and the measurement of food-specific IgE antibodies can be helpful [16]. The majority of infants diagnosed with cow's milk allergy will become tolerant before the age of 3 years [17]. The question remains what is the best possible moment to (re)challenge these infants? In **chapter 3** we showed that a positive SPT to milk and hen's egg at the time of diagnosis of cow's milk allergy were predictive for persistent allergy 6 and 12 months later, respectively.

Our findings corroborate other studies [18-20] and warrant suspension of oral food challenge in this particular patient group until the age of 2 years.

In **chapter 5** we evaluated whether the measuring of fractional exhaled nitric oxide (FE_{NO}) could be used a instrument to increase the sensitivity and/or specificity of the oral food challenge in infants. Our data showed no relationship between FE_{NO} and the results of the cow's milk food challenge. We hypothesized that the lack of correlation might be due to the absence of eosinophilic infiltration in the airway mucosa. Indeed, others found that in children with peanut allergy, FE_{NO} levels were also normal unless the children had coexistent asthma [21]. So far no other studies have addressed the question whether exposure to a food allergen could induce alterations in FE_{NO}. Consequently FE_{NO} may helpful in diagnosing and controlling asthma, but has no place in the diagnosis of food allergy.

Food allergy and atopic eczema/dermatitis syndrome (AEDS) in early infancy may be associated with the development of allergic rhinitis and asthma later in life [22]. The discovery in the late 1980's of the T-lymphocyte derived interleukin(IL)-4 being responsible for IgE switching of B-lymphocytes, indicated the T-lymphocyte as an essential player in the allergic response [23]. The classification into the T helper1 and T helper2 subsets based on cytokine profiles in mice [24] and men [25] led to the Th1-Th2-paradigm in allergic disease. This paradigm was a useful model but it has been difficult to identify patients that are at risk for persistent allergic disease using lymphocyte subsets as predictors. iNKT-cells are another specific subpopulation of T-lymphocytes expressing an invariant T-cell receptor and Natural Killer cell markers [26]. Upon activation by glycolipids presented by CD1d on antigen presenting (dendritic) cells, iNKTcells can produce both Th1- and Th2-type cytokines [27]. CRTH2 is the membrane receptor for prostaglandin D2 (PGD2). In AEDS and aeroallergen sensitized adults, increased levels of $CRTH2^{+}$Th-lymphocytes were found in peripheral blood [28,29]. In **chapter 4** we demonstrated reduced frequencies of iNKT-cells in infants with AEDS at the time of diagnosing cow´s milk allergy, and in food sensitized infants after 12 months of intervention with probiotics. Our results on iNKT-cells are in accordance to other publications on decreased circulating numbers of these cells in allergic adults [30,31] and children [32]. Additionally, these iNKT-cells differed in the quality of immunologic reaction mounting a skewed Th2-lymphocyte response [32]. Until yet it has been unclear if and how iNKT-cells play a part in the allergic immune response. As iNKT-cells respond to glycolipids[27] and not to protein allergens, we presume that these cells do not initiate, but act in synergy with protein specific $CD4^{+}$ T-cells to establish an allergic immune response. In a murine model co-administration of an iNKT-ligand during mucosal antigen exposure prevented the development of tolerance and stimulated a Th2-response [33], showing their potential role in allergy induction.

CRTH2 is preferentially expressed on Th2-lymphocytes [34] and activation elicits a broad spectrum of responses including chemotaxis. CRTH2 signals mediated by PGD2 enhanced Th2-lymphocyte polarization via inhibition of the D prostanoid receptor on Th1-lymphocytes [35].

In a subgroup with sensitization to food allergens we established an increased percentage of $CRTH2^+$leukocytes, suggesting that these are involved in early stages of sensitization.

In addition, after 12 months of intervention, hen's egg-sensitized infants showed lower frequencies of iNKT-cells and higher frequencies of $CRTH2^+$ lymphocytes compared to nonsensitized infants. In previous studies it was shown that early sensitization to hen's egg has a high positive predictive value for atopic disorders [36,37]. Thus, in comparison to the frequency of iNKT cells as a parameter for atopy in general, increased levels of $CTRH2^+$Th-lymphocytes seems a more selective marker in a subgroup. It remains to be shown if this marker has additional value compared to specific IgE against hen's egg.

Neither the numbers of iNKT-cells nor the frequency of $CRTH2^+$ lymphocytes in peripheral blood changed with the development of tolerance. This is in contrast to the diminished iNKT-cells in CMA infants described by Jyonouchi et al [32]. A possible explanation for this discrepancy may be the high cumulative percentage of tolerance acquisition in our study group, and the resulting lack of power.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is hypothesized to be the result of a dysregulated immune response towards the commensal intestinal flora. Like most immunologically driven diseases IBD is a multifactorial disease in which genetic susceptibility and environmental triggers are thought to play a causative role. Specific genetic defects in the innate immune system, e.g. NOD2 mutations [38], have been associated with the development of IBD. Progression of the inadequate innate responses initiates a vicious circle of harmful innate and adaptive immune responses resulting in clinical disease. The diagnosis of IBD is not based solely on clinical grounds. Endoscopic and histopathological criteria need to be met in order to make a correct diagnosis and discriminate disease subtypes. The search for novel, less invasive, diagnostic markers that accurately distinguishes a group of patients with IBD from those unaffected by the disease has become a focus in IBD research. Fecal and serological markers can potentially be used to determine the probability that a patient has IBD. Calprotectin and lactoferrin are the most frequently use fecal markers. Calprotectin is a marker of neutrophil infiltration [39], while lactoferrin is correlates to intestinal inflammation[40]. Serologic tests have been used in attempts to improve the diagnosis of IBD, such as tests for perinuclear antineutrophil cytoplasmic antibodies (pANCAs) and anti-Saccharomyces cerevisiae antibodies (ASCAs). In pediatric populations the specificity of serological markers for IBD is high, but low sensitivity making them less useful as diagnostic tests [41]. In contrast to invasive procedures like endoscopies and serology we demonstrate in **chapter 7** a novel and non-invasive marker in infant IBD: buccal epithelial cells. Intestinal epithelial cells can play a role in initiating and regulating mucosal immune responses through the secretion of cytokines. We hypothesized that the same mechanism could be found in buccal epithelium. Indeed, in children with Crohn's disease, buccal epithelial cells produced significantly

higher levels of CXCL-8, CXCL-9 and CXCL-10, in comparison to children with Ulcerative Colitis, to healthy controls or to adults with Crohn's disease. Enhanced buccal epithelial cell chemokine production was not noticed in all newly diagnosed children with Crohn's disease nor did we find an association with disease activity. We concluded that the enhanced chemokine production of buccal epithelium was limited to a subset of pediatric patients with a specific phenotype. This was supported by our finding that none of the adult IBD patients, all diagnosed in adulthood, showed enhanced buccal epithelial chemokine response.

Nevertheless we have to be cautious in extrapolating our results. We did not detect increased levels of CXCL-8 or -9 in healthy infants or adults, nor in cow's milk allergic infants. Nonetheless, this test can and probably will have false positive results in children with vague intestinal complaints and may therefore misguide the physician. Further research into the validity of our findings is necessary to pinpoint the place of this test in daily office practice.

In conclusion children presenting with intestinal symptoms, a high production of CXCL-8 or CXCL-9 by the buccal epithelium should raise the suspicion of Crohn's disease. This can be helpful in making the correct diagnosis, and may well provide us with the first soluble marker that is exclusively linked to pediatric Crohn's disease.

Probiotic intervention

More than a century ago, Metchnikoff observed the possible beneficial effects of fermented milk [42]. Consumption of the bacteria that cause fermentation was hypothesized to have a wholesome effect on the gut flora and modulate the mucosal immune system. Specific probiotic strains might influence immune function through different pathways including effects on local immune cells and T and B cells [43]. Allergic diseases are a major health problem in the industrialized world and intake of probiotic bacteria was proposed as a new strategy in prevention or treatment. As a consequence (and due to some brilliant marketing strategies) infant formulas became increasingly supplemented with probiotics.

In the last two decades numerous trials have been conducted on probiotics in the treatment [44-60] or prevention [61-74] of allergic diseases. Foremost, prevention studies failed to show clinically relevant effects, with the exception of atopic eczema dermatitis syndrome (AEDS) [64, 66,72,75]. The majority of intervention trials also focussed on AEDS. In several studies, including our intervention study discussed in **chapter 3,** the severity of AEDS improved similarly in treated and placebo groups [44,46,48,51,59]. Other studies reported improvement of disease severity [49,52,60], or no difference as a result of probiotic treatment [54,56]. Studies of Isolauri [49] and Majamaa [52] showed a benefit of probiotics over placebo. The age of the infants and the severity of AEDS at the start of intervention varied, but this did not affect the outcome. The benefits were however temporary, as the placebo group improved during the follow-up period and no difference remained at the end of follow-up.

We would like to address a possible bias in these studies, i.e. the "placebo" effect of extensively hydrolyzed formula (EHF). In 50% of the interventions, as in our study, EHF was identified as placebo formula, while the other studies do not mention whether EHF was consumed throughout the intervention. In infants with AEDS, EHF is commonly used, e.g. when they are also suspected of or proven cow's milk allergic. It would be medically unethical to withhold EHF in these infants. We conclude that probiotics as single therapy in all probability have no place in the treatment of AEDS in children.

Probiotics ingestion could alter the gut flora. Differences in flora composition before and after treatment were an outcome parameter in numerous studies [50-52,59,66,67,70,72]. In the majority of the trials the supplemented probiotic bacteria (or subgroup) increased in the gut flora during intervention [52,59,72] and disappeared after a follow-up period [66,67]. These results are in agreement with the fecal analyses in our cohort, as we showed in **chapter 3** and **6,** that probiotic administration did not affect the numbers of specific microbial strains but did affect the distribution of specific members within the genus of Bifidobacteria and Lactobacilli. However, independent of our intervention, we detected differences in the composition of the intestinal microbial flora between those infants who acquired tolerance and those with persistent cow's milk allergy. This confirmed results from other publications showing that raised levels of Bacteroides correlated with tolerance acquisition [76,77].

We hypothesized that supplementation of probiotics could alter gut homeostasis and thereby promote tolerance to cow's milk. In **chapter 3** we show that a combination of two specific probiotic strains (*Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb-12) to hydrolyzed formula fails to induce additional or accelerated tolerance during 12 months of treatment in cow's milk allergic infants. It remains unclear whether other probiotic strains might be more successful. Studies of probiotic supplementation in infants with (suspected) cow's milk allergy [52], [53,57-59] have been reported earlier (refs), but whether probiotic supplementation affected the clinical course of cow's milk allergy was never addressed before.

Through the work presented in this thesis we can conclude that the administration of these specific probiotics in early infancy did not result in any consistent clinical effects and did not have any adverse effects. Other probiotic intervention studies vary in methodological quality, the specific probiotics studied, the duration of the interventions, and the doses used and are therefore difficult to compare. In 2011 the Committee on Nutrition of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition did not recommend the routine use of probiotic-supplemented formula in infants [78].

Healthcare specialists and consumers can become biased by publication bias, as negative studies are less likely to be published than studies that show significant benefits [79]. At the end of 2005 Biogaia reported to end a research into the effects of probiotics on allergies [79]. BioGaia's clinical study was launched in 2001, with 232 newborn infants being given Biogaia's patented Lactobacillus reuteri bacteria. The incidence of AEDS did not decrease with the probiotic

supplement. To our knowledge the results of this study are not yet published in a peer-reviewed journal. . Hence we have to be critical towards the health claims of the major food industries.

Finally, it may be that benefits of specific probiotics are limited to specific phenotypes and that these do not appear in studies that consider large populations. It could be hypothesized that treatment and prevention of allergy using probiotics should be based on individual characteristics, including the unique composition of an individual's gut flora. Studies to identify of specific phenotypes that might benefit from any of the hundreds of probiotic strains seem hardly feasible.

CONCLUSION AND RECOMMENDATIONS

This thesis provides additional insight into mucosal immune regulation in early infancy. We describe a role for intestinal epithelial cells in the induction and maintenance of mucosal homeostasis, and we suggest that future studies in animal models and humans should focus on the cooperation between the innate responses of the epithelial cell layer and the underlying adaptive immune system. Furthermore we demonstrated that two specific subtypes of T-lymphocytes, iNKT and CRTH2, are associated with certain aspects of atopy in early infancy. These markers should however be validated in an independent cohort of atopic and non-atopic infants. Lastly we showed that intervention by means of certain probiotic bacteria was unable to modify the clinical evolution of cows milk allergy and the mucosal immune system. We propose that future probiotic intervention studies should use validated clinical outcome measures in randomized placebo controlled trials, with relevant inclusion/exclusion criteria and adequate sample sizes, and take individual characteristics including the gut microflora into account. Since the majority of probiotic intervention studies are food or dairy company funded, with an evident risk of bias, trials that are financed by non-profit organizations would be highly desirable.

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Chapter 10

Nederlandse samenvatting

In dit proefschrift proberen we meer inzicht te krijgen in de rijping van het mucosale immuunsysteem bij jonge kinderen. Het mucosale immuunsysteem bevindt zich in alle met slijmvlies bedekte organen, maar is het meest uitgebreid in het maag-darmstelsel. Ten eerste hebben we specifiek gekeken naar de relatie tussen de ontwikkeling van allergie en het uitrijpen van het mucosale immuunsysteem. Ten tweede hebben we onderzocht welk effect het gebruik van "goede" probiotische bacteriën heeft bij kinderen met een koemelkeiwitallergie.

In **hoofdstuk 1** geven we een overzicht van de verschillende aspecten van het immuunsysteem. Eén doel van ons immuunsysteem is het eradiceren van pathogene micro-organismen als er sprake is van een infectie. Daarnaast moet het systeem tolerant zijn voor het eigen lichaam, de darmflora en voeding. Het immuunsysteem bestaat uit een aangeboren en een verworven gedeelte. Het aangeboren en verworven immuunsysteem werken innig samen om elk moment van de dag de juiste keuze te maken of een antigeen (= molecuul dat in staat is een reactie van het afweersysteem op te wekken) goed of slecht is. In ons maag-darmstelsel bevinden zich verreweg de meeste cellen van het immuunsysteem, omdat het aanbod van antigenen zeer groot is. De belangrijkste taak van het maag-darmstelsel is voeding verteren en opnemen, zodoende worden er voedselantigenen geproduceerd. Daarnaast bevinden zich er ook nog eens 10^{14} bacteriën in de darmen met hun eigen specifieke antigenen.

Indien alle antigenen correct worden herkent en verwerkt wordt noemen we dat homeostase. Homeostase is een nauw evenwicht dat verstoord kan raken. Wanneer we intolerant worden voor bijvoorbeeld koemelkeiwitten kan dat leiden tot een koemelkeiwitallergie.

De afgelopen 3 decennia is de prevalentie van allergische ziekten verdubbeld in de Westerse wereld. Een mogelijke verklaring die hiervoor gegeven wordt is de Hygiëne Hypothese, die veronderstelt dat de afname van blootstelling aan micro-organismen door bijvoorbeeld betere riolering, vaccinaties en beschikbaarheid van antibiotica leidt tot een inadequate rijping van het immuunsysteem. Door de veranderde blootstelling aan micro-organismen verandert ook de darmflora. Onderzoek heeft aangetoond dat de darmflora van allergische kinderen verschillend is ten opzichte van niet-allergische kinderen. Probiotische bacteriën zouden de darmflora naar "normale en gezonde samenstelling" kunnen veranderen en allergie kunnen voorkomen of genezen.

De afgelopen 20 jaar is daarom steeds meer interesse ontstaan naar het toevoegen van probiotica aan zuigelingenvoeding of yoghurt(-drankjes) en wetenschappelijk onderzoek hiernaar. In **hoofdstuk 2** beschrijven we de kwaliteit van citaten van het vaakst geciteerde wetenschappelijk artikel over probiotica en allergie. Bijna 1/3 van de citaten over dit artikel in andere wetenschappelijke tijdschriften bleek incorrect. Daardoor kan het oorspronkelijke onderzoek verkeerd worden geïnterpreteerd door mensen die niet bekend zijn met de originele studie. Onjuist citeren is en blijft een voortdurend gevaar in wetenschappelijke publicaties.

In **hoofdstuk 3** presenteren we het KAMEEL-onderzoek (KoemelkeiwitAllergie Met Eliminatie En Lactobacilli), een dubbelblind, placebogecontroleerde studie naar de effecten

van *Lactobacillus casei* CRL431 en *Bifidobacterium lactis* Bb-12 op het natuurlijk verloop van koemelkeiwitallergie. In totaal deden 119 kinderen jonger dan 6 maanden met een bewezen koemelkallergie aan het onderzoek. De standaard behandeling van een koemelkeiwitallergie is een extensief caseïne hydrolysaat, in ons geval namelijk Friso 1 Allergy Care®. Gedurende 12 maanden kregen 60 kinderen de standaard behandeling, 59 kinderen kregen Friso 1 Allergy Care® met daaraan toegevoegd *Lactobacillus casei* CRL431 en *Bifidobacterium lactis* Bb-12 Na 6 en 12 maanden zagen we geen verschil in het percentage kinderen dat tolerant was geworden voor koemelk. Ook vonden we geen effect van probiotica op de ernst van atopisch eczeem, ziekenhuisopnames, respiratoire infecties of antibioticagebruik. De probiotica kon wel in de ontlasting worden aangetoond. Dit geeft aan dat onze probiotica wel in staat zijn het maag-darmstelsel te overleven maar geen klinische waarneembare effecten heeft kunnen induceren. Niettemin vonden we in het bloed lagere percentages van T-lymfocyten en T-helper lymfocyten in kinderen die probiotica kregen, zonder effect op de uitkomst van het onderzoek.

We concludeerden dat het toevoegen van deze specifieke probiotica geen effect heeft op het verloop van koemelkeiwitallergie.

Hoofdstuk 4 beschrijft de resultaten van de flow cytometry analyse van de leukocyten aan het begin en het einde van het KAMEEL-onderzoek en de associatie met atopie en allergie. Naast de in **hoofdstuk 3** beschreven lagere percentages van T-lymfocyten en T-helper lymfocyten in de probiotica groep, waren er geen andere verschillen tussen deze groepen. Opvallend was dat borstvoeding langer dan 3 maanden voor de start van het onderzoek eveneens leidde tot T-lymfocyten en T-helper lymfocyten. Onafhankelijk van de probiotische interventie hadden kinderen met mild tot ernstig atopisch eczeem significant lagere percentages van invariant Natural killer T-cells (iNKT-cellen). Aan het eind van het onderzoek hadden kinderen gesensibiliseerd voor kippeneiwit, hetgeen geassocieerd is met een grotere kans op astma op latere leeftijd, significant lagere percentages van invariant Natural killer T-cells (iNKT) en verhoogde percentages $CRTH2^+$(prostagladine D2 receptor) -lymfocyten.

We concludeerden dat probiotica en borstvoeding beiden leiden tot verlaagde percentages van T-lymfocyten en T-helper lymfocyten. Daarbij zijn verlaagde percentages van iNKT-cellen geassocieerd met atopie, terwijl we speculeren of $CRTH2^+$-lymfocyten een potentieel interessante marker is om te vervolgen in de ontwikkeling van allergie.

Hoofdstuk 5 beschrijft een studie naar de associatie tussen FeNO, een marker voor eosinofiele luchtwegontsteking, en de uitkomsten van een koemelkeiwit-provocatietest. Bij 44 kinderen van het KAMEEL-onderzoek werd gedurende een provocatie met koemelkeiwit op verschillende momenten FeNO gemeten. We hebben geen relatie kunnen aantonen tussen een positieve koemelkeiwit-provocatietest en de FeNO.

We concludeerden dat FeNO geen toegevoegde rol heeft in het stellen van de diagnose koemelkeiwitallergie.

In **hoofdstuk 6** hebben we onderzocht of de samenstelling van de darmflora beïnvloedt wordt door de supplementatie met probiotica. Daarnaast hebben we bovendien bepaald of kinderen die tolerant werden voor koemelk gedurende het onderzoek een veranderde darmflora hadden in vergelijking met persisterende koemelkallergische kinderen. We hebben gevonden dat de gesuppleerde *Lactobacillus casei* CRL431 en *Bifidobacterium lactis* Bb-12 uitsluitend werd gevonden in de ontlasting van de probiotca-groep. We konden geen andere verschillen aantonen. Onafhankelijk van de probiotische interventie konden we aantonen dat kinderen met een persisterende koemelkeiwitallergie, onder andere, significant meer *Bacteroidetes, Enterobacteriaceae* en *Streptococcus* in hun ontlasting hadden.

Zoals intolerantie voor voedselantigenen kan leiden tot een voedselallergie kan men ook onnodig intolerant reageren ten opzichte van de commensale darmflora. In genetische gevoelige individuen kunnen deze reacties leiden tot de ontwikkeling van inflammatoire darmziekten, zoals de ziekte van Crohn en Colitis Ulcerosa. In **hoofdstuk 7** beschrijven we de resultaten van een studie naar de immunologische reactie van wangslijmvlies in kinderen en volwassen met inflammatoire darmziekten. We hebben gevonden dat een gedeelte van de kinderen met de ziekte van Crohn een verhoogde productie had van inflammatoire cytokines, namelijk CXCL-8, CXCL-9 en CXCL-10. Geen van de volwassen patiënten met de ziekte van Crohn, kinderen met Colitis Ulcerosa en gezonde controles vertoonde deze verhoogde productie. Opvallend is dat de verhoogde productie van de cytokines geen relatie heeft met de ernst van de ziekte of behandeling. Mogelijk hebben deze kinderen met de ziekte van Crohn een specifiek (erfelijke) pathogenese.

Zoals **hoofdstuk 7** aantoonde is de tolerantie ten opzichte van de darmflora noodzakelijk voor mucosale homeostase. In **hoofdstuk 8** hebben we onderzocht of wangepitheel bij de geboorte reeds tolerant is voor bacteriële stimulatie of dat dit een verworven proces is. We hebben aangetoond dat neonataal wangepitheel spontaan CXCL-8 produceert en gevoelig is voor microbiële stimulatie. Reeds binnen enkele weken na de geboorte verliest het wangepitheel deze eigenschappen en wordt het ongevoelig voor microbiële prikkels, weergegeven door verhoogde spiegels van de nucleaire factor IκBα. IκBα remt translocatie van Nuclear Factor-kappa B (NF-κB) naar de celkern, waar NF-κB inflammatoire genen activeert. In Caco-2-cellen leidt microbiële stimulatie tot productie van Secretory Leukocyte Protease Inhibitor (SLPI), een gekende regulator van de NF-κB cascade. In het neonataal wangepitheel konden we geen SLPI aantonen, in tegenstelling tot het tolerante wangepitheel van gezonde individuen.

We concludeerden dat neonataal wangepitheel microbiële tolerantie verwerft in de periode kort na de geboorte. De ontwikkeling van tolerantie gaat gepaard met een accumulatie van het regulerende eiwit SLPI. SLPI speelt mogelijk een rol in het verwerven van mucosale homeostase. Of verminderde functie of verlaagde spiegels van SLPI betrokken zijn in de pathogenese van inflammatoire darmziekten dient verder uitgezocht te worden.

Samengevat geven de studies in dit proefschrift aanvullend inzicht in de mucosale immunologische regulatie op de vroege kinderleeftijd. We hebben aangetoond dat epitheelcellen van het maag-darmstelsel een rol spellen in de inductie en onderhoud van mucosale homeostase. Daarnaast hebben we gevonden dat twee specifieke subsets van lymfocyten, iNKT-cellen en CRTH2-cellen, geassocieerd zijn met aspecten van atopie op de vroege kinderleeftijd. Als laatste hebben we gedemonstreerd dat het toevoegen van probiotica aan de standaard behandeling voor koemelkeiwitallergie niet in staat is het verloop van de allergie te veranderen, noch het mucosale immuunsysteem. Mogelijk kunnen de resultaten in dit proefschrift in de toekomst een bijdrage leveren aan de ontwikkeling van nieuwe diagnostische parameters of therapieën voor patiënten met allergie of inflammatoire darmziekten.

List of abbreviations

AEDS	Atopic Eczema Dermatitis Syndrome
CAMEL	Cow's milk Allergy Modified by Elimination and Lactobacilli
CD	Crohn's Disease
CM	Cow's Milk
CMA	Cow's Milk Allergy
DBPCFC	Double Blind Placebo Controlled Food Challenge
DC	Dendritic Cell
EHF	Extensively Hydrolyzed Formula
ESPACI	European Society of Pediatric Allergy And Clinical Immunology
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology And Nutrition
ESR	Erythrocyte Sedimentation Rate
FE_{NO}	Fractional exhaled nitric oxide
IBD	Inflammatory Bowel Disease
ICC	Intraclass Correlation Coefficient
IEC	Intestinal Epithelial Cells
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IκBα	I kappa B alfa
IL	Interleukin
iNKT	invariant Natural Killer T
IPEX	Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked
IQR	Interquartile range
IRAK-1	IL-1 receptor associated kinase-1
IRAK-M	Interleukin-1 receptor-associated kinase- M
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
MDP	Muramyl Dipeptide
NF-κB	Nuclear Factor kappaB
NO	Nitric Oxide
NOD2	Nucleotide-binding Oligomerization Domain containing 2
Pam3Cys	Triacyl-lipopeptide
PAMP	Pathogen Associated Molecular Patterns
PCDAI	Pediatric Crohn's Disease Activity Index
PG	Peptidoglycan

PGD2	Prostaglandin D2
PMBC	Peripheral Blood Mononuclear Cells
ppb	parts per billion
PRR	Pattern Recognition Receptor
qPCR	quantitative Polymerase Chain Reaction
SCORAD	SCORing Atopic Dermatitis
SIGRR	Single Immunoglobulin IL-1R-Related molecule
SLPI	Secretory Leukocyte Protease Inhibitor
SPT	Skin Prick Tests
TGF-β	Transforming Growth Factor β
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Tollip	Toll Interacting Protein
UC	Ulcerative Colitis

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Curriculum Vitae

Jeroen Hol was born in Poortugaal on February 7th, 1975. In 1993 he passed his secondary school exam (Atheneum B) at the "Sint Montfort College" in Rotterdam. In the same year he started his medical training at the Medical Faculty of the Erasmus University of Rotterdam. During his study the author was member of the board of the "Medische Faculteitsvereniging Rotterdam" and several student committees.

After obtaining his medical degree in 1999, he worked for 2.5 years as a pediatric resident (ANIOS) at the Groene Hart Ziekenhuis in Gouda. During the last year of this period he began preparations for the CAMEL-study. In January 2003 he started as a PhD student at the department of Pediatric Respiratory Medicine (Prof.dr. H.J. Neijens and Prof.dr. J.C. de Jongste) and the laboratory of Pediatric Gastroenterology and Nutrition (Prof.dr. E.E.S. Nieuwenhuis and Dr. J.N. Samsom). The research performed during this period is presented in this thesis.

In March of 2008 he enrolled in the residency program for pediatrics, at the University Hospital Ghent (head of pediatrics Prof.dr. D. Matthys and Prof.dr. J. Vande Walle) and the Groene Hart Ziekenhuis in Gouda (Dr. F.G.A. Versteegh and Dr. J.S. Starreveld).

Jeroen is married to Nicole Wijnands and together they have one son, Jesper (2010).

List of publications

Damen GM, **Hol J**, de Ruiter L, Bouquet J, Sinaasappel M, van der Woude J, Laman JD, Hop WCJ, Büller HA, Escher JC, Nieuwenhuis EES. Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease. J Pediatr Gastroenterol Nutr. 2006 Feb;42(2):142-9.

Gabriele C*, **Hol J***, Kerkhof E, Elink Schuurman BEE, Samsom JN, Hop WCJ, Nieuwenhuis EES, de Jongste JC. Fractional exhaled nitric oxide in infants during cow's milk food challenge. Pediatr Allergy Immunol. 2008 Aug;19(5):420-5.

Hol J, van Leer EHG, Elink Schuurman BEE, de Ruiter LF, Samsom JN, Hop WCJ, Neijens HJ, de Jongste JC, Nieuwenhuis EE; Cow's Milk Allergy Modified by Elimination and Lactobacilli study group. The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomized, controlled trial. J Allergy Clin Immunol. 2008 Jun;121(6):1448-54.

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Hol J, van Leer EHG, Elink Schuurman BEE, de Ruiter LF, Schuren F, Nieuwenhuis EES, te Biesebeke RT. Fecal microbial composition correlates to the acquisition of tolerance towards cow's milk. Submitted

Hol J*, Menckenberg CL*, de Ruiter LF, Raatgeep HC, Simons-Oosterhuis Y, van Lierop PE, Groeneweg M, Elink Schuurman BEE, de Jongste JC, Samsom JN*, Nieuwenhuis EES* Human primary buccal epithelium acquires microbial hyporesponsiveness at birth, a process involving increased secretory leukocyte protease inhibitor (SLPI) expression. In preparation

* these authors contributed equally.

ERASMUS UNIVERSITEIT ROTTERDAM

PhD Portfolio

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Jeroen Hol Erasmus MC – Sophia Children's Hospital Department: Pediatric Pulmonology and Laboratory of Pediatric Gastroenterology and Nutrition	PhD period: January 2003 – March 2008 Promotors: Prof.dr. J.C. de Jongste / Prof.dr. E.E.S. Nieuwenhuis Supervisor: Dr. J.N. Samsom / Dr. E.H.G van Leer

PhD training

	Year	Workload (Hours)
General courses		
– Introduction into Data-analysis	2004	40 hrs
– Post graduate school Society of Mucosal Immunology: Introduction into Immunology	2005	8 hrs
– Molecular Medicine Postgraduate School: Immunology	2006	40 hrs
Oral Presentations		
– Koeien en Kamelen, het KAMEEL-onderzoek in de praktijk. *Onderzoeksdag Sophia Kinderziekenhuis Rotterdam*	2003	12 hrs
– Melk goed voor elk? *Nascholing consultatieburo-artsen, Dordrecht*	2004	12 hrs
– Chemokine production by buccal epithelial cells as a potential parameter for immunomodulation by probiotics, *European Society for Paediatric Gastroenterology, Hepatology and Nutrition*	2005	12 hrs
– Chemokine production by buccal epithelial cells as a potential parameter for immunomodulation by probiotics. *Society of Mucosal Immunology Boston*	2005	6 hrs
– Fraction exhaled nitric oxide is not a marker of positive reaction to cow's milk food challenge. *European Respiratory Society, Kopenhagen*	2005	6 hrs
– Activation induced unresponsiveness to peptidoglycan of intestinal epithelial cells upon short term exposure to Lactobacillus casei. *Nederlandse Vereniging voor Gastroenterologie, Veldhoven*	2005	12 hrs
– Acquiring innate tolerance. *Wetenschapsdag Erasmus MC, Rotterdam*	2007	12 hrs
Teaching		
Supervision doctoral student	2005	80 hrs