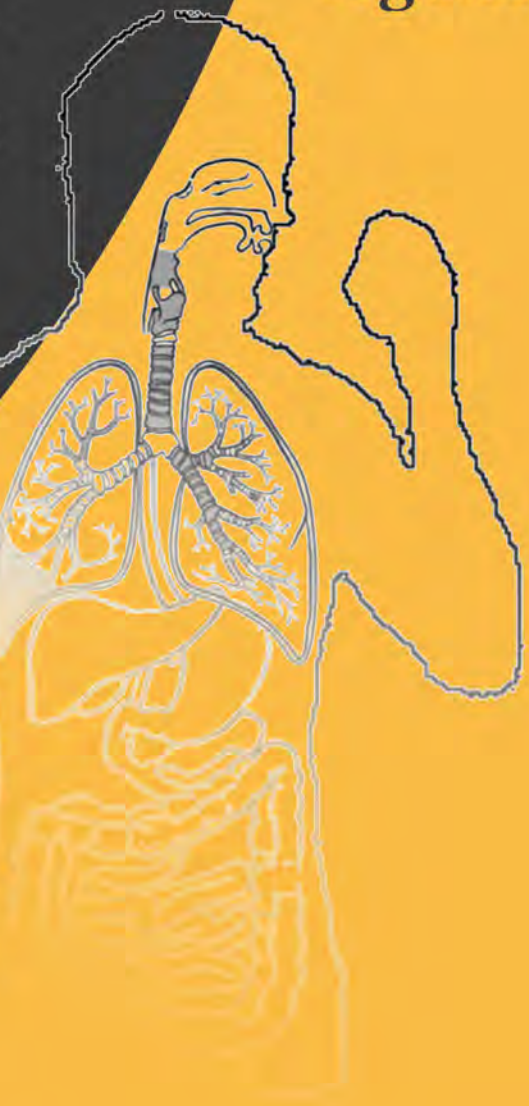
Relevance of IgA in allergy regulation by mucosal factors



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Gerco den Hartog

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Thesis committee

Promotor
Prof. dr. Huub F. J. Savelkoul
Professor of Cell Biology and Immunology

Co-promotor
Dr. R. J. Joost van Neerven
Senior scientist, FrieslandCampina Research, The Netherlands

Other members
Prof. dr. Jerry M. Wells, Wageningen University
Prof. dr. Stephan R. Durham MD, NHLI, Imperial College, London, United Kingdom / Royal Brompton Hospital London, United Kingdom
Prof. dr. Rudi W. Hendriks, Erasmus MC, Rotterdam, The Netherlands
Dr. Edward Knol, UMC Utrecht, The Netherlands

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regulation by mucosal factors

Gerco den Hartog

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Gerco den Hartog

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voor
Franciska
Matthias
Yaela

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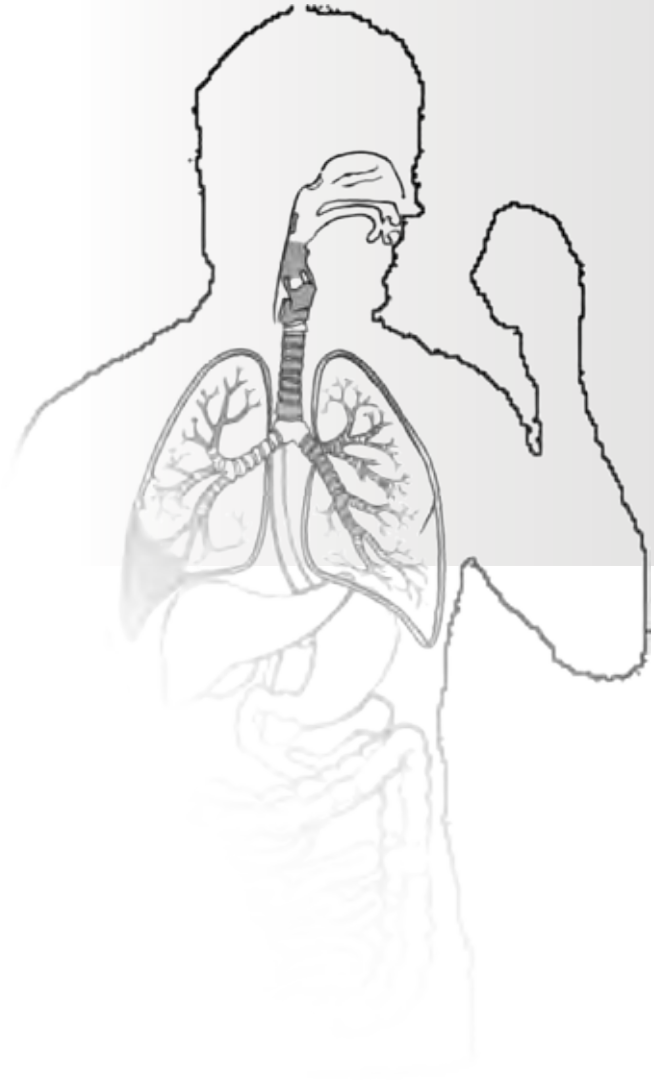

chapter 1

General Introduction

Gerco den Hartog

It has been shown convincingly by many studies that the immune system plays a crucial role in the induction and progression of allergy. In addition, evidence is accumulating showing the immunomodulatory capacity of food components. Both atopic allergy and immunomodulation by food involve the mucosal immune system of the gastro-intestinal tract, respiratory tracts and oral cavities.

In this introduction the mucosal immune system (I), allergy (II) and the research aim and thesis outline (III) are discussed.

I MUCOSAL IMMUNE SYSTEM

General features of mucosal immunity

The respiratory tract and the gastro-intestinal tract harbour large mucosal immune systems. The intestinal mucosal immune system constitutes the largest 'immune organ' in the body. The respiratory and especially gastro-intestinal tract encounters high loads of foreign materials, both innocuous and pathogenic. The majority of antigens the intestine is exposed to are food components and bacteria, commensal as well as potentially pathogenic. To prevent massive intestinal inflammation, it is crucial that food components and commensal bacteria are tolerated as they are in principle non-pathogenic and harmless. Apart from tolerance, induction of an inflammatory immune response is required when food components and bacteria cause pathology. Under homeostatic conditions; the mucosal immune system is geared towards inducing tolerance [1-4]. Key players in the induction of oral tolerance are tolerogenic dendritic cells, regulatory T cells (Treg) and IgA-producing B cells.

In addition, the mucosal immune system influences the composition of the intestinal microbiota, both pathogenic and commensal, thus preventing translocation over the epithelium and the induction of inflammatory responses towards pathogens [5, 6]. In addition to pathogens, commensal or mutualistic bacteria also need to be controlled as these bacteria that are harmless and often useful in the lumen may cause pathology once they have entered the internal milieu of the host.

In mucosal body defence an innate and an adaptive compartment can be distinguished. The innate mucosal immune system consists of a mucus layer exposed to the intestinal lumen, an epithelial cell layer with tight junctions, and neutrophils and antigen presenting cells underlying the epithelium (Figure 1). The cellular compartment of the adaptive immune system is limited to the inner milieu, and consists of T cells and B cells.

The innate immune system depends on features like extracellular and intracellular pattern recognition receptors (PRR) that recognize general molecular patterns. Different types of PRR have been described, identifying microbe-, pathogen- and danger-associated molecular patterns (abbreviated as MAMP, PAMP and DAMP respectively). PRR enhance ligation and phagocytosis of microbes or have signalling ability allowing activation of the cell. Ligation of the latter pattern recognition receptors can result in production of oxygen radicals with microbial killer activity and production of cytokines. Production of cytokines can affect epithelial integrity and chemo-attraction of other immune cells of both the innate and adaptive immune system [7-11]. Ligation of extracellular toll like receptors (TLR) by bacterial ligands like lipo-poly saccharide (LPS) (binds to

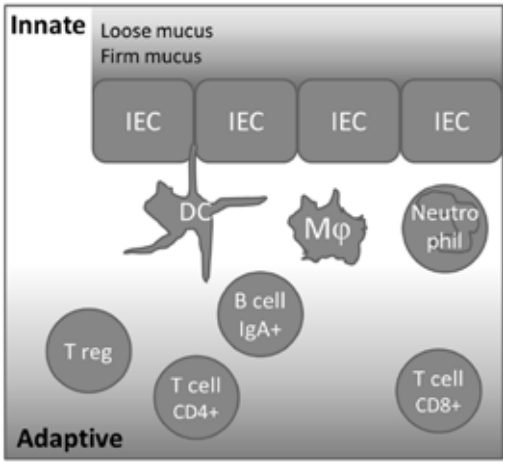

Figure 1. *Major innate and adaptive cells types of the mucosal immune system. IEC is intestinal epithelial cell, DC is dendritic cell, MΦ is macrophage*

TLR4), peptidoglycan (PGN) (TLR2) and flagellin (TLR5) results in MyD88-dependent production of inflammatory cytokines like interleukin (IL)-1ß, IL-6, IL-8, and partly TNF-α. In important part of the innate intestinal immune system is formed by Paneth cells that produce antimicrobial peptides that are secreted into the lumen.

Next to soluble antimicrobial activity by the production of antimicrobial peptides, the innate immune system has a cellular compartment that takes up antigens from the local environment. Antigen-uptake can be enhanced by extracellular type I and II C-type lectin PRRs [12]. Phagocytosis of bacteria is enhanced when these bacteria are opsonized by IgG that can be bound by Fc IgG receptors on APC. Similarly, soluble antigens can be bound by IgG, processed and presented. In the mucosa, luminal secretory (sIgA) binds to antigens. IgA-antigen complexes can be retro-transported from the lumen to the internal milieu, where the IgA-antigen complex can be bound by APC that in turn process and present the antigen.

Under inflammatory conditions neutrophils are attracted under the influence of interleukin (IL)-8, which can be produced by epithelial cells and APCs. Neutrophils can infiltrate the tissue in minutes upon infection and will remove bacteria present from the tissue. Bacteria taken up are killed intra-cellular by neutrophils; whereas antigen presenting cells (APC) also have the ability to present short linear peptides on MHC II after digestion. Antigenic peptides presented in the context of MHC II molecules can be recognised by peptide-specific $CD4^+$ helper T cells (Th) of the adaptive immune system. This is accompanied by production of different cytokine profiles and expression of co-stimulatory surface molecules (for instance CD80, CD86) that are required to induce and regulate downstream immune responses of T cells and B cells [13-16].

The $CD4^+$ T helper cell population expresses a wide repertoire of T-cell receptors (TCR) that can specifically recognize peptides presented in the context of MHC II class molecules. Activation of T helper cells further depends on expression of co-stimulatory molecules on the membrane of APCs and production of specific cytokines. Depending on the combination of different stimuli a naïve T cell receives, different Th cell subpopulations are being induced with different cytokine expression profiles. Initially the Th1 and Th2 cells were identified, of which Th1 enhanced

macrophage activation via interferon (IFN)-γ and Th2 was found to enhance B cell antibody production via IL-4 and IL-10. Since then several other Th types like Th17, Th9 and Th22 have been identified, characterized by production of IL-17, IL-9 and IL-22 respectively. It is still debated to what extent differentiated Th cells are stable and to what extent Th types harbour intrinsic plasticity. Activation and differentiation of Th cells is crucial for both inflammatory and regulatory (Treg mediated) immune responses. Tregs produce anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-ß. The Th cell phenotype specific cytokine profiles in turn affect APCs, B cells and antibody production, and other cells of the immune system (both innate and adaptive) [17]. Also the specific location of activation of immune cells determines the fate and functional effect of the cell.

Like T cells, B cells produce specific receptors in the form of immunoglobulins (Ig). Immunoglobulins can be expressed on the membrane of B cells or secreted. Secreted Ig can bind to soluble antigens and particulate antigens like bacteria. The advantage of soluble receptors in the form of Ig is that soluble proteins can easily be disseminated through the tissue and whole body (local versus systemic activation). By this, immune receptors reach places like the intestinal lumen that are relatively inaccessible for cells. In addition, Ig can be bound by receptors on the membrane of other immune cells resulting in downstream activation of cellular programs in other cell types like neutrophils and DCs. These downstream responses can be both inflammatory and tolerogenic, depending on the specific Ig-receptors involved and for instance the presence of antigen-specific T cells. B cells are able to produce a highly variable repertoire of Igs that recognize all kinds of antigens. In addition to the antigen-specificity of an Ig molecule, the different constant parts of the Ig isotypes (e.g. IgG and IgA and their subclasses IgG1-4 and IgA1 and 2) result in different downstream effector functions (discussed elsewhere).

Mucosal immune skewing

Different subsets of dendritic cells and macrophages are present in the mucosal tissues, with differential capabilities in directing Th cell differentiation. It has become apparent that next to classical immune cells, endothelial, epithelial and stromal cells affect the immune response [4, 18-20].

Epithelial cells are polarized, which enables them to discriminate the external lumen from the inner environment [21, 22]. TLR expression is polarized on intestinal epithelial cells and therefore the cells can discriminate basolateral from apical TLR activation [22]. Under homeostatic conditions intestinal epithelial cells are potent initiators of tolerogenic downstream immune responses [4]. To our current understanding of the intestinal immune system, the intestinal tolerogenic milieu depends on retinoic acid (RA), TGF-ß, and prostaglandin (PG)E_2 produced by epithelial and stromal cells (Figure 2). Also Treg derived IL-10 is a crucial regulatory cytokine by suppressing inflammatory immune responses.

Under the influence of specific stimuli naïve Th cells can differentiate and produce different cytokine profiles, which have different downstream consequences. Activation and maturation status of APC's is usually determined by flow cytometric analysis of expression levels of CD80 and CD86 respectively CD83. CD80 (B7-1) and CD86 (B70-2) are costimulatory molecules expressed on APCs (often referred to as signal 2 commencing after initial recognition of the antigen-derived peptide

as signal 1) and ligate to CD28 expressed on Th cells. A third signal of APC dependent activation of Th cells is mediated by the production of inflammatory cytokines like IL-1ß, IL-6, IL-12 and TNF-α, and the regulatory cytokines IL-10 and TGF-ß. Expression of these co-stimulatory molecules and cytokines can be inhibited by IL-10, resulting in reduced activation of Th cells [23]. Allergic disease is widely considered to be Th2 mediated. Differentiation of Th2 is inhibited by Th1 cells mainly by IFN-γ and Th1 cells can inhibit Th2 differentiation mainly by IL-4. Production of the Th1 skewing cytokine IL-12 by APC is also inhibited by IL-10. Next to absolute production levels, the ratio between IL-10 and IL-12 is used to identify if a stimulus is skewing towards Th1 or Th2. Precise evaluation of the cytokine production requires both absolute production levels and cytokine ratios. However in case of tissue damage, inflammation or translocation of intestinal microbiota, intestinal DCs can initiate differentiation of Th1 and Th17 cells, which are characterized by a more inflammatory phenotype. Apart from its harmful function in allergy and autoimmunity, Th2 cells are generally tolerogenic by inducing antibody secretion. There is evidence that colonic induction of Th2 cells is crucial in the prevention of development of inflammatory diseases like Crohn's disease [24]. Colonic DCs preferable induce Th2 rather than Th1 responses. $CD11c^+$ DCs isolated from blood are potent inducers of Th2 cells when cultured in the presence of thymic stromal lymphopoietin (TSLP) compared to DCs cultured in the presence of CD40L [20]. Epithelial cells produce TSLP, which seems to be lost in patients suffering from Crohn's disease. On the other hand, TSLP can be involved in the induction of allergy by its DC mediated Th2 promoting capabilities [25].

In a mouse model, co-stimulation with ovalbumin (OVA) and *Staphylococcus* enterotoxin B was shown to increase Th2 induction. This was dependent on expression of T-cell immunoglobulin-domain and mucin-domain (TIM)-4 by intestinal $CD11c^+$ DCs [26]. Together with expression of TIM-4, CD80 and CD86 expression on the cell surface increased and increased levels of IL-4 and IL-13 were produced. When cells or mice were only stimulated with OVA or SEB, no increase in Th2 induction was observed. This study did not report

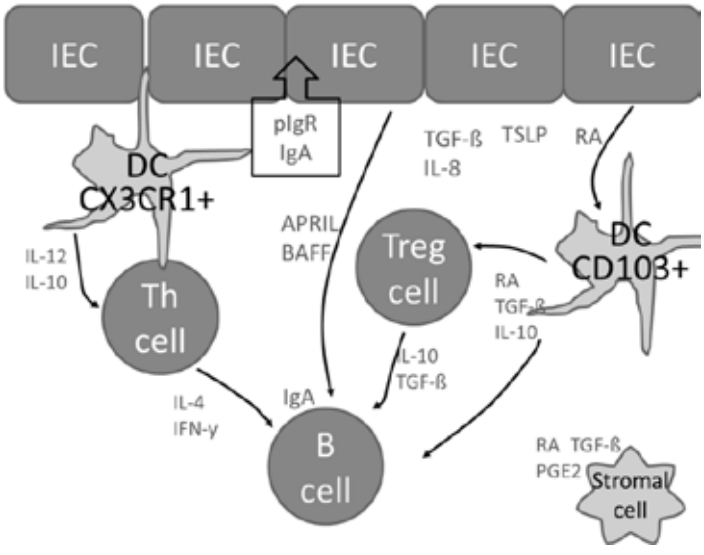

Figure 2. *Mucosal factors involved in induction of Tregs and IgA by B cells. IEC is intestinal epithelial cell, DC is dendritic cell, Th is T helper cell, B is B cell, pIgR is polymeric Ig receptor, RA is retinoic acid*

the specific subset of DC's involved in breaking tolerance and inducing Th2 cells.

Increased permeability of the intestinal epithelium may cause increased induction of allergy and anaphylaxis [27]. The relation between increased permeability of the gut and induction of allergy has recently been reviewed by Perrier et. al. [28].

The intestinal immune system is mainly located in the small intestine and colon with differences in the anatomical patterning and physiological functions [29]. It is important to be aware of the compartmentalization of the intestine even if the intestinal immune system associated with the small intestine has been subject to more studies and is the most widely described [30]. According to the compartment, differences in immune responses in response to local antigens can be easily understood: food antigens are more numerous in the small intestine while in the ileum and colon they have essentially been digested and absorbed. In contrast, commensal microbes are scarce in the duodenum but more numerous in the ileum and above all in the colon. The gut microbiota forms multiple complex interactions with each other and the host immune system [31]. The composition of the gut microbiota or presence of specific species has been associated with diseases. Several bacterial species and strains have been identified that reduce or increase inflammatory and allergic phenotypes. In the KOALA birth cohort study presence of *E. coli* was associated with increased eczema and *Clostridium difficile* with multiple atopic manifestations [32]. Though still topic of debate and subject to experimental variation, several probiotic strains have been identified that reduce allergic symptoms in either humans or mouse models [33-35]. However, recent reviews conclude that protection against asthma or allergy is strongly dependent on the specific probiotic used [36, 37]. The increased Th1 skewing as identified by IFN-y production could explain at least part of the protective effect by microbes on allergy development.

Mucosal B cell IgA production and secretion into the lumen is a crucial mechanism in both protection against infections and protection against inflammatory responses to antigen. Absence of IgA in the lumen is associated with increased mucosal leakiness and consequently increased numbers of bacteria can translocate over the epithelium into the sub-epithelial area [5, 38]. Production of IgA is induced by bacteria, as shown by introducing bacteria in germ free mice. In mice, a dual origin for IgA plasma cells in the small intestine has been shown. IgA plasma cells originate from two lineages of B cells designated B-1 and B-2, which differ according to their origins, anatomical distribution, cell surface markers, Ab repertoire and self-replenishing potential. B-1 cells are maintained by self-renewal of cells resident in the peritoneal cavity, and they utilize a limited repertoire that is mostly directed against ubiquitous bacterial Ags. B-2 cells, originated from bone-marrow precursors, are present in organized follicular lymphoid tissues, within Peyer's patches (PP), as precursors of plasma cells, and use a large repertoire of Abs and are believed to be more abundant in the distal ileum compared to colon [30]. Thus the sIgA response to specific protein-antigens requires a classical co-stimulation by Ag-specific T cells, an entero-enteric cycle as described previously, and are secreted by IgA plasma cells derived from B2 lineage precursors in the PP. By contrast, sIgA Abs against Ags from commensal bacteria can be induced independent from T cell help. Those IgA antibodies are polyspecific, and are secreted by IgA plasma cells derived from the peritoneal cavity B-1 cells. They protect the host

from the penetration of commensal bacteria. In mice, B-1 lineage could represent 40% of total IgA plasma cells. The contribution of peritoneal B cells to the intestinal lamina propria plasma cell population in humans is still a matter of debate [39]. IgA production is induced by Th cells and independent of T cell help as shown by CD40 and APRIL (role of APRIL is discussed below) knock out mouse models.

B cell immunity

An important reason why humoral immunity is effective in protecting against infections is the ability to produce different immunoglobulin isotypes. This provides the humoral immune system to fine tune downstream response by adapting the produced immunoglobulin (sub) class. Immunoglobulin class is partly associated with location, as IgG is dominantly present in systemic fluids, whereas IgA is dominantly produced in mucosal tissues, with a production between 3 and 5 g of IgA per day in the gastrointestinal system.

B cell immune maturation

Before an efficient B cell response is mounted, B cells need to mature either from naïve B cells or pre-activated B cells. The purpose of B cell activation is to produce the proper isotype and antigen recognition repertoire. Like with T cell repertoires, B cell repertoires are analysed based on the complementarity determining region 3 (CDR3) [40, 41]. The humoral immune system needs to mature after birth or hatch (in case of birds).

Naïve B cells produce IgM and a non-maturated antigen repertoire. Upon activation B cells can undergo class switch recombination (CSR) to other immunoglobulin isotypes (discussed below). The antigen specificity repertoire is for a large part determined by the CDR3. Upon co-stimulation with Th cells the expression of the enzyme activation induced deaminase (AID) can be initiated resulting in subsequent mutations to increase the affinity of the immunoglobulin produced by that B cell [42]. Intestinal body defence partly depends on affinity maturated B cells, but also on B cells expressing a more naïve repertoire that is determined by proximal V gene sequences on the germline. Antibodies produced by those B cells are referred to as natural antibodies (nAb), which recognize more general patterns with relatively low affinity. Systemic nAb are generally of the IgM isotype whereas mucosally a proportion of the IgA antibodies also might have nAb characteristics. nAb represent a more primitive defence layer and are useful for instance for the detection of molecular patterns of bacteria present in the intestinal lumen [43, 44].

Antibodies specific for an antigen, generally do not recognize the whole antigen but a part called an epitope. Epitopes can recognize linear or conformational sequences. Numerous studies have been performed identifying IgE-epitopes of allergens. IgE-epitope length varies from 3 to 30 amino acids in length [45]. Antibodies bind to antigens with varying affinity. Affinity of antibody-binding to epitopes is enhanced by activation of the B cell by specific Th cells, which induce somatic hypermutation by B cells.

Next to the antigen specificity of a B cell and the immunoglobulin isotype, differentiation into memory or plasma cells is an important feature of B cells. Memory cell formation is crucial for long term source of affinity maturated antibodies [46]. Typically, vaccination strategies aim at inducing affinity maturated B cells. Antigen experienced B cells that have undergone affinity maturation and isotype switching express CD27 [47]. Memory B

cells can secrete antibodies upon activation by for instance antigen encounter.

Antibody secreting plasma cells can be discriminated by detection of CD38 and CD138 on their membrane [48, 49]. Plasma cells are the major source of immunoglobulins present in systemic fluids as well as at mucosal surfaces. Plasma cells can reside in systemic immune organs like spleen and bone marrow, as well is in local mucosal tissues of both the intestinal tract and the respiratory tract.

To exert their effector function, B cells can be conditioned to migrate to specific sites in the body. This is regulated by the expression of for instance the integrins ß1 and ß7 and chemokine receptors (CCR)10 and CCR9 to migrate to peripheral organs (including the respiratory tract) respectively the intestines [50, 51]. CCR7 expression is crucial for homing from the periphery to the lymph nodes [52].

Antibody production and class switching

$CD4^+$ Th cells are potent activators of B cells by expressing CD40 ligand [13, 53]. Subsequent immunization or antigenic challenges result in Th dependent affinity maturation. Next to this, Th cells can direct CSR, which affects the effector function of the antibodies produced. Naïve B cells produce IgM but can switch to other isotypes under the influence of Th cells. Isotype switching requires activation of AID and can be directed by specific cytokines. B cells can switch directly or sequentially from IgM to any isotype [54, 55]. Most manifestations of allergy depend on the presence of allergen specific IgE. To our current knowledge, class switching to IgE depend on Th2 cell help and is mediated by production of IL-4 and IL-13 [55, 56]. Th2 cells are responsible for induction of both IgG4 and IgE. In the presence of IL-10 IgE production is inhibited and IgG4 production initiated [57]. Tregs can serve as an important source for IL-10.

Switching by definition is only possible downstream as intervening DNA is looped out during switch recombination [58]. The order of heavy chain isotypes on the human chromosome 14 is subsequently IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE and IgA2 (human genome, genbank build 37.3). Sequential switching has been reported for IgG to IgE and from IgG and IgM to IgA2 via IgA1 [18, 53]. The possibility of sequential class switching provides opportunities to change effector functions of B cells while retaining their epitope specificity. For instance, IgE-producing B cells might be able to switch downstream to IgA2.

The humoral immune system is a crucial part of the first line of defence at mucosal sites. The majority of secretory IgA is produced mucosally at high levels, even exceeding levels of IgG produced systemically [59]. IgA is produced in dimeric form and linked by a J-chain, which is bound by secretory component to be transported over the mucosal epithelia by the polymeric Ig receptor (pIgR) [60, 61]. Lack of expression of pIgR results in strongly reduced IgA levels in the intestinal lumen and in an increase in exposure of the sub-epithelial immune system to intestinal microbiota [5, 60, 62].

Recent evidence is accumulating showing that B cell activation and isotype switching is not solely induced under the influence of Th cells. Especially in the mucosa extra-follicular B cell activity is observed. Th cell independent class switching has been described several times for IgA [63-65]. As epithelial-DC axis mediated cytokines like APRIL and BAFF upregulate AID expression, B cells might be able to undergo any type of isotype switching under the influence of these cytokines,

independent of the presence of Th cells. Class switching to IgE has been reported to also occur in the mucosa [54, 66]. However it has not yet been revealed of this occurs solely dependent on Th2 cells or also might occur on the influence of innate cytokines like APRIL and BAFF.

Immunoglobulin effector functions

Isotypes have different capabilities to either bind to Fc receptors or activate the complement system. IgM and IgG cooperate with the complement system, but not IgE and IgA [67]. Like IgG, IgE can be bound be both high and low affinity isotype specific receptors [56, 68]. Otherwise, IgG4 is a non-cytophilic IgG subclass that is not bound by Fc receptors, hence can be involved in maintenance or induction of tolerance.

Several Fc domain binding receptors have been identified, with different affinities and isotype specificities. IgA is bound by the FcαRI (CD89) or transferrin receptor (CD70). IgE is bound by the high affinity FcεRI or low affinity FcεR (CD23). Binding of IgG occurs by high affinity FcγRI (CD64) and low affinity FcγRII (CD32) and FcγRIII (CD16). Expression of those receptors varies per cell type. Monocyte derived DCs may preferentially express the low affinity receptor CD32, whereas monocytes also express CD89, some of the high affinity receptor CD64 and minor levels of CD16 [69-71]. Ligation of CD89 by IgA-antigen complexes results in production of IL-10. Expression of CD16 is more prominent on neutrophils, which can become activated upon binding of immune complexes. Similarly, to initiate an intracellular signaling cascade via CD32, complexes may be required [69, 72]. It is important to note that not all IgG subclasses are bound by the Fc gamma receptors. Mainly IgG1 and IgG3 are bound, which are therefore called cytophilic IgGs. IgG2 and especially IgG4 are well-known for their relatively tolerogenic features in allergy, which could be explained by the lack binding of those subclasses by Fc receptors.

To be functionally active on mucosal surfaces that are exposed to the external milieu, transport over the epithelium is required. This transport is restricted to J-chain linked IgA or IgM that can be transported by the pIgR. Therefore pentameric IgM and dimeric IgA specifically are able to exert a protective role at mucosal surfaces. Next to pIgR, the neonatal Fc receptor (FcRn) can be expressed at the mucosa. The most well-known role of FcRn is to transfer maternal IgG present in breast milk over the intestinal epithelium [73].

Systemic and local immune responses

An important feature of the adaptive immune system is that a local antigenic challenge can result in a systemic memory Th cell pool [74, 75]. For primary activation of naïve Th cells professional APC's like DCs are required. Intestinal DCs migrate through the lymph to the MLN upon activation [76]. Maturation of naïve Th cells into a systemic matured Th cell population requires homing of activated peripheral cells to lymphoid organs and subsequent recirculation.

Activated APC's from the intestine travel to the mesenteric lymph node to present digested peptides to Th cells [52]. From the MLN cells recirculate through the bloodstream and contribute to systemic immunity. Typically, allergy is characterized by a systemic Th2 and an IgE producing B cell population. Peripheral B cells recognizing antigen are easily activated when matured Th cell with similar antigen specificity are encountered [77].

IgA is the dominantly produced immunoglobulin class at mucosal surfaces. Production of IgA

does not solely depend on Th cells, but can also be induced by local DCs. It even is hypothesized that large amounts of locally produced IgA is independent of T cell help [65, 78] and probably remain at the mucosa and is not disseminated through the body. This could relate to the nature of the effector function of mucosal IgA, to form a first line of defence by binding to general molecular patterns on bacteria. To exert this function, polyspecific IgA molecules are produced which do not depend on affinity maturation, in contrast to IgA responses to specific pathogenic or toxic structures. Induction of IgA can occur via the epithelial-DC axis. Epithelial cells express toll like receptors an can produce the cytokine APRIL [18].

II ALLERGIES

Type I allergy is an IgE mediated hypersensitivity to in principle harmless food proteins and inhalant allergens like pollen en dust. Production of allergen specific IgE depends on Th2 cells that produce the cytokines IL-4 and IL-13. Those cytokines induce production of IgE. IgE is secreted and recirculates through the body and is bound by high affinity IgE receptors expressed on among others basophils and mast cells. Allergen bound by IgE immobilized on those cells can cause degranulation resulting in allergic symptoms within half an hour or even minutes after encountering the allergen.

Allergy is associated with reduced numbers of Tregs, which inhibit production of IgE by producing IL-10. Tregs also have the ability to enhance production of allergen specific IgG4, which can compete with IgE epitopes present on allergens and thereby prevent activation of mast cells and basophils. Below different diagnostic approaches and the role of the different immunoglobulin isotopes in relation to allergy are discussed in more detail.

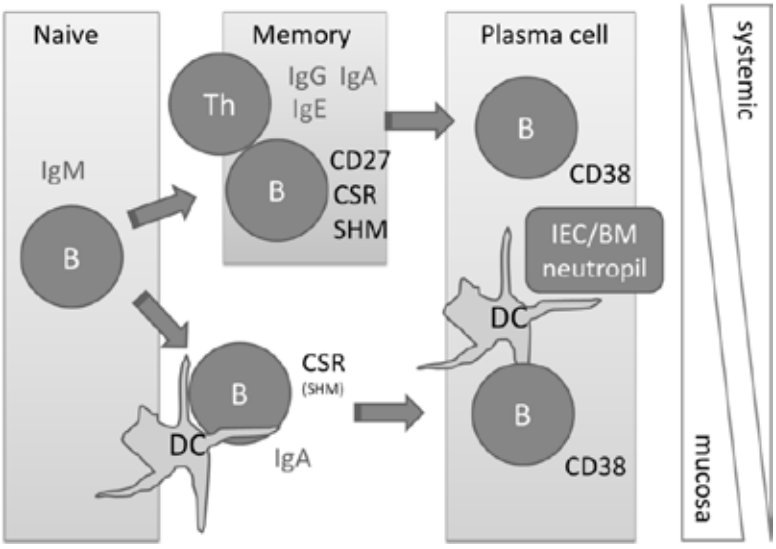

Figure 3. *Different stages of B cell differentiation, under the influence of Th cells or DCs. Th cell are more potent inducers of SHM and may more often induce CSR to IgE and IgG compared to DCs, which preferably induce IgA under homeostatic condition in the mucosa. These different routes of B cell differentiation may differ in frequency between systemic and mucosal tissues. Terminal differentiation to plasma cell is enhanced by mucosal factors and plasma cells are also maintained in the bone marrow.B is B cell, Th is T helper cell, CSR is class switch recombination, SHM is somatic hypermutation, DC is dendritic cell, IEC, intestinal epithelial cell, BM is bone marrow.*

Inhalant and food allergy

The gastro-intestinal and respiratory tracts have a number of features in common, but also differences exist with implications for allergy. The gasto-intestinal tract harbours high loads of microbial antigens and more than 100 grams of food proteins are encountered on a daily basis. Compared to this, the antigenic load and composition is very different in the respiratory tract. The intestines are also characterized by more abundant lymphoid follicles and Peyers patches (several hundreds in a human gut) compared to the respiratory tract. It may be partly due to these differences in antigenic content that explain a better correlation between allergen specific IgE and clinical status of patients for inhalant allergens compared to food allergens.

Types of immune cells found in the respiratory tract seem to resemble those found in the intestine. Both have epithelial cells producing TSLP that is involved in induction of Th2 cells, mucus producing goblet cells and B cells producing IgA. However, inhalant allergy may result in development of chronic asthma, mediated by infiltrated inflammatory cells like eosinophils. A number of inhalant allergens, like house dust mite, fungi and pollen can have protease activity and coackroach and house dust mite allergens are accompanied with endotoxin from faeces. Though the mechanisms haven't been elucidated, the protease activity of allergens and presence of endotoxin may enhance the allergenicity of airway allergens. In addition, doses of exposure may vary between inhalant and food allergen. Finally, in contrast to inhalant allergens, food allergens are exposed to food digestions processes, including pH changes and enzymatic modifications.

In conclusion, though the gastro-intestinal tract and respiratory tract harbour similar immune cells and structures, food and inhalant allergy have important different features.

Diagnosis of allergy

Primary diagnosis of allergy is performed by anamneses by a trained physician, aiming at identifying if the patient is truly suffering from allergy, and subsequently what type of allergy. History of exposure to allergens and clinical manifestations are used to indicate what type of allergen or allergens may cause the allergic reactions. In many cases several diagnostic tools are employed to obtain a more precise diagnosis. Diagnosis of allergy aims at discriminating allergic sensitization from clinically relevant allergy. The diagnostic tests described below mainly discuss the assays commonly applied in research settings, which assess allergic sensitization, but do not link to clinical status or at best remotely relate to clinical features of allergy [79].

One of the most common used analysis is serological detection of allergen specific IgE. In general, the presence of specific IgE is a prerequisite to diagnose allergy, except for very young children. Though the presence of allergen specific IgE is generally considered to be a prerequisite for allergy, it often doesn't correlate with severity of the symptoms encountered. More laborious or invasive diagnostic tools, like skin prick testing, double blind provocations or a mediator release assay are used to enhance the clinical relevance of the diagnosis. As skin prick testing is performed on the subject, presence of specific IgE is not the only determinant of the response measured. One of the essential differences is that for skin prick testing degranulation of for instance mast cells is required to from a wheal. Mast cell degranulation does not only depend on the presence of specific IgE on its membrane, but also on the epitopes detected and the location of those epitopes on an allergen [80]. Several IgE molecules are required to bind an allergen at the same time to result in degranulation. Detection of allergen specific

IgE does not provide information of the likeness of mast cell degranulation. To circumvent this, mediator release assays are used [81]. A commonly used assay is the basophil activation test (BAT), which requires allergen crosslinking of IgE loaded on the cell membrane *in vivo* to result in activation of the cell.

In specific cases, IgE does not explain the allergic symptoms observed. Recent research indicates the role of IgG in activating for instance basophils [82]. But also neutrophils could be activated by allergen specific IgG that binds to FcγRII [83]. The IgG subclass 4 is linked to protection against allergy, by blocking formation of allergen-IgE complexes on either basophils and mast cells or B cells [84].

To study cross-reactivity between allergens, inhibition ELISA's or western blots are used [85]. In both assays binding of allergens by antibodies are detected. Binding by antibodies reflects immunogenicity of the allergens, while the above mentioned ability of allergens to crosslink mast cell bound IgE and cause mediator release is called allergenicity of the allergen. Serum samples can be pre-incubated by another allergen resulting in decreased levels of unbound allergen specific IgE. In case pre-incubation with the other allergens results in reduced detection of antibodies binding to the immobilized allergen, it is concluded that the same antibody is involved in binding both the immobilized and the pre-incubated allergen.

The most physiological and reliable (when performed properly) diagnostic tool is nasal (inhalant allergen) or oral (food allergen) provocation, preferably performed doubled blinded and randomized [86]. Disadvantage of this method is the risk and discomfort for the patient and the costs. Even though double blind placebo controlled provocation is considered the best available diagnostic tool, interpretation of outcomes still can be dubious. As described later in this introduction, nasal and oral provocation also involves potential protective effect of locally present IgA antibodies, which are less abundantly present in serum.

A problematic group of patients to diagnose are for instance young (one year of age) children allergic to cow's milk as often no specific IgE can be detected [87, 88].

Allergy and immunoglobulins

Immunglobulin isotypes in relation to allergy

Most allergies depend on the presence of allergen specific IgE. This IgE bound by the high affinity IgE receptor was still present after six weeks in a mouse model [89]. Specific IgE was injected in B cell deficient mice. IgE was undetectable after six days, but animals could still develop allergic symptoms after challenge. The receptors for IgE on mast cells and basophils are of high affinity that rapidly binds IgE in solution. This kinetics likely affects the relation between serologically detectable IgE and cell-bound IgE resulting in absence of soluble IgE whilst IgE is still present on the membranes of the mast cells and basophils. Probably, the finding that IgE still can cause clinical symptoms allergies while soluble IgE is undetectable can explain the absence of detectable IgE in for instance young cow's milk allergic children.

Allergen specific IgE levels do not correlate very well with clinical symptoms, especially for food allergy [79, 90]. IgE levels regulate the amount of IgE receptors on basophils and mast cells [91]. Therefore it is unlikely that expression levels of the high affinity IgE receptor could explain the poor correlation between specific IgE levels and severity of the clinical symptoms. Still, the threshold for activation of mast cells and basophils could differ

between individuals. For activation of mast cells and basophils, accessibility of the IgE epitopes is crucial. This accessibility can be affected by other immunoglobulin classes that have similar specificity compared to IgE and thereby can block or compete with IgE for binding allergens. Blocking antibodies can be of IgG4 class and probably of IgA as discussed below.

Recently IgD was shown to bind to basophils and cause production of a number of cytokines in including IL-4, antimicrobial factors and B cell-activating factors [92]. Induction of IgD production was reported to occur in the respiratory mucosa [92]. Thus far, the specificity of IgD immunoglobulins has mainly been linked to microbes, and also to inhalant allergens [93]. IgD levels have been linked to atopic allergy [94]. Smoking has been associated with increased IgD levels, though data is conflicting [95, 96]. Taken together, IgD is induced in the respiratory tract and associated with atopy. The precise mechanism is unknown, but data suggests a role for IgD in airway allergy.

Immunoglobulin production and immunotherapy

IgG4 is often used as parameter to assess effectiveness of immunotherapy. IgG4 has been proven to inhibit interaction between IgE and allergens. IgE and IgG4 could recognise the same or different epitopes. To functionally protect, IgG4 needs to prevent crosslinking on cell-bound IgE. This can be achieved by blocking several epitopes, or by steric hindrance. Not necessarily all epitopes have to be blocked, as a single interaction between IgE and allergen strongly reduces mast cell and basophil activation compared to cross-linking IgE-allergen complexes. Studies have shown that epitopes for IgE and IgG4 are partly overlapping [97-99]. An elegant assay to identify the induction of blocking IgG-antibodies by immunotherapy is the facilitated allergen presentation assay (FAP-assay) that identifyies the presence of blocking antibodies by reduced binding of allergen-IgE complexes to CD23 on B cells [100-102].

IgG levels are high in systemic fluids, but low at external mucosal surfaces under homeostatic conditions [103]. Therefore, protection against allergy by IgG4 is mainly expected to be relevant in the internal milieu. At the mucosal surface, antibody-mediated protection against allergy may depend on IgA binding allergens. The majority of IgA is being produced locally in the mucosa. Therefore assessing a relationship between allergen specific IgA is likely more relevant on mucosal fluids and not systemic fluids like serum. In addition, the ratio between IgA subclasses is not the same in different body compartments [104]. Recent efforts have shown an association between increased mucosal IgA levels and protection against allergy [105, 106].

Shellfish allergy

Allergies to shrimp, lobster, crab, mussel, oyster and others are referred to by the common name shellfish allergy. Prevalences of self-reported mollusc allergy ranges from about 0.15% (4/2716) in school children in France [107] to about 0.4% (or 20% of all seafood allergic cases) in a household survey of 14,948 individuals in the USA [108]. In a meta-analysis, shellfish allergy prevalence ranged from 0 till 1.4% or was on average 2.5%, depending on whether numbers were based on self-reported or skin prick testing or IgE sensitization [109]. This allergy is persistent, severe and presents itself with eczema, urticaria (hives), allergic asthma, gastro-intestinal problems, and anaphylaxis. In westernized countries shrimp allergy occurs most frequently [110]. Most occurring route

of exposure to shellfish is the gastro-intestinal tract. However, there are also some reports about inhalant exposure by shellfish workers [111, 112].

The group of shellfish allergens is composed of crustacean and molluscan species, which are biological distinct groups. This is also reflected when tropomyosin sequences are aligned, which result in separate clusters for crustacean and molluscan [113]. The fact that crustacean and molluscan are different is also reflected in much more sensitizations to for instance shrimp compared to mussel or oyster [113, 114]. Of course, also consumption patterns vary, and levels of allergens in those species.

Tropomyosin

The major shell fish allergen is identified as tropomyosin [115]. The protein tropomyosin has an uncomplicated tertiary structure; two chains are intertwined in parallel direction. Tropomyosin is involved in muscle action and is able to resist heat treatment relatively well. Tropomyosin levels differ between different seafood species and the specific amino acid composition of tropomyosin is species specific. Tropomyosin is referred to as a highly cross-reactive pan-allergen of shellfish and logically, its amino acid composition is conserved [116, 117]. As a result, initial sensitization to tropomyosin of one species could result in allergy to a whole group of foods.

Next to sea food, tropomyosin is present in terrestrial species like cockroaches and probably more important, house dust mite [118]. Although house dust mite doesn't belong to crustacean (eg shrimp, lobster, crab), its tropomyosin sequence is highly similar (approximately 80%). It has been reported that shrimp allergic patients also had specific IgE against tropomyosin of house dust mite [119].

House dust mite allergy

House dust mites are widely present in the environment in-house of Westernize societies. Major exposure to allergens from house dust mite originates from faeces present in for instant pillows and mattresses. Related to house dust mite are storage mites. House dust mite is an important cause for asthmatic symptoms in children and exposure was associated with wheeze and asthma [120, 121]. Several allergens have been identified, including tropomyosin. Though tropomyosin is not a major house dust mite allergen, it still could be a potential sensitizer and cause cross-reactivity with shellfish species.

As tropomyosin of house dust mite is very similar to tropomyosin of crustacean species, there is a potential risk for house dust mite to be a primary sensitizer resulting in subsequent sensitization to other crustaceans. This has been reported for lobster, but was not caused by tropomyosin [122]. Cross reactivity between shrimp and house dust mite has been reported, but the primary sensitizer is not always identified [119].

House dust mite allergy is an inhalant allergy, therefore primarily affecting nasal and oral surfaces. Cross-reactivity to food allergens like shrimp and lobster will result in exposure of the intestinal mucosa with subsequent intestinal related allergic symptoms. It should be noted that inhaled allergens can be swallowed and subsequently reach the gastro-intestinal mucosa.

Mucosa and allergy

The majority of known allergens are primarily encountered at the respiratory or intestinal mucosa. Inhaled allergens that reach the nasal mucosa and trachea can be transferred to the gastro-intestinal tract by cilia that can clear allergens from the respiratory tract. Consequently,

inhaled allergens can also be encountered at the mucosa of the gastro-intestinal tracts. The allergenicity of an allergen may depend partly on the IgA present at the site of exposure. Allergen specific IgA might prevent binding of the allergen by IgE and therefore reduce the allergic reaction in number and/or severity.

Assays to monitor immunotherapy or perform diagnosis of allergy rely on systemically present antibodies, mainly IgE and IgG4. IgG4 can be induced by immunotherapy resulting in reduced sensitivity to the allergen [123]. However, IgG4 is not actively transported in large amounts to the mucosal surfaces or locally produced in the mucosa, like IgA. Therefore the protective role of IgG4 against allergy might be mainly at the systemic levels, whereas IgA might prevent allergy at mucosal surfaces.

Data describing systemic processes in allergy and tolerance induction by specific immune therapy is becoming widely available [124, 125]. Tregs have been identified as mediators for re-introducing tolerance to allergens, involving both IL-10 and TGF-ß [126]. Those Tregs can be allergen specific and produce IL-10 that inhibits production of IgE and enhances production of IgG4 and IgA. Next to an active site for Treg induction by tolerogenic DCs, the mucosa are sites of local antibody class switch recombination. Local features of class switching to IgE and IgA have recently been identified [18, 54, 65, 127]. Class switching to IgE was observed after allergen exposure in the nasal mucosa.

Though still debated, reports are increasingly showing an association between IgA levels and protection against allergy [106, 128]. IgA is mainly present at the mucosa and to a large extent produced locally. Therefore clinical relevance of Ig detection may be enhanced by combining serological detection of allergen specific IgE with detection of (allergen) specific IgA in mucosal secretions. Also IgA levels in breast milk have been related to allergy in the offspring [129, 130]. Many attempts to relate IgA levels to allergy have been unsuccessful or could only partly be related [131, 132]. This may be explained by the notion that IgA in serum doesn't resemble mucosally produced IgA. This is confirmed by reports showing that salivary IgA does correlate with protection against allergy [106, 133]. In addition to the fluid used for IgA detection, discriminating IgA1 and IgA2 may enhance specificity of the assay. This is in line with a report showing allergen specific IgA2 in nasal tissue after allergen specific immunotherapy [105]. Another indication is the organization of the human immunoglobulin operon, where IgA2 is located downstream of IgE, which therefore is the only class switch possibility for IgE producing B cells (human genome, genbank build 37.3).

III RESEARCH AIM AND THESIS OUTLINE

Research aim

Type I allergy is an IgE mediated disease that is dependent on activation of allergen specific Th2 cells. IgE needs to be specific for an allergen, and in addition different IgE-molecules have to bind to the allergen at multiple locations to efficiently cross-link and activate mast cells and basophils. The first part of the work described in this thesis aimed at identifying IgE-reactivity to allergens, either in relation to primary sensitization or in relation to potential cross-reactivity to related allergens.

As allergens are mainly encountered at mucosal sites, we also studied if the presence of mucosal IgA can protect against symptoms of allergy. To this aim, allergen-specific IgE and IgA levels to house dust mite allergens were related to clinical status. As humans produce two subclasses of IgA, IgA1 and IgA2, we studied if allergen specific levels were different for IgA1 and IgA2 and whether IgA1 and IgA2 levels were different between serum (systemic) and saliva (mucosal). Differences in IgA1 and IgA2 levels to allergens in relation to clinical status of patients might have implications for diagnostics in the clinic, and for strategies for immunotherapy. Especially IgA2 production may be initiated by DCs that are conditioned by epithelial cell-derived TGF-ß and RA. Therefore we aimed at identifying the effect of TGF-ß and RA on DC phenotype and functionality and at identifying mucosal factors derived from epithelial cells and DCs that could selectively induce production of IgA2 in a T-cell independent manner. Allergies can develop early in life and have been associated with microbiota composition in the intestines. Knowledge on the development IgA production and antigen specificity repertoire is limited. Therefore, also the ontogeny of intestinal IgA repertoire and production in relation to microbiota composition may contribute to our understanding of the role of the mucosal IgA production in allergy. Dairy products form an important part of the western diet. Bovine milk contains cytokines and antibodies. We wanted to know whether these cytokines and antibodies could interact with the human immune system and thereby modulate the mucosal immune responses.

Thesis outline

Activation of mast cells requires cross-linking of several IgE molecules bound by the high affinity IgE receptor (FcεR1) on the membrane by binding to allergens (**Chapter 1**). This cross-linking requires the presence of at least two IgE binding epitopes on the allergen. **Cross-reactivity** of IgE molecules with allergens from related species also requires multiple IgE epitopes on the allergen to induce cross-linking and to become clinically relevant. If the allergen to which IgE binds in a cross-reactive manner only has a single epitope to which igE can bind, the allergenicity of the cross-reactive allergen is significantly reduced. Location of IgE epitopes and likeliness of cross-reactivity was studied using tropomyosin from shellfish (**Chapter 2**).

For diagnostics of cross-reactive allergies elucidation of the primary sensitizing allergen may be required. To this extent **inhibition assays** are applied, which point to the primary sensitizer as the allergen that inhibits most of the IgE reactivity. We used ImmunoCap allergens as inhibitory source for fig and ficus cross-reactive allergy as described in **chapter 3**.

Serological detection of allergen-specific IgE is a widely used method to identify the allergens a patient is sensitized to. Though for most allergies presence of specific IgE is required, it poorly relates to clinical status of patients. As the vast majority of allergens is encountered on mucosa surfaces, we studied if allergen-specific levels of the mucosal immunoglobulin class **IgA** is related to **clinical status of patients**. In **chapter 4** we correlate allergens specific IgA levels to clinical status of house dust mite allergic patients. In addition, the difference between the IgA subclasses IgA1 and 2 was studied.

As the majority of IgA is produced at the mucosa and several mechanisms of IgA induction have been described, we wondered if IgA1 and IgA2

production is differentially regulated. Recent data has shown a role for **DCs** in IgA induction. Tolerogenic CD103$^+$ DCs are able to produce TGF-ß and RA that induce Tregs but also may enhance IgA production. As data on the role of DCs in regulation of IgA subclass production is not available in the literature, we aimed at the role of DCs in production of **IgA subclasses** rather than total IgA. To this extent, we characterized the effect of mucosal factors RA and TGF-ß1 on DC phenotype and functionality (**chapter 5**) and the role of DCs and factors produced by DCs on IgA production (**Chapter 6**).

Environmental factors in early life influence the development of allergies, both early and later in life. As production of IgA has to be initiated during ontogeny by environmental factors, those environmental factors may affect IgA antigen specificity development. Those environmental factors consist for al large part of bacterial ligands. The interplay between initiation of the IgA immune response and microbiota in early life is poorly studied. In **chapter 7** we present data about the interplay between microbiota and IgA production and repertoire after hatch in layer hens.

The intestinal immune system is exposed to about 100 grams of dietary proteins daily. Dietary proteins can modulate the immune system. Dairy products form an important part of the diet, especially for infants. Like breast milk, cow's milk contains proteins of the immune system including cytokines and antibodies. **In chapter 8** the immune regulating effect of **bovine IL-10** on the human immune system is studied. Likewise, in **chapter 9** we studied the interaction of **bovine IgG** with the human immune system.

The findings discussed in chapters 2 till 9 are discussed and related to the literature. Implications of the results obtained and directions for future research are discussed in **chapter 10**.

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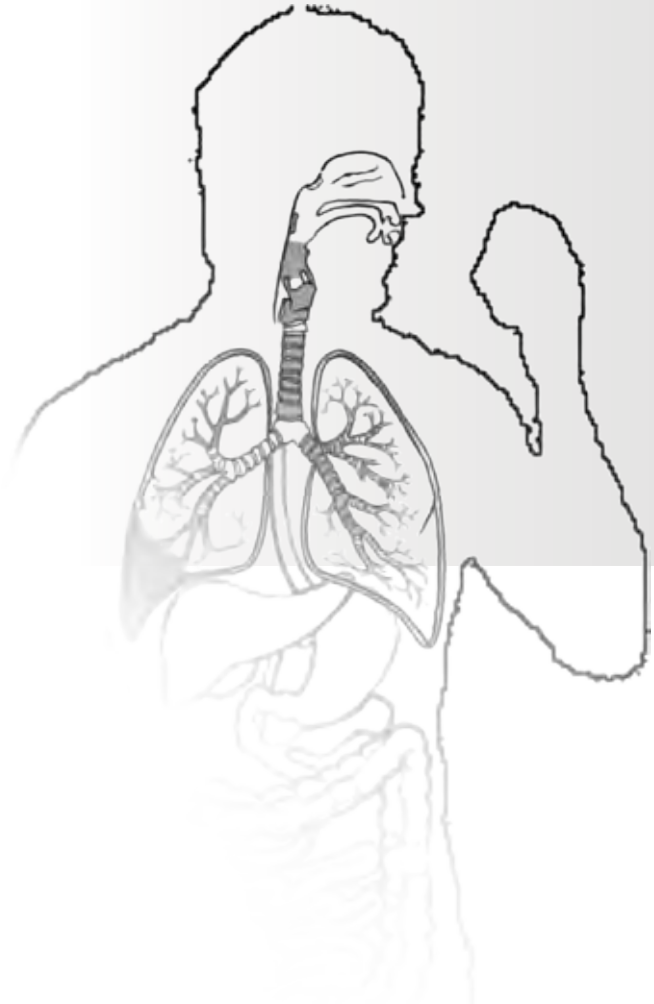

chapter 2

Limited cross-reactivity of shellfish tropomyosin-specific IgE to molluscan tropomyosin

Gerco den Hartog MSc, Lara Cornel MSc, Ad P. H. Jansen MD, Theo H. W. M. Roovers MD, R. J. Joost van Neerven PhD, Janneke Ruinemans-Koerts PhD, Aalt D. J. van Dijk PhD, Aad Smaal PhD and Huub F. J. Savelkoul PhD

ABSTRACT

Seafood-allergic patients react more frequently, and with more severe allergic symptoms, to consumption of crustacean than to consumption of mollusc. Our objective was to explain this difference between allergy to crustaceans and molluscs.

IgE reactivity to the major seafood allergen tropomyosin was studied by comparing the occurrence and composition of IgE-epitopes, and sequence similarity between different crustacean (shrimp, lobster, crab) and mollusc (mussel, squid, oyster) seafood species. Data on IgE-epitopes were collected from the literature and related to tropomyosin-specific IgE levels of seafood allergic patients. By 3D modelling tropomyosin, the location of IgE-binding epitopes relevant to patients clinically allergic to seafood was analysed.

Phylogenetic analysis showed that tropomyosin full-length sequences, as well as the IgE-binding epitopes, are divergent between crustacean and mollusc species. Likewise, IgE from seafood-allergic patients could discriminate mollusc from crustacean tropomyosin. IgE levels to allergens of different seafood species were associated with similarity of IgE-binding epitopes of tropomyosin of those species. Analysis of IgE-epitopes and amino acid residues essential for IgE binding totropomyosin of the most studied crustacean species shrimp revealed that the epitope located in the C-terminus (position 249 – 281) most likely is the most important cross-reactive IgE epitope in seafood tropomyosin.

We conclude that tropomyosin IgE-binding epitopes and IgE reactivity patterns are different between crustacean and mollusc allergens. Consequently, crustacean and mollusc allergy may have to be diagnosed differentially.

INTRODUCTION

Consumption of seafood, whether species of crustaceans or molluscs, can cause severe hypersensitivity reactions including IgE mediated anaphylactic reactions. The major seafood allergen identified is the muscle protein tropomyosin, which is highly conserved throughout the animal kingdom. IgE-cross-reactivity between tropomyosin of different species has been reported frequently, mainly for crustaceans [1-8]. Though different seafood allergies share the major seafood-allergen tropomyosin, shrimp allergy has been studied and described most often and seems to occur most frequently as evidenced by epidemiological studies [9-11].

Cell culture experiments in our laboratory with cells from healthy donors could not explain differences in sensitization to shrimp (crustacean) and mussel (mollusc) (data not shown). Dendritic cells were cultured in the absence or presence of T cells and stimulated with different concentrations of shrimp or mussel extracts. No increased DC activation or DC or T cell cytokine production was observed upon stimulation with shrimp extract compared to mussel extract.

In addition to sensitization frequency, severity of symptoms vary between patients allergic to crustaceans or molluscs[10]. The observation that frequency

and severity of mollusc and crustacean allergy varies might suggest that cross-reactivity of IgE binding to allergens of those animal (sub)phyla plays a limited role in the clinical picture. This would be in accordance with the fact that mollusc and crustacean species are clearly distinct groups in the animal kingdom. However, until now the role of tropomyosin in cross-reactivity between crustacean and mollusc allergy is still controversial. In addition, some clinics distinguish crustacean and mollusc allergy, while others refer to seafood only. To resolve this controversy, a detailed comparison of IgE-reactivity and epitopes between tropomyosin of both animal classes is needed.

Because clinical relevance of exposure to mollusc allergens and cross-reactivity of IgE-binding to crustacean allergens is unclear in Western countries, we aimed at identifying differences in IgE reactivity to mollusc and crustacean allergens in seafood-allergic patients. The molecular basis for tropomyosin cross-reactivity between crustaceans and molluscs was linked to IgE levels and sensitization prevalence data. Tropomyosin sequences of different species were analysed and related to IgE-reactivity and IgE-epitope specificity. Finally, IgE-binding epitopes were visualised using a 3D model of tropomyosin. Together, our data may help to explain the clinically observed heterogeneity between crustacean and mollusc allergies.

MATERIALS AND METHODS

Patients

Databases of two large allergy centres were screened for case records containing positive (≥0,35 kU/L) results of serum specific IgE tests to any of the following seafood allergen sources: mussel, oyster, squid, octopus, snail, shrimp, lobster, crab and the allergen tropomyosin. From a total of approximate 7500 records over the past 5 years, there were specific IgE measurements noted in 141 cases.

Independent of the retro perspective screening of databases, serum was collected from seven patients with seafood allergy. Their allergies were all based on a convincing medical history of anaphylactic reactions to these foods, reports and clear descriptions of these reactions from emergency department records and on positive IgE sensitization to seafood as documented by skin tests and/or specific IgE levels. Anaphylactic reactions were mainly documented upon ingestion of crackers and shrimp. Because of the anaphylactic character of their allergic reactions no food challenges with seafood were done in these patients. Serum was collected during a follow up visit for further diagnosis in serum collection tubes (Greiner), centrifuged and stored at -20ºC.

Detection of specific IgE

The seven patient sera were analysed for the presence of allergen-specific IgE with ImmunoCAP 100 or 250 (Phadia/Thermo Scientific, Sweden) using default procedures and allergen-CAPs. The test panel (codes behind allergens indicate Phadia ImmunoCAPs used) consisted of: house dust mite (d1), recombinant tropomyosin from house dust mite (d205), shrimp (f24), recombinant tropomyosin of shrimp (pen a1, f351), oyster (f290), lobster (f80), crab (f231) blue mussel (f37) and pacific squid (f58) and scallop (f338).

Western blot

Frozen shrimp or life mussels were obtained by a local fishmonger, Wageningen, The Netherlands. Abdomen of shrimp (most common species: European shrimp Crangon crangon) or abductor muscle of mussel (blue mussel, Mytilus edulis)

was minced, sonicated and centrifuged (20 min 20 000g, 4°C) and stored in aliquots at -20°C until use. Gel electrophoresis was performed using 12.5% SDS-PAGE gels in a mini-protean II gel system (Bio-Rad, USA). 20 µg of non-reduced protein extract was added per lane and 7µl marker (Precision Plus Protein Dual Color Standard, #161-0374EDU, Bio-Rad, USA). Samples were prepared by adding loading buffer. Gels were run for 30 minutes at 80V and subsequently for 60 minutes at 130V.

For western blotting the samples were transferred to a nitrocellulose membrane using a semi-dry transfer system (Bio-Rad) at 15V and 0.25mA per membrane for 40 minutes. The membrane was blocked with 2.75% biotin-free casein (SDT-reagents, Germany) and subsequently five times diluted patient serum was added, and incubated for 1 hour at room temperature on a shaker. For inhibition assays, 500 µl extract (1 mg/ml) was added to 500 µl undiluted serum and incubated for 30 min. before addition to the blot.

The membrane was washed three times with TBS-tween (10mM Tris-Cl, 150mM NaCl, 0.05% tween-20, pH 7.5) and incubated with 250 times diluted biotinylated monoclonal mouse anti human IgE (1 mg/ml, BD Biosciences) for 1 hour on a shaker at room temperature. Subsequently, the membrane was washed (three times) and incubated with 5000 times diluted streptavidin poly HRP80 (SDT-reagents, Germany) for one hour while shaking at room temperature. The developing reaction was carried out using ECL western blotting reagent (GE Healthcare, UK) and subsequently a film (Lumi-Film Chemiluminescent Detection Film, 11666657001, Roche USA) was exposed for 5 minutes or one hour in the dark. After exposure films were developed and fixed (Sigma).

To confirm identity of the proteins at 37 kDa, coomassie stained bands were excised and analysed by nanoLC`-ESI-Iontrap-MS/MS followed by a Mascot database search (Panatecs, Tübingen, Germany).

Tropomyosin sequence analysis

The literature databases Scopus, ISI Web of Science and NCBI PubMed were used to search for papers reporting seafood allergy. Of each paper the method of allergen determination, country, IgE-binding epitopes and epitope determination method (if reported), cross-reactivity, clinical symptoms and age and nationality of the patients was recorded.

Tropomyosin sequences used are indicated in Table S1, including genbank accession numbers. The relation shown in the trees were inferred using the p-distance model Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates and the percentage of replicate trees in which the associated taxa clustered together in a bootstrap test is shown next to the branches. Phylogenetic analyses were conducted in MEGA4 [12].

3D modelling of tropomyosin

To predict the 3D structure of mussel and shrimp tropomyosin, homology modeling using the program Modeller was performed[13]. To obtain a suitable template structure, blast searches were performed against the PDB; based on E-value and resolution of the structure, entry 1c1g was selected [14]. Default parameters were applied in Modeller using the automodel class. Out of 1,000 tropomyosin structures the best one based on objective score was selected for further analysis. For analysis of tropomyosin – IgE Fab fragment interactions, multiple copies of the IgE Fab fragment in PDB entry 2r56 were assembled onto the modelled tropomyosin structure. This was performed by overlaying the bovine Beta-Lactoglobulin allergen bound to IgE in PDB entry 2r56 with the various tropomyosin

epitopes. Figures were generated using MolScript [15] and Raster3D [16].

Statistical analysis

IgE levels to allergens obtained from retro-perspective database searches were entered in IBM SPSS Statistics version 19. IgE level to crustacean and mollusc allergens were compared using non-parametric independent samples median test (Figure 1).

Differences in specific IgE levels to allergens of the seven patients were tested using non-parametric mean comparison, or correlations untransformed or log transformed when needed to obtain normally distributed data.

Correlation between specific IgE levels and epitope or critical amino acid similarity was tested with the non-parametric Wilcoxon rank test.

RESULTS

Differences in diagnosis frequency and IgE reactivity between crustacean and mollusc allergens

Databases of two allergy centres were screened in retrospect for reports about seafood allergic patients. 141 patient reports were found with documented specific IgE levels to any seafood of at least 0.35 kU/L . Of 27 (19%) of those patients mollusc specific IgE levels of more than 0.35kU/L were reported. 135 (96%) of the 141 patients had IgE levels to crustacean of 0.35 kU/L or more. For 25 patients both mollusc and crustacean-specific IgE levels were determined, and only 19 patients were reported to be sensitized to both crustaceans and molluscs, of which the majority (17 out of 19, 89%) had higher levels of specific IgE to crustaceans in comparison with molluscs.

The majority of patient sera were tested for levels

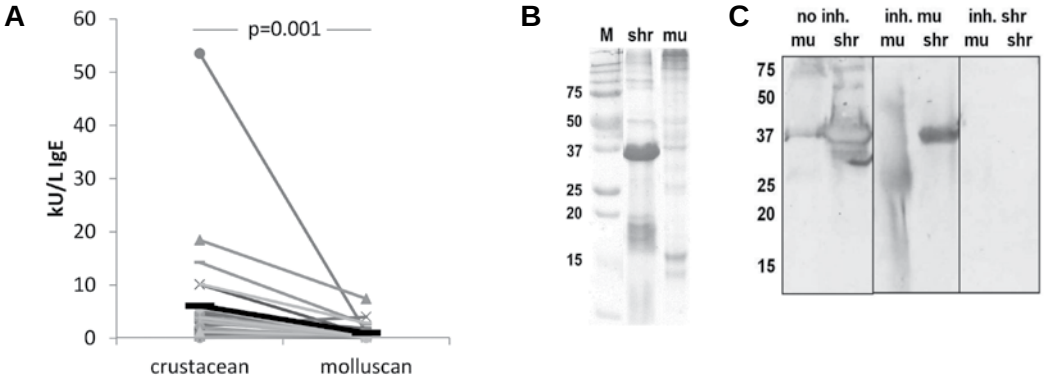

Figure 1. *Specific IgE levels of shellfish allergic patient show that molluscan and crustacean allergens are differentially recognized. Results are shown from serological analysis of 25 patients (A). Each line represents data from one individual. The thick black line with large black bar indicates average specific IgE levels. SDS-PAGE (B) and western blot showing IgE-specific bands (C) of mussel and shrimp extract using serum of shellfish-allergic patients, indicating tropomyosin as the major allergen and very limited cross-reactivity between mussel and shrimp. Mussel (mu), shrimp (shr) and marker (M) were separated on size and transferred to nitrocellulose for western blot analysis. Serum was incubated directly (no inh.) or inhibited with mussel (inh. mu) or shrimp (inh. Shr) extract prior to addition to the nitrocellulose membrane. Western blot analysis was performed for seven patients, yielding similar results.*

of specific IgE against shrimp (120 patients, 85%). 25 of the 27 records containing data on IgE to mollusc also contained data on specific IgE levels to crustacean allergen extracts. Of the 25 patient records containing specific IgE level data against both crustacean and mollusc allergen extracts, the IgE levels between those extracts were compared. Comparison of specific IgE levels against crustacean and mollusc allergens indicated that patient IgE differentially recognized crustacean and mollusc allergens (Figure 1A). Specific IgE levels (range 0.4- 94.9 kU/L) to crustacean allergens were significantly higher compared to IgE levels against mollusc allergens (p=0.001).

To determine the dominant seafood allergens for clinically diagnosed seafood-allergic patients, mussel and shrimp extracts were separated on size (SDS-PAGE) and analysed for IgE-reactivity by western blot. The dominant allergen is around 37 kDa. The protein band at 37 kDa of both shrimp and mussel extract was excised and confirmed to be tropomyosin. We compared several batches of shrimp and mussel extract, and tropomyosin concentrations are consistently higher in shrimp extracts compared to mussel extracts (example shown in Figure 1B). IgE-binding was much higher to shrimp than to mussel extracts. The dominant shrimp allergen bound by patients' IgE was tropomyosin, whereas binding of IgE to tropomyosin of mussel was observed some but not all patients. IgE-binding could be almost completely inhibited when serum was incubated with shrimp extracts, whereas incubation of serum with an excess of mussel extract did not inhibit binding of IgE (Figure 1C), indicating that patients were primarily and mainly sensitized to shrimp and not to mussel allergens, and that IgE from sensitized individuals discriminate between shrimp and mussel allergens.

Literature review of IgE-binding to tropomyosin

As specific IgE levels were observed to discriminate between crustacean and mollusc tropomyosin, a literature search to IgE-binding activity and cross-reactivity to the major seafood allergen tropomyosin was performed. If specified in the publications, we also indicated the primary sensitizing allergen, which is indicated at the top of Figure S1A in red. Cross-reactivities are published between IgE reacting to the class Arachnida, class Malacostraca (subphylum Crustaceans) and class Insecta, and also the phylum Mollusca (mainly classes Bivalvia, Cephalopoda, Gastropoda), which are detailed below.

IgE cross-reactivity is caused by similarity of IgE epitopes present on tropmyosin and is frequently observed between tropomyosin of crustacean species [4, 17]. Cross-reactivity of IgE directed against tropomyosin has been reported across animal classes; between house dust mite and snail [18, 19] and between shrimp, house dust mite and cockroach [20]. Also cross-reactivity between IgE binding to crustacean (shrimp) tropomyosin and different mollusc species has been reported [2]. Those reports also describe cross-reactivity of IgE against tropomyosin between squid (mollusc) and lobster (crustacean) [21]. Most reports about cross-reactivity between crustacean and mollusc originate from Asian countries, whereas reports from American and European countries are less frequent (Table S2).

Sequence analysis and IgE epitopes of tropomyosin

The tropomyosin amino acid sequence is available for many species. Therefore we compared these tropomyosin sequences and studied the IgE-binding epitopes reported in literature. As cross-

reactivity is not defined by the overall sequence identity but by the structural similarities in IgE-binding epitopes, we also compared IgE-binding regions (epitopes) and the reported single amino acids that are essential for IgE reactivity. Epitopes substitution studies showed that the amino acids in the middle of the IgE-epitope is most important for IgE binding and that one or two substitutions could already inhibit most of the IgE reactivity [20, 22]. Tropomyosin sequences were aligned with ClustalW and a neighbor joining tree was constructed (Figure 2A). Crustacean and mollusc tropomyosin sequences form non-overlapping clusters, indicating that sequence similarity within crustacean species and within mollusc species is higher than homology between crustacean and mollusc species. All known tropomyosin allergens in our analysis have a sequence identity with human tropomyosin of less than 60%. Cockroach and mite tropomyosin clustered together with crustacean tropomyosin sequences but not with molluscs. A number of shrimp species (*Litopenaeus vannamei, Penaeus monodon, Farfantepenaeus aztecus* (pen a1)) have identical amino acid sequences of tropomyosin, whereas others differ (*Metapenaeus ensis*, *Crangon crangon*).

When only IgE epitopes (Figure 2B, green shaded) were included for construction of the neigbor joining tree, the division between crustacean and molluscs was maintained, as was the general distribution of species (Figure 2B). When this analysis was performed for amino acid residues critical for IgE-reactivity to tropomyosin of shrimp, crustacean and molluscs sequences still clustered separately, but bootstrap values indicated decreased reliability of the dendogram (Figure 2C).

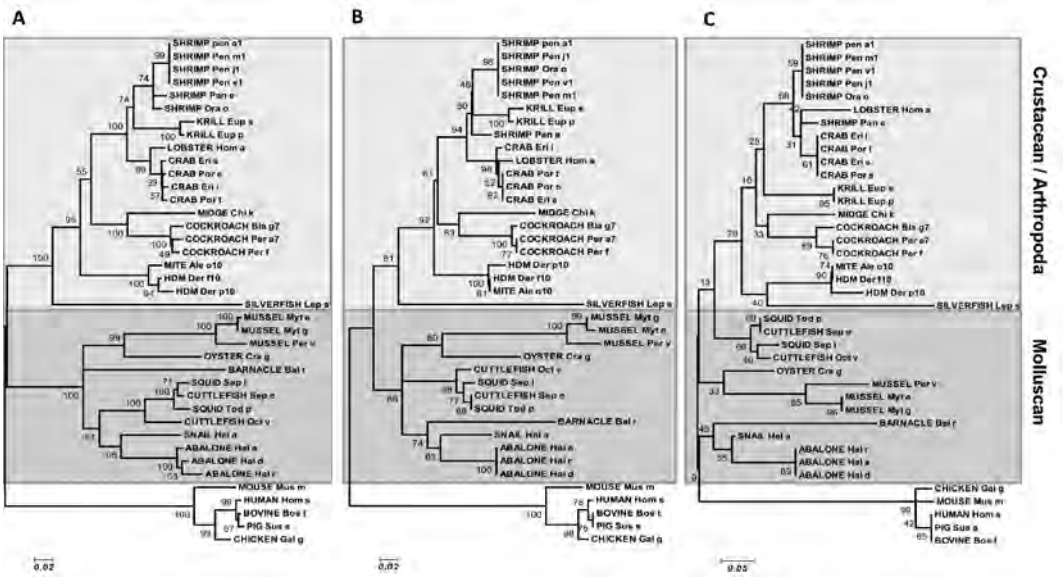

Figure 2. *Neigbor joining tree of (A) the full tropomyosin amino acids sequences (284 positions), (B) the IgE-binding epitopes, including critical amino acids (95 positions) and (C) amino acids critical for IgE reactivity only (20 positions). Especially the topology of A and B is very similar, and the topology of C still distinguishes molluscan (blue shaded) and crustacean (green shaded) sequences. Reliability of the trees is indicated by the bootstrep values (%) shown for each branch.*

Tropomyosin 3D model

At least two IgE molecules have to bind to the distinct sites of cross-reactive allergen to cause mast cell and basophil degranulation resulting in clinically relevant symptoms of allergy. The IgE epitope differences and amino acids reported to be essential for IgE-reactivity were modelled (Figure 3) to identify distribution of the IgE epitopes and assess likeliness of cross-reactivity by modelling amino acids substitutions between species.

Tropomyosin consists of two parallel oriented alpha helices, resulting in a relatively simple tertiary structure (Figure 3). Reported shrimp IgE-binding epitopes and amino acids essential for binding of IgE are indicated in Figure 3A and 3B (coloured residues), including non-homologous substitutions (blue) between shrimp and mussel. Evaluation of the epitope differences indicated high percentage (79%) of identical or homologous amino acids across species for epitope V, low similarity for epitope III (sequence identity 41%) and intermediate similarity for epitopes I, II and IV (sequence identity 46%, 58% and 55% respectively). The lowest observed similarity for epitope III is even more interesting in light of the observation that there is an Asparagine that could putatively be glycosylated (based on the occurrence of an Nx[ST] motif) immediately preceding epitope III in shrimp tropomyosin, which is not present in mussel tropomyosin. Glycosylation could result in increased affinity of binding to IgE [23, 24]. Such putative glycosylation motif is also present at similar positions in various other tropomyosin sequences (octopus (*O. vulgaris*), oyster (*C. gigas*) and sea snail (*T. cornutus*)). The size of the IgE-bound antigen surface in the available structure of an IgE Fab fragment in complex with the major birch pollen allergen (Bet v1) 25 is roughly 20 by 10Å (PDB entry 2r56; data not shown). The IgE-binding epitopes on tropomyosin depicted in figure 3, have similar sizes as determined by measuring the surface that could be approached from one side of the epitope by the IgE Fab. The size of the putative interaction surface ranges from 18 by 10Å for the smallest epitope (epitope IV) to 40 by 10 Å for the largest one (epitope V). In addition, the modelled structures revealed that the different epitopes occur at a quite regular distance from each other, at approximately 70Å (Figure 3C). The roughly ~50Å diameter of the above mentioned IgE Fab structure, impli-

Table 1. *Summary of clinical symptoms of allergic patients.*

age	gender	allergen causing anaphylaxis	Muller	time	other known sensitizations and allergies
15	f	shrimp, lobster	III	-	fruits, grass pollen, tree pollen, animal dander
50	m	shrimp and/or mussel	I - III	45 min	HDM, cat
25	f	mixed (Asian) sea food, but SPT only positive for shrimp	I	< 1 hour	cat, dog
71	m	shrimp	IV	1 hour	HDM
45	f	shrimp	II - III	-	-
42	f	shrimp, crab	II	45 min	tree pollen, grass pollen, apple
53	m	shrimp cracker (exercise induced)	I	<15 min	HDM, grass pollen, tree pollen, animal dander

Time indicates lag between consumption and occurrence of clinical symptoms. – indicates data unavailable; SPT, skin prick test; HDM, house dust mite.

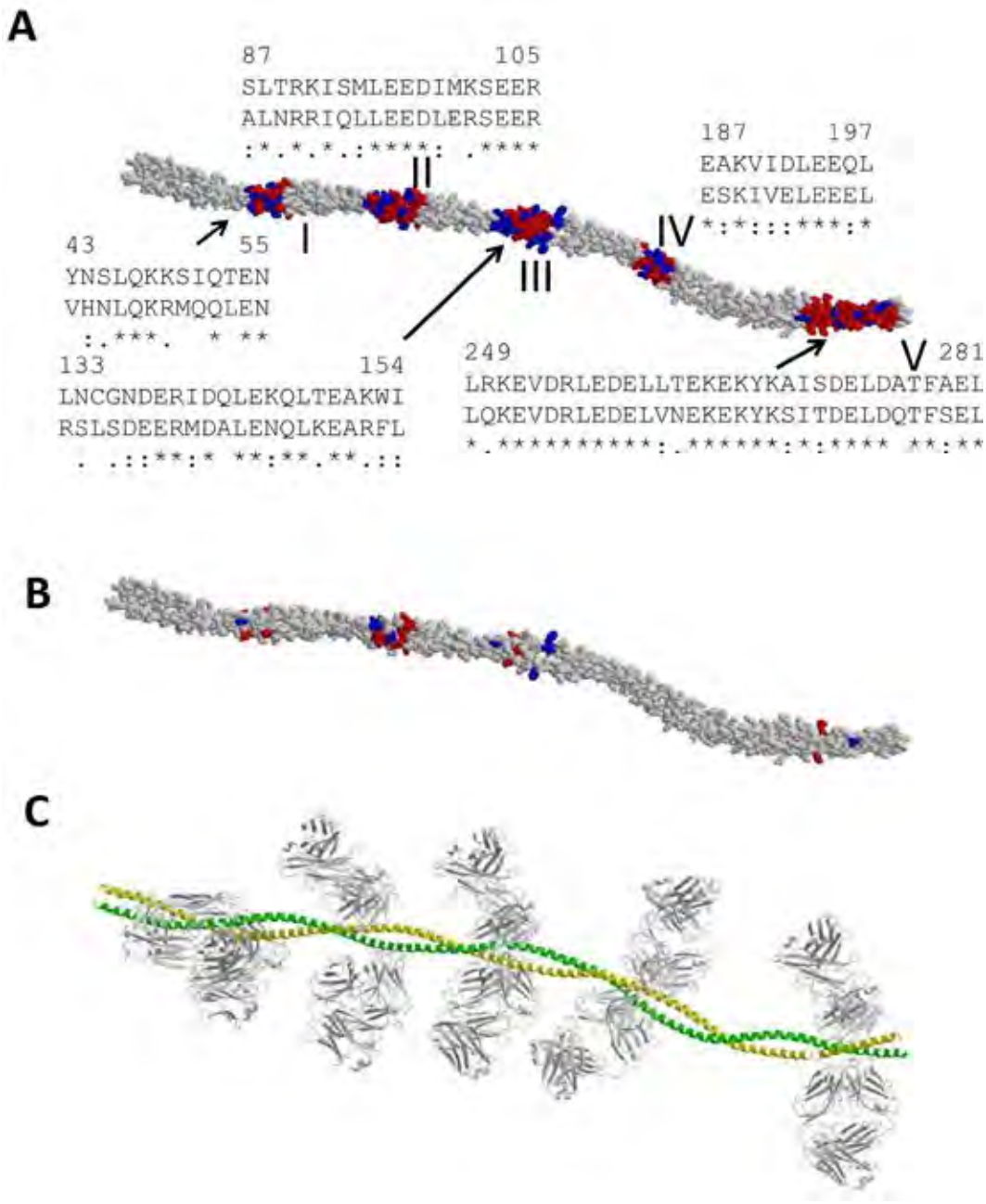

Figure 3. *Structural context of tropomyosin epitopes. (A) Mussel tropomyosin structure model, with epitopes indicated (I-V). Epitope residues in mussel are conserved (red) or non-conserved (blue) when compared with shrimp. Parts of sequence alignment of mussel and shrimps are shown. (B). Critical amino acids for mussel tropomyosin, using similar color scheme as in panel B. (C) Mussel tropomyosin structure model showing two parallel helices (green and yellow) and illustrating potential binding modes of IgE Fab fragments.*

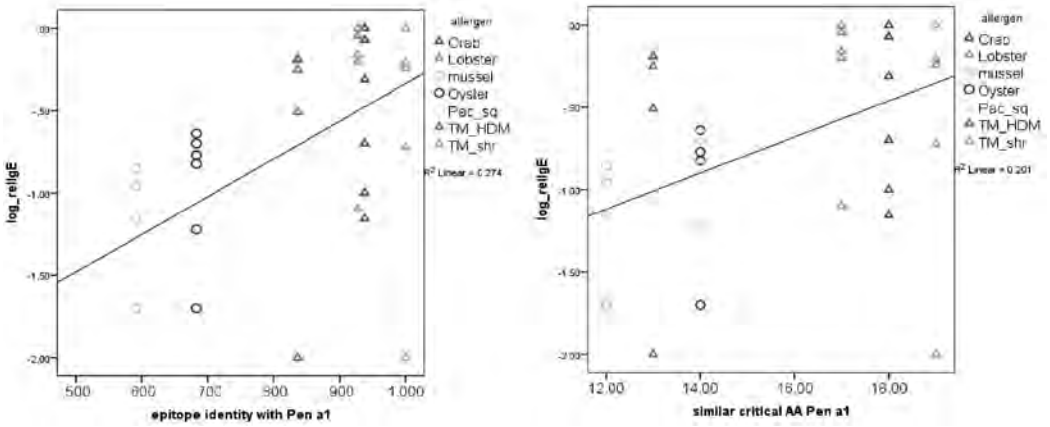

Figure 4. *Allergen specific IgE levels from patients were plotted against similarity compared to shrimp tropomyosin epitopes (A) and amino acids identified to be essential for IgE reactivity of shrimp allergic patients (B). Abbreviations in legend denote pacific squid (Pac_sq), house dust mite and shrimp tropomyosin (TM_HDM respectively (TM_shr). Log_rellgE on y-axes indicates log transformed specific IgE levels.*

cates that shrimp tropomyosin can simultaneously be bound by multiple IgE molecules and thereby cause cross-linking of IgE immobilized on mast cells and basophils.

Specific IgE levels correlate with tropomyosin epitope similarity

Sera from seven patients with a clinically relevant seafood allergy were obtained, independently from the sensitization data used for Figure 1. Seafood allergic patients with anaphylactic symptoms mainly respinded to crustacean allergens, and often were also sensitized or allegic to other allergens (Table 1). The sera were analysed for specific IgE levels to house dust mite (HDM, *Dermatophagoides pteronyssinus*), house dust mite tropomyosin (der p 10), shrimp tropomyosin (pen a 1), crab, lobster, oyster, blue mussel and pacific squid. Consistent with data obtained in retrospect from allergist databases (shown in Figure 1), all patients showed only minor specific IgE levels to mollusc extracts compared to levels to crustacean extracts (p=0.027; data not shown). A non-parametric correlation analysis was performed to test correlations between specific IgE levels to different mollusc and crustacean extracts. Total house dust mite-specific IgE levels did not correlate with any of the other specific IgE levels. IgE levels to tropomyosin of shrimp and house dust mite correlated significantly with the other crustacean-specific (crab, lobster) IgE levels, but not with the mollusc-specific IgE levels (Table 2). Crab- lobster-, pacific squid- and oyster-specific IgE levels correlated significantly with each other, but not with blue mussel. Specific IgE levels to the mollusc pacific squid correlated significantly with IgE levels to the mollusc oyster, but not blue mussel.

Correlations between tropomyosin-specific IgE levels were compared with overall sequence identity of tropomyosin. When specific IgE levels to different species showed a significant correlation, the tropomyosin sequences showed relatively high sequence identity (Table 1). In addition to sequence identity, we correlated IgE-epitope identity with specific IgE levels (Figure 4). A significant correlation was observed between IgE levels and

Table 2. *Analysis of sIgE correlations and corresponding tropomyosin sequence identity.*

		HDM	TM HDM	TM Shrimp	Crab	Lobster	Pacific squid	Oyster	Blue mussel	
HDM			.973	.590	.137	.459	.203	.247	.910	**P-values**
TM HDM		0.021		.001	.051	.018	.124	.957	.861	
TM Shrimp		0.281	0.993		.050	.010	.054	.465	.774	
Crab		0.621	0.877	0.811		.018	.001	.054	.344	
Lobster		0.338	0.939	0.916	0.840		.004	.040	.349	
Pacific squid	**correlation**	0.548	0.775	0.804	0.958	0.910		.014	.151	
Oyster		0.506	-0.034	0.374	0.746	0.776	0.858		.036	
Blue mussel		0.060	0.110	0.179	0.472	0.468	0.663	0.842		
TM HDM										
TM Shrimp			0.802							
Crab			0.82	0.926						
Lobster			0.82	0.933	0.971					
Pacific squid	**TM Sequence**		0.63	0.63	0.63	0.626				
Oyster			0.602	0.616	0.616	0.619	0.753			
Blue mussel			0.552	0.57	0.573	0.573	0.704	0.788		

IgE epitope similarity ($p<0.001$). This was also observed when IgE levels were correlated with amino acids critical for IgE reactivity to tropomyosin (Pen a 1) of shrimp ($p<0.001$).

DISCUSSION

Allergy to crustacean is more prevalent than allergy to mollusc and generally causes more severe clinical reactions. The current report aimed at studying the role of the major allergen tropomyosin in the distinct IgE reactivity to crustacean and mollusc allergens. In order to study this, a detailed analysis of IgE binding patterns and sequence homology of the major allergen tropomyosin was performed, in combination with studying in vitro IgE reactivity of crustaceans and mollusc species.

Among seafood-sensitized patients the prevalence of crustacean sensitized (mainly shrimp) patients was highest in databases of the allergy centres and reports of epidemiological surveys in the United States and Australia[9, 10]. Moreover it is reported that shrimp allergic patients have far more often anaphylactic reactions than patients with mollusc allergy [10].

An extensive literature study was performed to review reported incidences of seafood allergy and the frequency of cross-reactivity between different species. IgE binding to crustacean was more often reported to be cross-reactive with tropomyosin allergens from other species. Furthermore, different mollusc species are studied in Asia (mostly Cephalopda) compared to Europe (mostly Gastropoda) (Table S2). The fact that different allergen sources are studied in different countries could be caused by different sensitization patterns. IgE-cross-reactivity between mollusc and crustacean has mainly been reported by Asian laboratories. Dietary and genetic differences, in line with indications that link ethnicity with IgE levels and atopy may help explain different allergen-sensitizations reported between Asian and Western countries [26, 27]. Ethnic background of patients may be associated with different sensitization patterns and clinical allergic manifestations. Recently, the literature of Asian-Pacific seafood allergies have been reviewed by Lee et. al. [28]. The authors indicate that unnecessary avoidance is advised by clinicians that are not aware of the biological and allergenic differences between seafood phyla. Consequently, in many cases patients allergic for crustacean may safely consume mollusc animals, and vice versa.

Mollusc and crustacean tropomyosin sequences and IgE epitopes clustered separately in a dendograms. This suggests that IgE cross-reactivity between those (sub)phyla might be limited too. Tropomyosin identity between human and seafood is below 60%. But also tropomyosin identity between mussel and shrimp is below 60%, which indicated that cross-reactivity might be quite limited due to the large differences between tropomyosin of crustaceans and molluscs. Consistent with other studies, the differences observed in IgE reactivity to mollusc and crustacean allergen extracts significantly associated with the overall sequence similarity of tropomyosin, as well as the similarity in amino acid-sequences of the IgE-binding epitopes (Table 1 and Figure 6)[29]. The 3D protein model of tropomyosin presented here visualizes the location of amino acid substitutions in IgE epitopes. Tropomyosin has a relatively simple structure and because of the high similarity between the shrimp and mussel tropomyosin sequences with the template sequence, the position of amino acids in the model is expected to relatively accurately predict surface exposure of epitopes to IgE. Modelling IgE epitopes has an additional added value because of the prediction of spatial distribution of epitopes

30. Crosslinking of multiple IgE molecules on mast cell or basophils may be more or less likely depending on the spatial distribution of distinct IgE epitopes. In this context, given the possibilities for binding multiple IgE molecules that we observed based on the structure model, it is interesting to note that the middle epitope (residues 133 – 154) is most different between shrimp and mussel. If substitution between shrimp and mussel observed in this middle epitope results in reduced binding of cross-reactive IgE, also the risk of clinically relevant cross-linking is reduced. In addition, we propose that a putative glycosylation site which is present in shrimp but not in mussel might result in differential sensitization to shrimp and mussel tropomyosin [31]. Although the occurrence of a glycosylation motif does not necessarily mean that glycosylation occurs, it is relevant to note that a similar site occurs in various other tropomyosin sequences, and that evidence exists for both glycosylation in crustaceans [32] and glycosylation of myofibrillar proteins, in particular myosin and tropomyosin [33].

Interestingly, specific IgE to tropomyosin from house dust mite correlated strongly with shrimp tropomyosin, crab and lobster IgE levels. This could mean that the widely abundant house dust mite tropomyosin is a potential sensitizer for crustacean seafood allergy. But also, shrimp tropomyosin-allergic patients may be sensitized to house dust mite tropomyosin [20].

Most tropomyosin IgE-epitope studies are performed on shrimp tropomyosin. However, we did find a few studies reporting IgE epitopes for mollusc species. The IgE-binding epitopes reported for the mollusc species *Crassostrea gigas, Turbo cornutus* and *Octopus vulgaris* overlapped with the positions of the epitopes reported for shrimp tropomyosin [34-36]. Those studies originate from Asian laboratories and suggest that in those countries mollusc allergy might be more frequent compared to Western countries. The epitope of the *T. cornutus* and *O. vulgaris* (aa 92- 105, equivalent to epitope II) is a partly conserved part of tropomyosin that based on our 3D model may cause cross-reactivity. The other IgE-binding epitopes identified for *O. vulgaris* also overlap with the epitopes identified for shrimp tropomyosin, but those epitopes contain more non-homologous substitutions that could interfere with IgE binding. For clinically relevant IgE cross-reactivity, more than one cross-reactive epitope needs to be recognized. Therefore, the finding that other epitopes contain numerous substitutions at the surface of the protein is a likely explanation for limited IgE cross-reactivity between crustaceans and molluscs. As a consequence, shrimp-allergic patients should not be advised to avoid eating mollusc seafood out of for fear for food allergy.

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Table S1 *Tropomyosin allergens*

Allergen	**Species**	**Genbank no**
abalone hal a	*Haliotis asinina*	Q7YZR0
abalone hal d	*Haliotis diversicolor*	Q9GZ71
abalone hal r	*Haliotis rufescens*	Q25145
barnacle bal r	*Balanus rostratus*	BAF46896
cockroach bla g7	*Blattella germanica*	Q9NG56
cockroach per a7	*Periplaneta americana*	Q9UB83
cockroach per f	*Periplaneta fuliginosa*	Q8T6L5
crab eri j	*Erimacrus isenbeckii*	BAF47269
crab eri s	*Eriocheir sinensis*	ABS12234
crab por s	*Portunus sanguinolentus*	ABL89183
crab por t	*Portunus trituberculatus*	
cuttlefish oct v	*Octopus vulgaris*	Q2V0V0
cuttlefish sep e	*Sepia esculenta*	Q2V0V4
hdm der f10	*Dermatophagoides farinae*	Q23939
hdm der p10	*Dermatophagoides pteronyssinus*	B6E457
krill eup p	*Euphausia pacifica*	A7VKE1
krill eup s	*Euphausia superba*	A7VKE0
lobster hom a	*Homarus americanus*	O44119
midge chi k	*Chironomus kiiensis*	CAA09938
mite ale o10	*Aleuroglyphus ovatus*	AAX37287
mussel myt e	*Mytilus edulis*	Q25457
mussel myt g	*Mytilus galloprovincialis*	P91958
mussel per v	*Perna viridis*	Q9GZ70
oyster	*Crassostrea gigas*	BAH10152.1
shrimp ora o	*Oratosquilla oratoria*	A9CSJ9
shrimp pan e	*Metapenaeus ensis*	Q25456
shrimp pen a 1	*Farfantepenaeus aztecus*	AAZ76743.1
shrimp pen j1	*Penaeus japonicus*	A2V731
shrimp pen m1	*Penaeus monodon*	A1KYZ2
shrimp pen v1	*Litopenaeus vannamei*	B4YAH6
silverfish lep s	*Lepisma saccharina*	Q8T380
snail hel a	*Helix aspersa*	O97192
squid sep l	*Sepioteuthis lessoniana*	Q2V0V3
squid tod p	*Todarodes pacificus*	Q2V0V2
human hom s	*Homo sapiens*	P09493
bovine bos t	*Bos taurus*	Q5KR49
pig sus s	*Sus scrofa*	P42639
chicken gal g	*Gallus gallus*	P04268
mouse mus m	*Mus musculus*	P58771

Table S2 *Overview country of origin of research papers about tropomyosin allergy*

	Papers					Sub-					
continent	**no**	**%**	**Phylum**	**#**	**%**	**Phylum**	**#**	**%**	**Class**	**#**	**%**
Europe	10	15.4	*Mollusca*	4	40.0				*Gastropoda*	3	75.0
									Bivalvia	1	25.0
									Cephalopda	0	0.0
			Arthropoda	4	40.0	*Crustacea*	0	0.0	*Maxillipoda*		
									Malacostraca		
						Chelicerata	1	25	*Arachnida*	1	100.0
									Insecta	3	75.0
			Nematoda	2	20.0						
Asia	41	63.1	*Mollusca*	18	43.9				*Gastropoda*	2	11.1
									Bivalvia	7	38.9
									Cephalopda	9	50.0
			Arthropoda	23	56.1	*Crustacea*	17	73.9	*Maxillipoda*	2	11.8
									Malacostraca	15	88.2
						Chelicerata	3	13.0	*Arachnida*	3	100.0
									Insecta	3	13.0
			Nematoda	0	0.0						
Africa	2	3.1	*Mollusca*	2	100.0				*Gastropoda*	2	100.0
									Bivalvia	0	0.0
									Cephalopda	0	0.0
			Arthropoda	0	0.0	*Crustacea*	0		*Maxillipoda*		
									Malacostraca		
						Chelicerata	0		*Arachnida*	0	
									Insecta	0	0.0
			Nematoda	0	0.0						
N-America	7	10.8	*Mollusca*	1	14.3				*Gastropoda*	0	0.0
									Bivalvia	0	0.0
									Cephalopda	1	100.0
			Arthropoda	6	85.7	*Crustacea*	5	83.3	*Maxillipoda*	0	0.0
									Malacostraca	5	100.0
						Chelicerata	1	16.7	*Arachnida*	1	100.0
									Insecta	0	0.0
			Nematoda	0	0.0						
S-America	5	7.7	*Mollusca*	0	0.0				*Gastropoda*		
									Bivalvia		
									Cephalopda		
			Arthropoda	2	40.0	*Crustacea*	0	0.0	*Maxillipoda*		
									Malacostraca		
						Chelicerata	0	0	*Arachnida*		
									Insecta	2	100.0
			Nematoda	3	60.0						
Total (World)	65	100	*Mollusca*	25	38.5				*Gastropoda*	7	28.0
									Bivalvia	8	32.0
									Cephalopda	10	40.0
			Arthropoda	35	53.8	*Crustacea*	22	62.9	*Maxillipoda*	2	9.1
									Malacostraca	20	90.9
						Chelicerata	5	14.3	*Arachnida*	5	
									Insecta	8	22.9
			Nematoda	5	7.7						

Table S3. *Sequence identity of tropomyosin sequences*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1 HUMAN Hom s		0.99	0.99	0.96	0.90	0.58	0.57	0.56	0.56	0.56	0.57	0.57	0.55	0.48	0.55	0.54	0.55	0.55	0.59	0.57	0.51	0.57	0.57	0.57	0.56	0.57	0.56	0.54	0.54	0.54	0.55	0.55	0.55	0.53	0.56	0.51	0.51
2 BOVINE Bos t	0.99		0.99	0.96	0.90	0.58	0.57	0.56	0.56	0.56	0.57	0.57	0.55	0.48	0.55	0.54	0.55	0.55	0.59	0.57	0.51	0.57	0.57	0.57	0.56	0.57	0.56	0.54	0.54	0.54	0.55	0.55	0.55	0.53	0.56	0.51	0.51
3 PIG Sus s	0.99	0.99		0.95	0.90	0.58	0.57	0.56	0.56	0.56	0.57	0.57	0.55	0.48	0.55	0.54	0.55	0.55	0.58	0.57	0.51	0.57	0.57	0.57	0.56	0.56	0.56	0.54	0.54	0.54	0.55	0.54	0.54	0.53	0.55	0.51	0.51
4 CHICKEN Gal g	0.96	0.96	0.95		0.92	0.58	0.57	0.58	0.58	0.59	0.58	0.58	0.56	0.49	0.56	0.56	0.56	0.59	0.58	0.52	0.58	0.58	0.58	0.57	0.58	0.58	0.56	0.54	0.54	0.55	0.56	0.55	0.55	0.54	0.53	0.56	0.52	0.52
5 MOUSE Mus m	0.90	0.90	0.90	0.92		0.58	0.57	0.57	0.57	0.58	0.58	0.58	0.56	0.48	0.55	0.54	0.55	0.59	0.58	0.51	0.58	0.58	0.58	0.56	0.57	0.57	0.54	0.54	0.55	0.55	0.54	0.53	0.56	0.52	0.52	0.52	
6 HDM Der f10	0.58	0.58	0.58	0.58	0.58		0.98	0.82	0.82	0.82	0.82	0.83	0.78	0.65	0.81	0.81	0.81	0.98	0.83	0.61	0.83	0.83	0.83	0.77	0.78	0.83	0.64	0.65	0.65	0.64	0.64	0.63	0.65	0.56	0.57	0.57	
7 HDM Der p10	0.57	0.57	0.57	0.57	0.57	0.98		0.80	0.80	0.80	0.81	0.82	0.76	0.64	0.80	0.80	0.80	0.96	0.82	0.60	0.82	0.82	0.82	0.75	0.77	0.81	0.63	0.63	0.64	0.63	0.63	0.62	0.61	0.64	0.55	0.55	0.56
8 SHRIMP Pen m1	0.56	0.56	0.56	0.58	0.57	0.82	0.80		1.00	1.00	0.97	0.93	0.79	0.68	0.82	0.82	0.83	0.81	0.93	0.58	0.92	0.93	0.92	0.90	0.92	0.98	0.63	0.63	0.63	0.64	0.63	0.63	0.61	0.64	0.56	0.57	0.57
9 SHRIMP Pen v1	0.56	0.56	0.56	0.58	0.57	0.82	0.80	1.00		1.00	0.97	0.93	0.79	0.68	0.82	0.82	0.83	0.81	0.93	0.58	0.92	0.93	0.92	0.90	0.92	0.98	0.63	0.63	0.63	0.64	0.63	0.63	0.61	0.64	0.56	0.57	0.57
10 SHRIMP Pen j1	0.56	0.56	0.56	0.58	0.57	0.82	0.80	1.00	1.00		0.97	0.93	0.79	0.68	0.82	0.82	0.83	0.81	0.93	0.58	0.92	0.93	0.92	0.90	0.92	0.98	0.63	0.63	0.63	0.64	0.63	0.63	0.61	0.64	0.56	0.57	0.57
11 SHRIMP Pan e	0.57	0.57	0.57	0.59	0.58	0.82	0.81	0.97	0.97	0.97		0.93	0.79	0.69	0.82	0.82	0.83	0.81	0.92	0.59	0.92	0.93	0.92	0.88	0.90	0.96	0.64	0.63	0.64	0.65	0.64	0.63	0.62	0.64	0.57	0.58	0.58
12 LOBSTER Hom a	0.57	0.57	0.57	0.58	0.58	0.83	0.82	0.93	0.93	0.93	0.93		0.80	0.68	0.83	0.82	0.83	0.83	0.97	0.60	0.96	0.97	0.97	0.86	0.87	0.94	0.63	0.63	0.64	0.64	0.63	0.62	0.61	0.63	0.57	0.57	0.58
13 MIDGE Chi k	0.55	0.55	0.55	0.56	0.56	0.78	0.76	0.79	0.79	0.79	0.80			0.64	0.87	0.86	0.87	0.78	0.80	0.58	0.80	0.80	0.80	0.75	0.77	0.80	0.61	0.62	0.62	0.62	0.60	0.60	0.59	0.62	0.55	0.56	0.56
14 SILVERFISH Lep s	0.48	0.48	0.48	0.49	0.48	0.65	0.64	0.68	0.68	0.68	0.69	0.68	0.64		0.64	0.65	0.65	0.65	0.68	0.48	0.67	0.68	0.68	0.66	0.66	0.68	0.58	0.57	0.57	0.59	0.55	0.55	0.54	0.56	0.48	0.49	0.49
15 COCKROACH Bla g7	0.55	0.55	0.55	0.56	0.55	0.81	0.80	0.82	0.82	0.82	0.83	0.87	0.84		0.97	0.98	0.81	0.83	0.57	0.83	0.83	0.83	0.77	0.79	0.83	0.60	0.61	0.62	0.62	0.61	0.60	0.62	0.56	0.57	0.57	
16 COCKROACH Per a7	0.54	0.54	0.54	0.56	0.54	0.81	0.80	0.82	0.82	0.82	0.82	0.86	0.85	0.97		0.98	0.81	0.83	0.58	0.83	0.83	0.83	0.77	0.79	0.83	0.61	0.61	0.62	0.62	0.62	0.61	0.60	0.63	0.56	0.57	0.57	
17 COCKROACH Per f	0.55	0.55	0.55	0.56	0.55	0.81	0.80	0.83	0.83	0.83	0.83	0.87	0.85	0.98	0.98		0.81	0.84	0.58	0.84	0.83	0.84	0.78	0.80	0.83	0.61	0.61	0.62	0.62	0.61	0.60	0.63	0.56	0.57	0.57	
18 MITE Ale o10	0.59	0.59	0.58	0.59	0.59	0.98	0.96	0.81	0.81	0.81	0.81	0.83	0.78	0.65	0.81	0.81	0.81		0.83	0.61	0.83	0.82	0.83	0.76	0.78	0.82	0.65	0.65	0.66	0.65	0.64	0.63	0.65	0.56	0.57	0.57	
19 CRAB Por s	0.57	0.57	0.57	0.58	0.58	0.83	0.82	0.93	0.93	0.93	0.92	0.97	0.80	0.88	0.83	0.83	0.84	0.83		0.59	0.99	1.00	1.00	0.85	0.87	0.93	0.63	0.63	0.64	0.64	0.63	0.62	0.61	0.63	0.57	0.57	0.58
20 BARNACLE Bal r	0.51	0.51	0.51	0.52	0.51	0.61	0.60	0.58	0.58	0.58	0.59	0.60	0.58	0.48	0.57	0.58	0.58	0.61	0.59		0.59	0.59	0.59	0.57	0.58	0.60	0.73	0.74	0.74	0.73	0.77	0.76	0.75	0.74	0.67	0.67	0.67
21 CRAB Eri i	0.57	0.57	0.57	0.58	0.58	0.83	0.82	0.92	0.92	0.92	0.92	0.96	0.80	0.87	0.83	0.84	0.83	0.99	0.59		0.99	0.99	0.85	0.86	0.93	0.63	0.63	0.64	0.64	0.63	0.62	0.61	0.63	0.57	0.57	0.58
22 CRAB Eri s	0.57	0.57	0.57	0.58	0.58	0.83	0.82	0.93	0.93	0.93	0.93	0.97	0.80	0.88	0.83	0.83	0.83	0.82	1.00	0.59	0.99		0.99	0.85	0.87	0.94	0.63	0.63	0.64	0.64	0.63	0.62	0.61	0.63	0.57	0.57	0.58
23 CRAB Por t	0.57	0.57	0.57	0.58	0.58	0.83	0.82	0.92	0.92	0.92	0.92	0.97	0.80	0.88	0.83	0.83	0.84	0.83	1.00	0.59	0.99	0.99		0.85	0.87	0.93	0.63	0.63	0.64	0.64	0.63	0.62	0.61	0.63	0.57	0.57	0.58
24 KRILL Eup s	0.56	0.56	0.56	0.57	0.56	0.77	0.75	0.90	0.90	0.90	0.88	0.86	0.75	0.86	0.77	0.77	0.78	0.76	0.85	0.57	0.85	0.85	0.85		0.98	0.90	0.63	0.63	0.63	0.64	0.62	0.62	0.60	0.63	0.56	0.56	0.56
25 KRILL Eup p	0.57	0.57	0.56	0.58	0.57	0.78	0.77	0.92	0.92	0.92	0.90	0.87	0.77	0.66	0.79	0.79	0.80	0.78	0.87	0.58	0.86	0.87	0.87	0.98		0.92	0.63	0.63	0.63	0.64	0.63	0.62	0.61	0.63	0.56	0.56	0.57
26 SHRIMP Ora o	0.56	0.56	0.56	0.58	0.57	0.83	0.81	0.98	0.98	0.98	0.96	0.94	0.80	0.88	0.83	0.83	0.83	0.82	0.93	0.60	0.93	0.94	0.93	0.90	0.92		0.64	0.64	0.64	0.64	0.64	0.63	0.62	0.65	0.57	0.58	0.58
27 SQUID Tod p	0.54	0.54	0.54	0.55	0.54	0.64	0.63	0.63	0.63	0.63	0.64	0.63	0.61	0.58	0.60	0.61	0.61	0.65	0.63	0.73	0.63	0.63	0.63	0.63	0.64	0.64		0.96	0.98	0.91	0.81	0.80	0.78	0.82	0.69	0.70	0.71
28 SQUID Sep l	0.54	0.54	0.54	0.54	0.54	0.65	0.63	0.63	0.63	0.63	0.63	0.62	0.57	0.61	0.61	0.61	0.65	0.63	0.74	0.63	0.63	0.63	0.63	0.63	0.64	0.96			0.98	0.92	0.82	0.81	0.79	0.84	0.70	0.71	0.71
29 CUTTLEFISH Sep e	0.54	0.54	0.54	0.55	0.55	0.65	0.64	0.63	0.63	0.63	0.64	0.64	0.62	0.57	0.61	0.62	0.61	0.66	0.64	0.74	0.64	0.64	0.64	0.63	0.63	0.64	0.98	0.98		0.92	0.82	0.81	0.78	0.84	0.70	0.71	0.71
30 CUTTLEFISH Oct v	0.55	0.55	0.55	0.56	0.55	0.65	0.63	0.64	0.64	0.64	0.65	0.64	0.62	0.59	0.62	0.62	0.62	0.65	0.64	0.73	0.64	0.64	0.64	0.64	0.64	0.91	0.92	0.92		0.83	0.82	0.79	0.83	0.70	0.71	0.71
31 ABALONE Hal a	0.55	0.55	0.54	0.55	0.55	0.64	0.63	0.63	0.63	0.64	0.63	0.60	0.55	0.62	0.62	0.62	0.65	0.63	0.77	0.63	0.63	0.63	0.62	0.63	0.64	0.81	0.82	0.82	0.83		0.99	0.96	0.87	0.74	0.75	0.75
32 ABALONE Hal d	0.55	0.55	0.54	0.55	0.54	0.64	0.62	0.63	0.63	0.63	0.63	0.60	0.55	0.61	0.61	0.61	0.64	0.62	0.76	0.62	0.62	0.62	0.62	0.62	0.63	0.80	0.81	0.81	0.82	0.99		0.97	0.86	0.74	0.74	0.74
33 ABALONE Hal r	0.53	0.53	0.53	0.53	0.53	0.63	0.61	0.61	0.61	0.61	0.62	0.61	0.59	0.54	0.60	0.60	0.60	0.63	0.81	0.75	0.61	0.61	0.61	0.60	0.61	0.62	0.78	0.79	0.78	0.79	0.96	0.97		0.85	0.71	0.72	0.72
34 SNAIL Hel a	0.56	0.56	0.56	0.56	0.56	0.65	0.64	0.64	0.64	0.64	0.64	0.63	0.62	0.56	0.62	0.63	0.63	0.65	0.63	0.74	0.63	0.63	0.63	0.63	0.65	0.82	0.84	0.84	0.83	0.87	0.86	0.85		0.70	0.70	0.71
35 MUSSEL Per v	0.51	0.51	0.51	0.52	0.52	0.56	0.55	0.56	0.56	0.56	0.57	0.57	0.55	0.48	0.56	0.56	0.56	0.56	0.57	0.67	0.57	0.57	0.57	0.56	0.56	0.57	0.69	0.70	0.70	0.70	0.74	0.74	0.71	0.70		0.94	0.94
36 MUSSEL Myt e	0.51	0.51	0.51	0.52	0.52	0.57	0.55	0.57	0.57	0.57	0.58	0.57	0.56	0.49	0.57	0.57	0.57	0.57	0.57	0.67	0.57	0.57	0.57	0.56	0.56	0.58	0.70	0.71	0.71	0.71	0.75	0.74	0.72	0.70	0.94		1.00
37 MUSSEL Myt g	0.51	0.51	0.51	0.52	0.52	0.57	0.56	0.57	0.57	0.57	0.58	0.58	0.56	0.49	0.57	0.57	0.57	0.57	0.58	0.67	0.58	0.58	0.58	0.56	0.57	0.58	0.71	0.71	0.71	0.71	0.75	0.74	0.72	0.71	0.94	1.00	

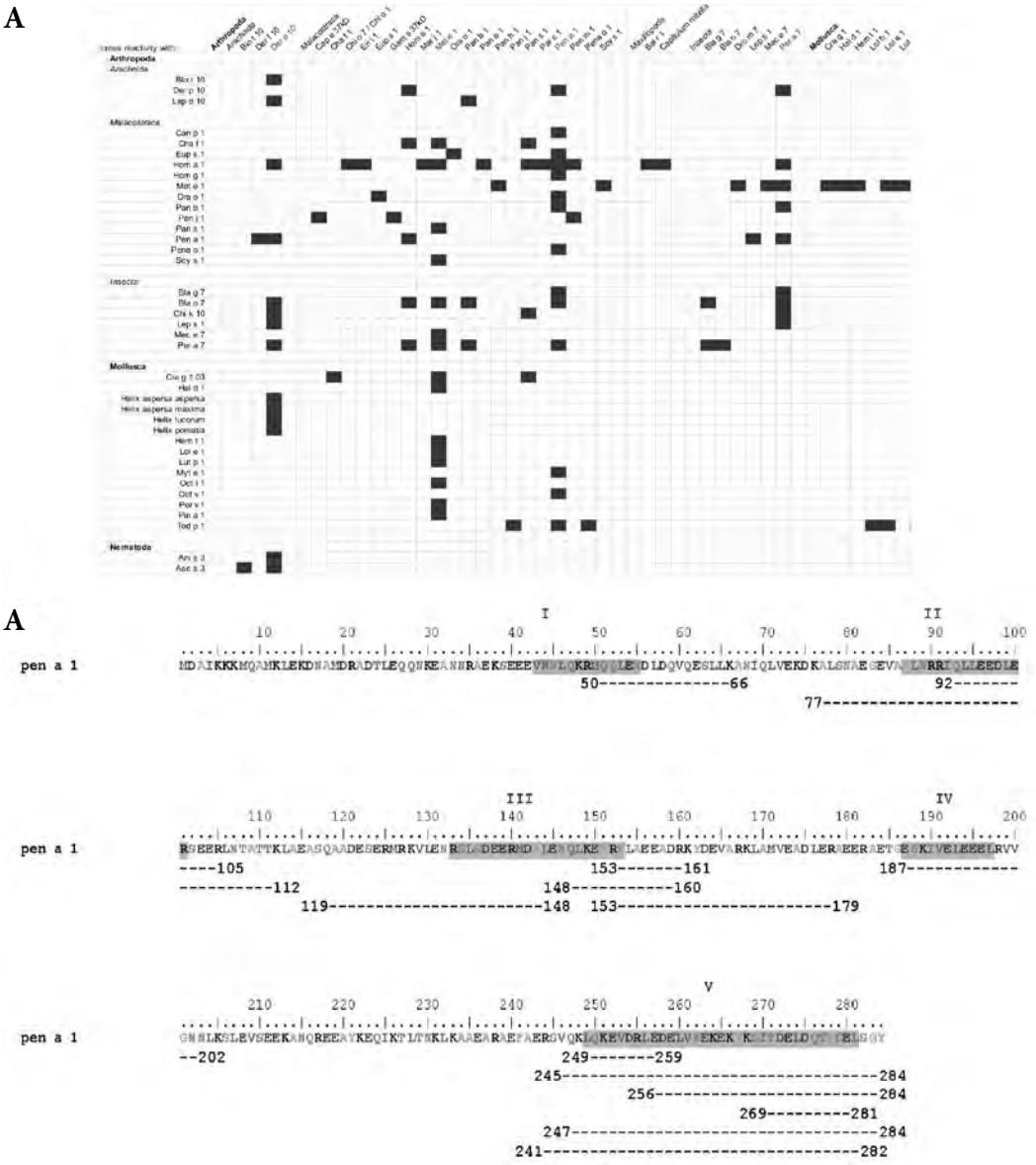
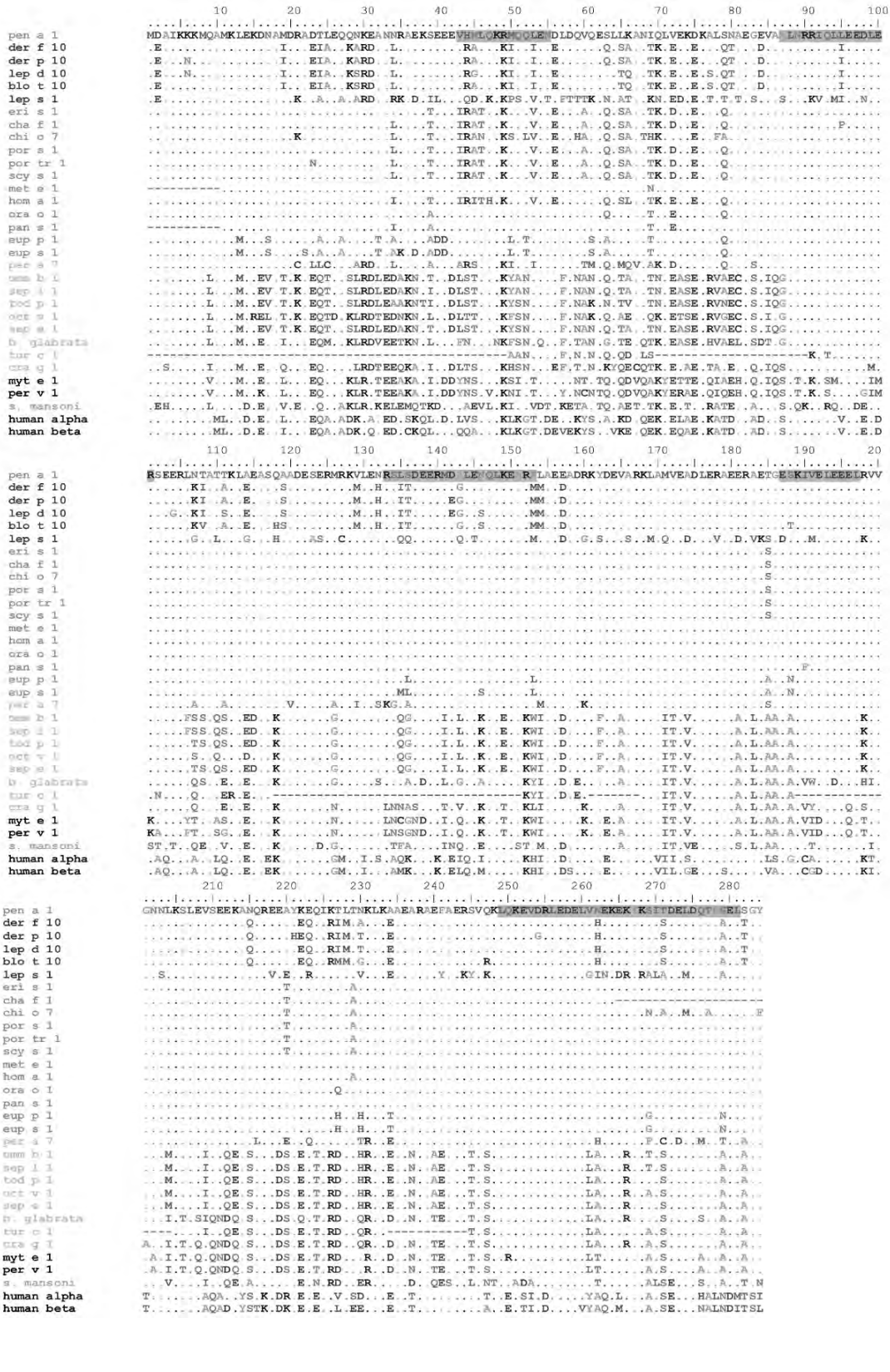


***Figure S1**. Overview of tropomyosin cross-reactivities (A) and IgE epitopes (B) as reported in literature. (A) The allergens that are primarily binding IgE antibodies are listed vertically. These same IgE antibodies also react in a secondary manner to the allergens listed in the top row (indicated by a filled box). The most prominent allergens that IgE antibodies show secondary reactions to, are indicated with red letters (Der p 10, Met e 1, Pen a 1, and Per a 7). (B) Several epitope regions have been identified, which are indicated with roman letter and grey shading of the shrimp (pen a 1) sequence.*

***next page: Figure S2.** ClustalW Alignment of amino acid sequences of tropomyosin. Shrimp (pen a1) is used as template; amino acids identical to shrimp are indicated with a dot.*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der p 10
lep d 10
blo t 10
lep s 1
eri s 1
cha f 1
chi o 7
por s 1
por tr 1
scy s 1
met e 1
hom a 1
ora o 1
pan s 1
eup p 1
eup s 1
per a 7
tmm b 1
sep i 1
tod p 1
oct v 1
sep e 1
b. glabrata
tur c 1
cra g 1
myt e 1
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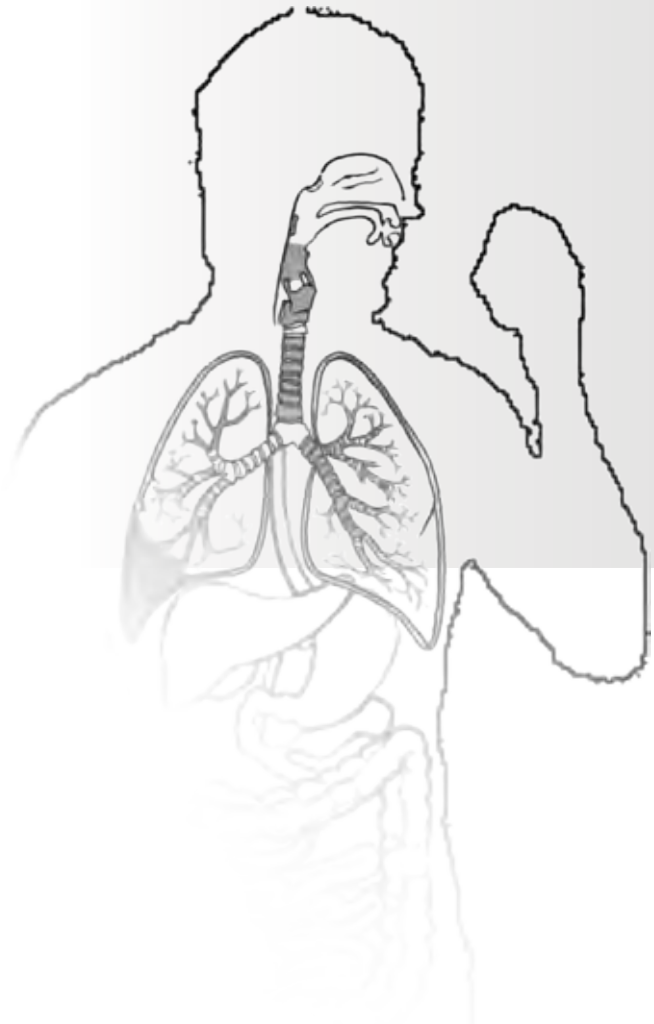
s. mansoni
human alpha
human beta



chapter 3

An alternative inhibition method for diagnosis of allergy: Two patients with a ficus (F*icus benjamina*) and fig (*Ficus carica*) sensitization

Gerco den Hartog, Ad P. H. Jansen, Yvonne Schmidt, Annelies Verlaet, Nicolette W. de Jong, Lisette Berendsen, Huub F. J. Savelkoul, Janneke Ruinemans-Koerts

ABSTRACT

Inhibition assays are a useful tool to identify the allergen of primary sensitization of cross-reactive allergens. Inhibition assays often depend on extracts made at the laboratory that may be subject to significant batch differences and are laborious to obtain. We developed an ImmunoCap based inhibition method that uses commercially available Caps as allergen source. This ImmunoCap inhibition method was used to study cross-reactivity between fig (*Ficus carica*) and ficus (*Ficus benjamina*). The newly developed method was validated against a classically applied inhibition method.

The ImmunoCap inhibition method showed reproducible results, and were consistent with the classical inhibition method. We conclude that ImmunoCaps are a useful allergen source for inhibition assays.

INTRODUCTION

Cross-reactivity between allergens from different species is a common phenomenon in IgE-mediated allergy. When the primary sensitizing agent is in question, inhibition assays are applied to determine the primary sensitizing allergen. Classically, information about cross-reactive allergens is obtained by adding allergen extract to the patients' serum and detecting the remaining unbound IgE (1, 2). This method requires the availability of the cross-reactive allergen extracts. As availability of commercial allergen extracts is limited and making allergen extracts that generate reproducible results is laborious, a simple and reliable alternative inhibition method is desirable.

Specific IgE measurement against more than 400 allergens can be performed on the ImmunoCap system (Phadia, Thermofischer Scientific) with allergen-coated cellulose sponges (Caps). We developed a procedure to use these Caps as an alternative allergen source in inhibition assays and tested this method for two patients with both a ficus (*Ficus benjamina*, also called weeping or Benjamin's fig) and fig (common fig having consumable fruits, *Ficus carica*) sensitization. Exposure to ficus is likely mainly through inhalation, whereas exposure to the fruits of common fig is mainly through ingestion (3, 4). In addition, ficus and fig have been reported as cross-reactive allergens before (5,6).

Results of the alternative inhibition method were compared to a classical inhibition method (IgE inhibition with allergen extracts). Differences in the results of the inhibition experiments between the patients were correlated to clinical symptoms and results of the in vitro functional Basophil Activation Test (BAT).

MATERIALS AND METHODS

Allergen Extracts

For SPT, fig fruit (*Ficus carica*) extract was prepared from ripe figs with peel. Ficus (*Ficus Benjamina*) extract was prepared from parts of leaves and stem. The parts were cut into small pieces, minced in 25 % (w/v) extract in phosphate-buffered saline, pH 7.4, containing 0.03 % human serum albumin and 0.5 % phenol (PBS) and centrifuged. Supernatants were passed through a 0.22 µm filter (Millipore). The same procedure was followed extract For inhibition assays, except that 10% w/v extracts were prepared in

phosphate buffered saline, pH 7.4, with 0.2% NaN3, 0.2% tween, 0.3% BSA (PBS-AT).

IgE-based inhibition with allergen coated CAP-sponges

Eight ficus (k81) and four fig (f328) coated celullose sponges (Phadia, Uppsala, Sweden, now part of Thermofisher Scientific) were carefully removed from the capsule. The preserving liquid was first removed by drying the sponge between filtering paper twice. Subsequently, the sponge was washed 3 times with 0.5 ml wash solution and the remaining liquid in the sponge was squeezed out with a stire wire, followed by drying between filtering paper. Then the sponge was placed back in a clean and dry capsule and the capsule was placed in an eppendorf cup.

Patient serum (40 µl) was pipetted to 4 ficus sponges and incubated at 37 °C for 30 min (Figure 1). The sponges were centrifuged at 12.000g for 3 min. The 4 eluates were pooled and 40 µl was added to 3 ficus sponges, incubated and centrifuged. The 3 eluates were pooled and 40 µl was added to two fig sponges and 1 ficus sponge. The two eluates of the fig sponges were pooled and 40µl was added to a new fig sponge. The eluate of the ficus sponge was not further analysed. All the incubated sponges were placed back in a new clean capsule and placed in the ImmunCap250. IgE binding was measured according to the standard procedure of the ImmunoCap250 and buffer was used as sample instead of serum (see also Figure 1A).

This procedure was repeated to study inhibition by fig allergens as visualized in figure 1B.

IgE-based inhibition with allergen extracts

A dose-dependent inhibition study was performed by incubating ficus or fig extract with patient serum at room temperature for 2h. Final concentration of extract protein in the patient serum was 400 µg/ml protein for fig and 711 µg/ml protein for ficus. After incubation with the allergens, the remaining unbound IgE was measured by the ImmunoCap method. The percentage of inhibition at concentration inhibitor x was calculated as % $\text{inhibition}_x = (IgE_{0\%} - IgE_x)/(IgE_{0\%})*100$. Incubation of serum with PBS was taken as 0% inhibition ($IgE_{0\%}$).

Basophil Activation Test (BAT)

The BAT was performed with the Flow2-CAST (Bühlmann, Basel, Switzerland) according to the instructions of the manufacturer. CD63 expression on basophils > 6% was regarded as a positive test result. Concentration of ficus and fig allergen in the stimulation was up to 100 and 5000 ng/ml respectively.

RESULTS

Clinical description of patients

Patient A is a 61 year old woman with intermittent moderate to severe allergic rhinitis due to a three pollen allergy. She frequently ate dried figs until she experienced an acute anaphylactic shock while eating it. She recuperated swiftly after treatment with adrenaline in a local emergency department. She has no history of asthma or food allergy. She never had known contact with F. benjamina.

Patient B is a 53 year old woman with persistent mild allergic rhinitis and IgE sensitisation to house dust mite, but not to grass or tree pollen. In her living room, she has several *F. benjamina* plants, but she has no complaints while tending to these plants. She has often eaten dried figs without any adverse reaction. While on holiday in Turkey, she ate a fresh fig for the first time. Within minutes, she experienced an acute hypersensitivity reaction

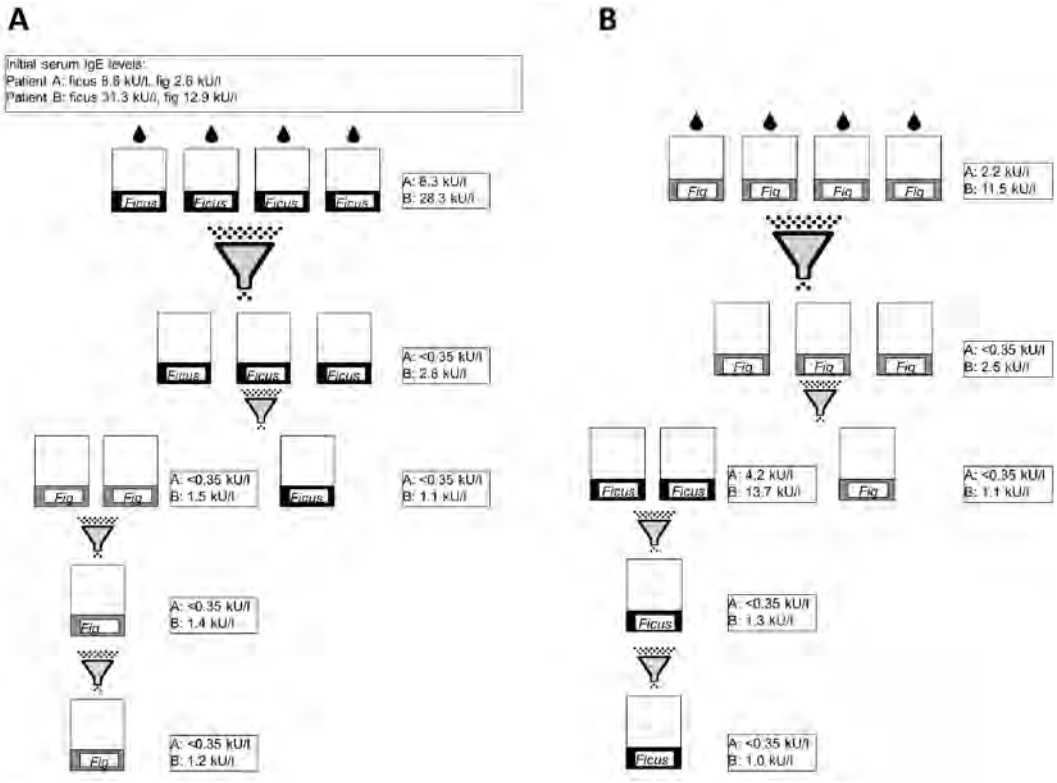


Figure 1. *Inhibition of IgE-binding to fig and ficus allergens by ficus (A) and fig (B). Allergens on CAPS are used as allergen source for inhibition. IgE levels bound by the inhibitor-CAP are analysed and remaining allergen-specific Ig is detected on new CAPS using the default ImmunoCAP procedure.*

with generalized urticaria, vomiting, diarrhea, facial angio-edema and asthma. This reaction was treated with adrenaline in a local emergency department.

IgE-based inhibition with allergen coated sponges

Inhibition of serum of patient A with one ficus sponge resulted in complete inhibition of ficus specific IgE. However, for patient B after two inhibition rounds with ficus sponges about 3.5% of the initial IgE level of ficus IgE was still detected (also observed after repeating the analysis) (Figure 1A). Pre-incubation of serum with ficus sponges completely or 88% inhibited detection of fig specific IgE in serum of patient A respectively B.

Inhibition with one fig sponge resulted in complete inhibition of fig IgE, and 50% inhibition of ficus IgE for patient A. For patient B, inhibition with one fig sponge was not complete and with a second and third sponge 8.5% of the initially detected fig specific IgE was still detected (Figure 1B). Inhibition with three fig sponges resulted in about 55% inhibition of ficus specific IgE.

IgE-based inhibition with allergen extracts

Inhibition with fig extract resulted in 100% inhibition of fig specific IgE, but partly (around 30%) inhibition of ficus specific IgE (Figure 2A). The shape of the inhibition curves is similar for both patients.

Inhibition with ficus extract resulted in 80-

100% inhibition of both ficus and fig specific IgE. However, the shape of the inhibition curves of patient A and B differ clearly. Inhibition of ficus and fig specific IgE was observed at a lower concentration for patient A than for patient B.

Basophil Activation Test (BAT)

Ficus extract resulted in 90% and 80% of activated basophils for patient A and B respectively (Figure 2C). The allergen concentration at which 50% activation was detected was 10 ng/ml for patient A and 20 ng/ml for patient B respectively.

Addition of fig extract in the BAT resulted in 60% of activated basophils for both patients, However, the allergen concentration at which 50% stimulation was reached was significantly different between the two patients; 4000 ng/ml for patient A and 1200 ng/ml for patient B.

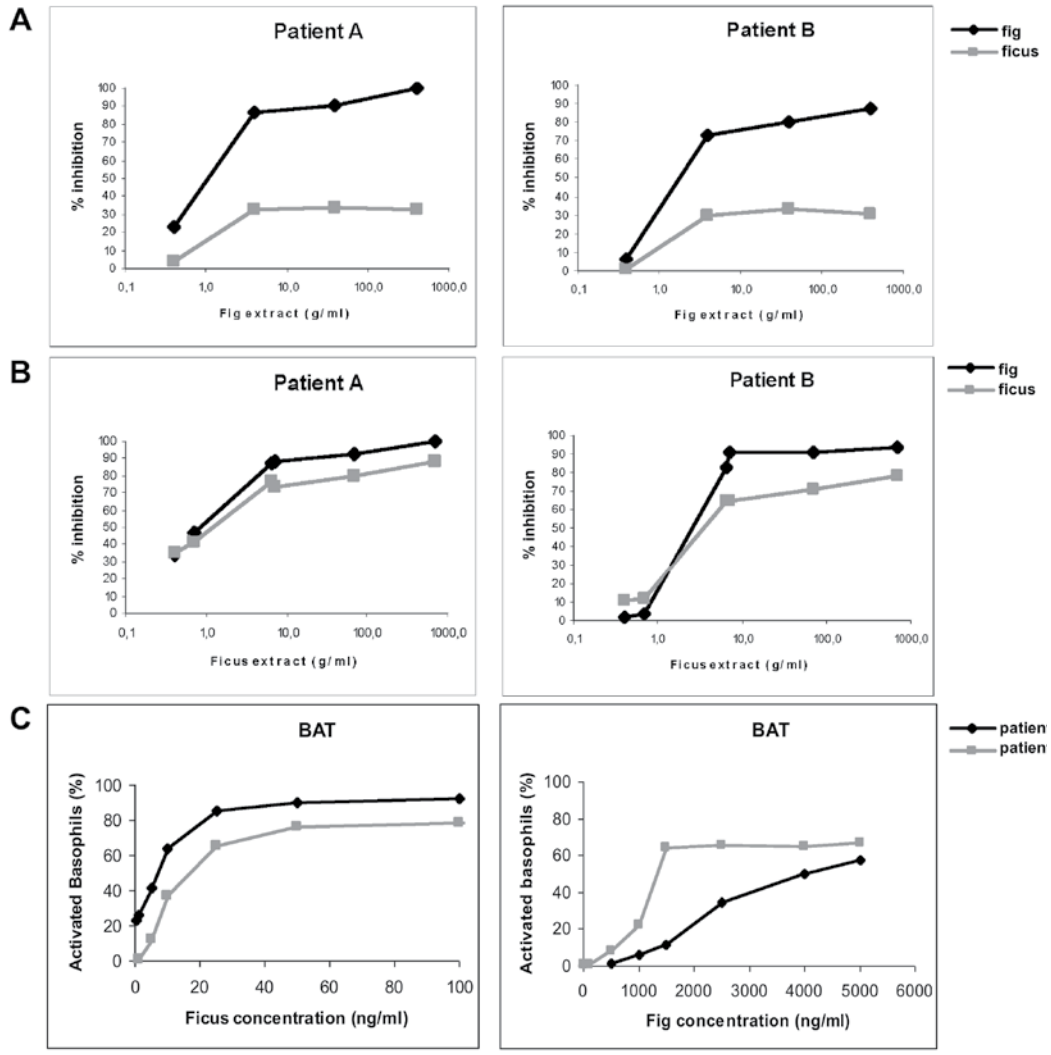


Figure 2. *ELISA inhibition assay with fig (A) and ficus (B) extract, and BAT (C). Values on x-axes show concentration of inhibitor allergen (A and B), and concentration of allergen added to basophils (C). Y-axes indicate percentage of inhibition (A and B) or percentage of activated basophils (C).*

DISCUSSION

In this study we aimed at applying an ImmunoCap-based inhibition method to identify the allergen of primary sensitization. Both the classically applied inhibition method and the ImmunoCap based inhibition method showed that pre-incubation with ficus resulted in almost complete inhibition of detectable fig specific IgE in serum. When fig was used during pre-incubation, only partial inhibition of detectable IgE specific for ficus was achieved. Based on this we conclude that ficus was the allergen of primary sensitization. This is consistent with the different levels of specific IgE; specific IgE to ficus was higher compared to fig.

Although both patients were primarily sensitized to ficus, IgE inhibition results showed that for patient B, higher amounts of ficus and fig allergen were required to inhibit binding of remaining IgA to ficus or fig allergen. In addition, binding of IgE of patient B could not be completely inhibited in both inhibition assays. This indicates that IgE of patient A and B may recognize different epitopes of the allergens and that the affinity of IgE may be different for the fig and ficus allergens. This difference in IgE profile might explain the difference in results of the BAT and clinical symptoms for patient A and B; patient A is allergic to dried fig, patient B only to fresh fig (dryed fig was tolerated) and lower concentrations of fresh fig extract in the BAT resulted in a higher response of activated basophils for patient B compared to patient A.

In conclusion, we developed a reproducible alternative inhibition method using ImmunoCaps. This novel method makes use of commercially available allergens of a validated diagnostic instrument and provides result consistent with a classical inhibition assay, which can give information of the primary sensitizing allergen and differences in IgE profiles.

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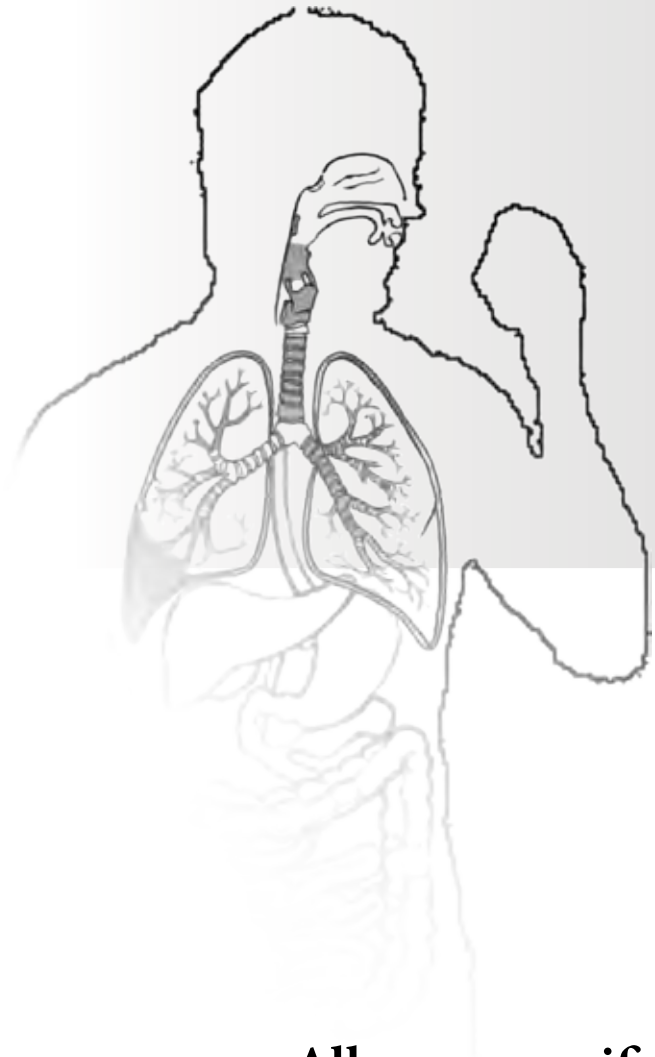

chapter 4

Allergen-specific IgA2, but not IgA1, is associated with protection against eczema in house dust mite allergic patients

Gerco den Hartog MSc, R. J. Joost van Neerven PhD, Johan D. Boot PhD,
Ad P. H. Jansen MD, Huub F. J. Savelkoul PhD

ABSTRACT

Upon inhalation, house dust mite (HDM) allergens are deposited at the nasal and oral mucosa, where IgA is produced abundantly.
It is currently not known if and how the human IgA subclasses IgA1 and IgA2 contribute to the clinical status of house dust mite-allergic patients.
HDM-allergic patients were subjected to a standardized nasal provocation tests (SNPT) with multiple concentrations of HDM. Peak nasal inspiratory flow (PNIF) measurements were performed and the allergen threshold was determined by a nasal composite symptom score (NCSS). Saliva and serum samples were collected, and HDM-specific IgG4, IgE, IgA1 and IgA2 levels were determined.
As expected, HDM-specific serum IgE levels correlated positively with serum IgG4 levels. HDM-specific levels of IgA1 in serum were similar to levels measured in non-allergic controls, but HDM-specific levels of IgA2 in serum were decreased in allergic subjects. Division of the HDM-allergic patients into groups of patients that suffered from eczema and patients that did not have eczema showed that this decrease in IgA2-levels was most prominent in HDM-allergic patients that also suffered from eczema alone, or in combination with asthma. In addition, HDM-specific IgA2 levels in saliva were decreased significantly in HDM-allergic patients compared to non-allergic controls. The ratio of HDM-specific serum IgE over HDM-specific IgA2 levels in saliva correlated significantly with the combination of the HDM provocation threshold and PNIF.
Taken together, our findings indicate that HDM-specific IgA2, but not IgA1, levels in serum and saliva are reduced in HDM-allergic patients suffering from eczema.

INTRODUCTION

Detection of allergen-specific IgE in serum is a widely used method to evaluate sensitization to allergens. However, levels of allergen-specific IgE in serum often correlate poorly with the intensity of clinical manifestations of allergy [1, 2]. Increasing evidence is showing that production of allergen-specific IgG4 antibodies correlate with protection against allergy [3]. Moreover, IgG4 is able to block crosslinking of IgE on mast cells and basophils [4]. IgG4 production is increased after allergen-specific immunotherapy, and increased production of allergen-specific IgG4 correlates with clinical efficacy [5]. Allergen-specific IgG4 can also decrease allergen-IgE complex binding to CD23 and subsequent activation of T cells [6, 7]. This has been shown to correlate better to the clinical status of allergy patients than allergen-specific IgE levels per se.

In addition to IgG4, IgA has been postulated in the past to protect against allergy, and recently salivary IgA was shown to be associated with protection against allergy [8-12]. IgA is the dominant isotype present at mucosal surfaces, and therefore is the first antibody isotype to come into contact with allergens that are deposited in the airways or the gastro-intestinal tract. The presence of allergen-specific IgA at mucosal surfaces may therefore prevent allergens from entering the body by inducing aggregates, or by blocking IgE-binding epitopes. In line with this, allergen-binding IgA2- but not IgA1-producing B cells in nasal tissue have been observed after successful birch-pollen

immunotherapy [13].

Despite this hypothetical mechanism of IgA-mediated protection, attempts to correlate allergen-specific serum-IgA levels with the presence or absence of allergy or with severity of allergic symptoms have generally been unsuccessful [14-16]. The majority of mucosal IgA is produced locally in the mucosal tissue, and does not originate from systemic sources [17-23]. This may explain why studies relating allergen-specific IgA in saliva to inhalant allergies were more successful than studies measuring IgA in serum [8, 9].

Humans produce two subclasses of IgA, namely IgA1 and IgA2 [24]. The distribution of IgA subclasses is throughout the body differs between IgA1 and IgA2; IgA1 levels are higher compared to IgA2 in serum and most mucosal sites including saliva, whereas jejunum and colon are dominated by IgA2 [25-27]. IgA2 is more resistant to bacterial proteolysis due to the shorter hinge region of the antibody [28]. The structural difference between IgA1 and IgA2 as well as the different distribution throughout the body suggest a different role in mucosal immunity for the two IgA subclasses.

The vast majority of studies on the role of IgA in allergy only report allergen-specific levels of total IgA without differentiating between IgA1 and IgA2. This may contribute to the lack of correlation between serum-IgA levels and allergy. Therefore the aim of this study was to relate allergen-specific levels of IgA1 and IgA2 in serum and saliva to clinical symptoms in house dust mite allergic patients.

Table 1. *Nasal combined score (NCS)*

Nasal Combined Score	HDM concentration (AU/mL)	PNIF
1	10	>80
2	10	40-80
3	10	<40
4	1	>80
5	1	40-80
6	1	<40
7	100	>80
8	100	40-80
9	100	<40

The NCS is derived from peak nasal inspiratory flow (PNIF) and maximum tolerated house dust mite (HDM) dose. Increasing clinical symptoms is reflected by higher NCS.

MATERIALS AND METHODS

Patients and controls

HDM-allergic patients, male and female and aged 14 years or older, with a positive medical history of allergic rhinitis with or without concomitant clinically stable asthma (FEV_1 > 80% predicted), and with or without mild to moderate atopic dermatitis (SCORAD < 2) were skin tested with common inhalant allergens (HAL Allergy BV, Leiden, the Netherlands). The mean HDM-specific IgE levels of patients was 14.5 kU/L (standard deviation +/- 22). Patients with a positive HDM skin test underwent a standardized nasal provocation test (SNPT) to multiple increasing allergen doses as defined in table 1. Patients were screened for contraindications such as severe asthma or use of interfering medication. If applicable, patients had to stop the use anti-histamines three days and steroid nasal sprays one week before the SNPT. Immediately after reaching the provocative nasal threshold, saliva (using salivettes, 51.1534.500, Sarstedt, Nümbrecht, Germany) and serum samples (serum vacutainers, BD, Franklin Lakes, NJ USA) were collected and stored at -20°C until use. All SNPTs were done outside the pollen seasons to correct for active hay fever symptoms.

All procedures were performed as specified in the EAACI position article and guidelines [29].The investigations were approved by the local Medical Ethical Committee.

Control subjects did not suffer from asthma or eczema. In the inclusion of control subjects (seven males, seven females), no clinical symptoms of allergy or sensitization to house dust mite was allowed. Four of the fourteen controls suffered from seasonal rhinitis due to pollen allergy. Samples from patients and control subjects were collected outside the pollen season.

Standardized titrated nasal provocation test (SNPT)

The SNPT on the HDM-patients was performed according to a protocol described elsewhere [30]. In short, after an hour acclimatization period the baseline NCSS (according to Lebel) and baseline nasal inspiratory flow (PNIF) (highest value of three measured by nasal inspiratory flow meter (Clemens Clarke, Harlow, UK) were noted. Symptoms were recorded using the following scoring system: sneezes ≤ 2 = 0 points, sneezes 3-4 = 1 point, sneezes ≥ 5 = 3 points, anterior rhinorrhoea = 1 point, posterior rhinorrhoea = 1 point, difficult breathing = 1 point, one blocked nostril = 2 points, two blocked nostrils = 3 points, nasal itch = 1 point, pruritus in palate or ear = 1 point, conjunctivitis = 1 point (total score range: 0-11 points). The pre-diluent NCSS was not allowed to be ≥ 3 points. The NCSS was considered positive once a total score ≥ 6 was reached.

The SNPT started with the allergen's diluent followed by spraying of 1 puff into each nostril of a standardized HDM extract (HAL Allergy, Leiden, the Netherlands) in stepwise incremental concentrations of 100, 1,000 and 10,000 AU/ml. The upper airway response was quantified by NCSS and PNIF 15 minutes after each challenge.

Nasal combined score

To relate HDM-specific IgA levels to NCSS, PNIF and the maximum tolerated HDM dose and the measurements of SNPT were combined into a nasal combined score (NCS), as described in Table 1. Patients able to tolerate a higher dose and less nasal obstruction received a low score, whereas patient tolerating a low dose of allergen and having a reduced PNIF received a high score, reflecting more severe clinical symptoms.

Detection of HDM-specific IgE and IgG4

HDM (*Dermatophagoides pteronyssinus*, CAP d1) specific IgG4 and IgE levels in serum and saliva were determined with ImmunoCap 250 (Thermo scientific, Uppsala, Sweden), using the standard procedure.

Detection of HDM-specific IgA1 and IgA2

HDM extract (0.2 µg/ml, HAL allergy, Leiden, The Netherlands) was coated overnight at 4°C. Plates (medium binding, Greiner Bio-one) were blocked with biotin free Universal Casein Diluent/Blocker (SDT reagents, Germany) for one hour at room temperature. Plates were washed three times with PBS containing 0.05% tween-20. All samples were tested in duplicate and diluted 5 and 20 times. Plates were washed (3 times) and 2000 times diluted biotinylated mouse anti human IgA1 or IgA2 (Southern Biotech, Birmingham, AL, USA) was added and incubated for 1 hour at room temperature. Plates were washed (5 times) and streptavidin poly HRP 80 (SDT reagents) was added and incubated. Plates were washed six times and stable soluble 1-component TMB was added (SDT reagents). 1% HCl was used as stop solution. Plates were read at 450 nm with 650 nm

as reference. HDM-specific IgA2 levels did not correlate with IgA total levels or HDM-specific IgA1 levels, indicating that the assay show HDM-specific levels of IgA subclasses.

Statistics

ELISA data were entered in PASW Statistics version 19. The independent samples T-test was used to test concentration differences between groups and the procedure Generalized linear models was used to evaluate the nasal combined score in relations to detected allergen specific Ig levels.

RESULTS

Patient description

In total 19 HDM-allergic subjects, six males and 13 females, were included in the study and were subjected to the SNPT. All 19 patients suffered from rhinitis. 10 patients also had eczema, and four of these also suffered from asthma. One patient had symptoms of asthma but not eczema (Table 2). All 19 clinically diagnosed HDM-allergic patients had significantly increased serum levels of HDM-specific IgE compared to non HDM-allergic subjects, who did not have detectable levels of HDM-specific IgE (Table 2). Serum of HDM-allergic patients and non-allergic subjects contained HDM-specific IgG4, however levels were significantly higher in serum of HDM-allergic patients (Table 2).

Nasal provocation of HDM-allergic patients with HDM-allergens mainly caused rhinorrhoea in patients with HDM allergy and concomitant pollen-related hay fever, and symptoms of blocked sinus in patients suffering from HDM-allergy only. HDM-allergic patients that also had pollen-related hay fever had increased levels of HDM-specific IgE ($p=0.068$) and IgG4 ($p=0.053$) in serum (data not shown).

HDM-specific IgA1 and IgA2 levels in serum and saliva

As detection of combined allergen-specific IgA levels is inconclusive in relation to the clinical status of patients, we analysed subclass-specific levels of HDM-specific IgA in serum. HDM-allergic as well as non HDM-allergic control subjects had detectable levels of HDM-specific IgA1 and IgA2 levels in their serum. HDM-specific levels of serum IgA1 were similar between HDM-allergic patients and non HDM-allergic control subjects (Figure 1). HDM-specific levels of IgA2 in serum were decreased in HDM-allergic patients ($p=0.082$). This difference between HDM-specific IgA1 and IgA2 levels in serum suggests that the two IgA subclasses may play different roles in allergy.

As locally produced IgA may be associated with protection against allergy, HDM-specific IgA2 levels were measured in saliva as readily accessible mucosal secretion. Levels of HDM-specific IgA2 in saliva were lower in HDM-allergic patients

Table 2. *Clinical symptoms of HDM-allergic patients and controls*

	Specific Ig levels							
	IgE kU/l	**IgG4** mg/l		**eczema**	**asthma**	**Eczema and asthma**	**No eczema or asthma**	**Total**
HDM-allergic	*14.5*	*0.27*	***Female***	5	0	4	4	**13**
	SD 22	*SD 0.16*	***Male***	1	1	0	4	**6**
Control	*<0.35*	*0.13*	***Female***	0	0	0	5	**5**
	SD 0.0	*SD 0.11*	***Male***	0	0	0	5	**5**

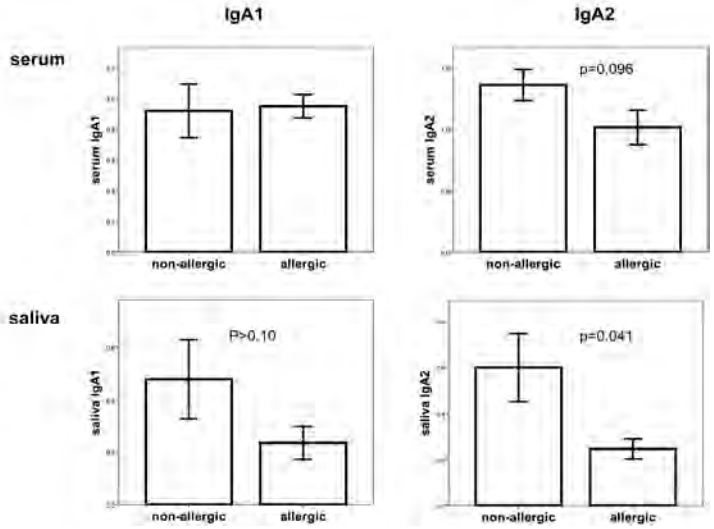

Figure 1. *HDM-specific levels of IgA1 (left) and IgA2 (right) in serum (top) and saliva (bottom) of HDM-allergic and non HDM-allergic individuals. Error bars indicate standard error, and p-values of significant differences are indicated.*

compared to non HDM-allergic control subjects p=0.084). HDM-allergic patients showed an insignificant trend of having decreased levels of HDM-specific IgA1 (Figure1). Taken together, decreased levels of HDM-specific IgA2 is associated with having HDM allergy.

Clinical symptoms eczema and asthma are associated with decreased IgA2 levels

Eczema has previously been reported to be inversely associated with IgA levels [12, 31, 32]. Therefore we compared HDM-specific levels of IgA1 and IgA2 in serum and saliva in the absence or presence of eczema in HDM-allergic patients. Levels of HDM-specific serum-IgA1, saliva-IgA1 and saliva-IgA2 were not different between HDM-allergic patients with or without eczema (Figure 2). However, HDM-specific levels of IgA2 in serum were significantly lower when HDM-patients also suffered from eczema. Levels of HDM-specific

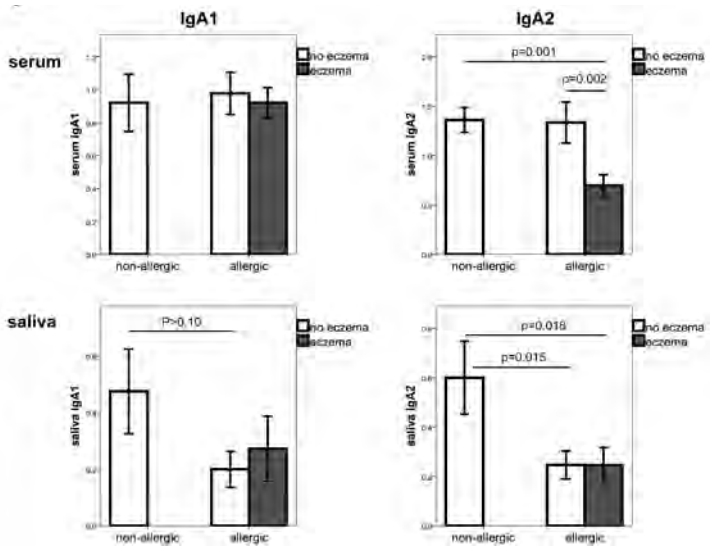

Figure 2. *HDM-specific levels of IgA2 in serum, but not IgA1 or IgA levels in saliva are decreased in HDM-allergic patients that also have eczema (grey bars). Error bars indicate standard error, and p-values of significant differences are shown.*

IgA2 were similar between non-HDM-allergic subjects and HDM-allergic patients not suffering from eczema. Therefore the different serum-IgA2 levels between HDM-allergic patients and control subjects seem to be caused by the sub-group of patients also suffering from eczema. Four of the ten HDM-allergic patients that suffered from eczema also suffered from asthma. Therefore we also analysed the relation between HDM-specific IgA2 and the combination of eczema and asthma. Levels of HDM-specific IgA2 were further decreased in patients suffering from eczema and asthma compared to patients suffering from eczema alone (p=0.063) and HDM-allergic patients not suffering from eczema or asthma (p=0.001) (Figure 3). Based on these observations we conclude that serum IgA2 is associated with the severity of allergic symptoms noted in HDM-allergic patients.

The ratio of HDM-specific serum IgE over HDM-specific IgA2 in saliva is linked to clinical status

To link the clinical parameters PNIF and maximally tolerated HDM dose (in SNPT) to levels of HDM-specific immunoglobulins, the two parameters were combined into the 'nasal combined score' (NCS, Table 1). The NCS did, however, not correlate significantly with HDM-specific levels of serum IgE, IgG4, IgA1 and IgA2 or salivary IgA1 and IgA2 alone (data not shown). As we hypothesized that the presence of IgA subclasses, especially IgA2 in the mucosa, is related to clinical status in addition to IgE, the ratio of HDM-specific IgE over IgA2 in saliva was calculated. The ratio of HDM-specific IgE over IgA2 correlated significantly (p=0.033) with clinical status as reflected by the 'nasal combined score'. This correlation was significant for the ratio serum-IgE over saliva IgA2 levels, but not for serum-IgE over serum-IgA2 or serum-IgE over serum-IgG4 levels (Figure 4).

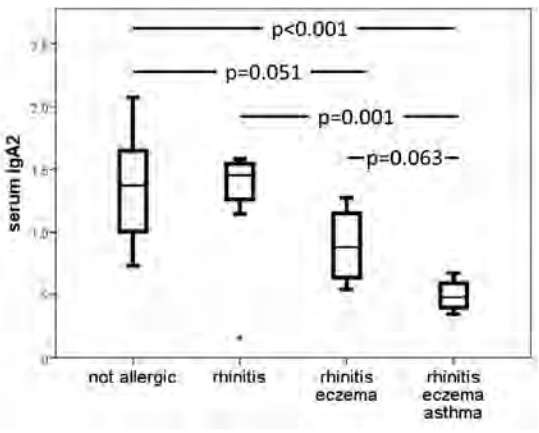

Figure 3. *Increased symptoms of HDM-allergic patients in associated with decreased levels of HDM-specific IgA2 in serum, as determined by absence or presence of eczema and asthma. Box plots indicate median (horizontal bar), 50% data range (box) and 1st and 4th quartile range (error bars), including all data points, except for a single outlier (*, allergic only).*

DISCUSSION

he aim of this study was to identify if and how IgA subclasses relate to the clinical status of house dust mite-allergic patients. HDM-allergic patients had significantly lower levels of IgA2 in serum, which was most prominent in the subgroup of HDM-patients that also suffered from eczema. Levels of HDM-specific IgE and IgA (IgA1 or IgA2) alone did not correlate to clinical status, but the ratio of allergen-specific serum-IgE over saliva-IgA2 was associated with the clinical status (nasal combined score) of the patients. The data reported here demonstrate that the location of IgA (serum or saliva) and HDM-specific IgA2, but not IgA1, levels are highly relevant parameters to measure in

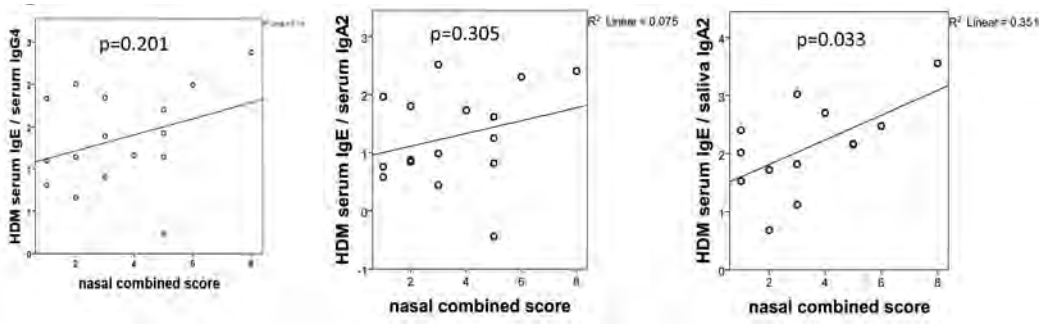

Figure 4. *The nasal combined score (NCS) of HDM-allergic patients is associated with the ratio of serum IgE and saliva IgA2. The NCS (table 1) is derived the PNIF recorded upon intranasal challenge in combination with maximally tolerated HDM-allergen dose that was injected in the nostrils with incremental doses. Increasing levels of serum IgE or decreasing levels of salivary IgA2 are correlated with increased allergic symptoms.*

relation to HDM allergy.

Both the PNIF and maximally tolerated dose of HDM-allergens were not directly associated with any of the concentrations of HDM-specific antibody classes. However, when PNIF and the maximally tolerated dose of HDM-allergens were combined as a measure of nasal hyperresponsiveness, a significant correlation was observed with the ratio of HDM-specific serum-IgE over saliva-IgA2. The fact that not IgA2 levels alone but the ratio of serum-IgE over salivary-IgA2 correlated significantly with clinical symptoms in the nose suggests that IgA2 did not neutralize all allergens. In line with our hypothesis, local levels of allergen-specific IgA2, but not systemic serum levels of IgA2 correlated with nasal symptoms upon allergen challenge. This is in line with literature showing reduced development of wheezing in children later in life when higher secretary IgA levels in saliva were observed [33].

After immunotherapy allergen-specific IgG4 levels in serum are increased and protect against allergy whilst IgE is still present [6, 34]. Increased levels of allergen-specific IgG4 induced after immunotherapy can block binding of allergen-IgE complexes to CD23 (IgE receptor) on B cells and prevent histamine release by basophils [35-37]. The ability of allergen-specific IgG4 to block IgE-CD23 interaction depends on IgE repertoire complexity, as well as on the ratio of IgE over IgG [38]. Consequently, IgG4 that inhibits CD23-dependent IgE-allergen binding correlates better with clinical outcome than the ratio between allergen-specific IgE and IgG4 [39]. Allergen-specific serum-IgA is not able to block IgE-allergen-complex formation [13, 36]. Specific IgA2 levels, but not IgA1levels seem to be correlated with presence or absence of eczema, whereas the majority of serum IgA is of the IgA1 subclass, which may explain why serum IgA was unable to inhibit IgE-allergen complex formation. In contrast to serum IgA, IgA from mucosal secretions may inhibit histamine release, as shown in patients receiving ragweed-immunotherapy [40]. Increased levels of salivary, but not serum, IgA after receiving sublingual peanut immunotherapy was associated with higher peanut consumption [41]. Thus, allergen-specific IgA may block entry of allergens at mucosal sites or result in non-inflammatory IL-10 production when IgA binds to the Fcalpha receptor (CD89) [13]. In addition to IgA-mediated mucosal protection, systemic protection is mediated by allergen-specific IgG4 induced by allergen-specific immunotherapy. We hypothesize that mucosal

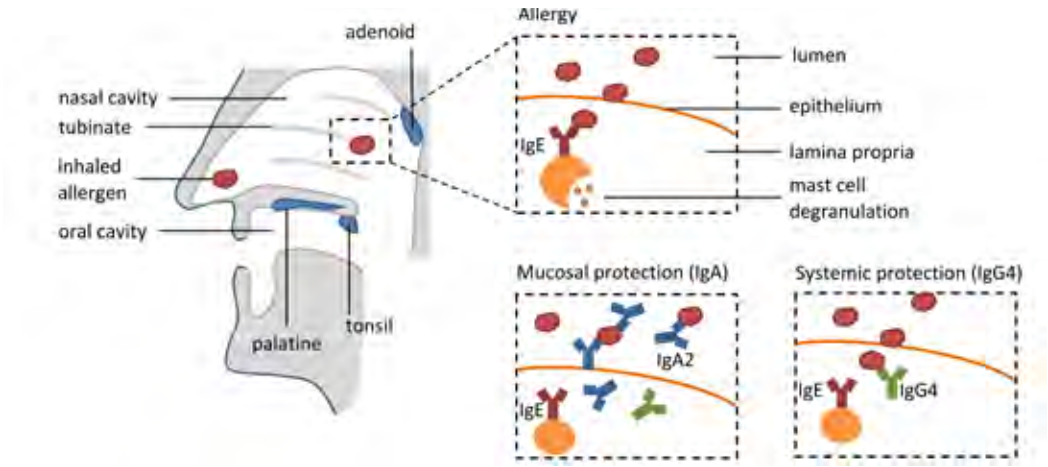

Figure 5. *Model explaining the different roles of mucosal IgA and serum IgG4 in prevention of mast cell or basophil degranulation. The increased levels of mucosal (salivary) IgA may prevent allergen-entry or result in non-inflammatory downstream immune responses upon binding of IgA-allergen complexes with myeloid cells. Allergen specific IgG4 can inhibit formation of IgE-allergen complexes that can be bound by mast cells, basophils or CD23 on B cells and thereby prevent cell-degranulation and Th2 cell activation.*

IgA2 may protect against allergy at mucosal surfaces of the respiratory tract and that systemic protection is mediated by IgG4 (Figure 5).

The data presented here support the notion that detection of allergen-specific IgA in serum should be IgA1 and IgA2 subclass specific, and be accompanied by measurements in saliva. Eczema correlated with reduced levels of systemic (serum) HDM-specific IgA2, but not serum-IgA1 or mucosal IgA1 and IgA2 (saliva). Interestingly, the association between eczema and decreased IgA levels in serum has been reported by Soothill and colleagues in the seventies of the previous century, but has been topic of debate since then and the underlying mechanism still needs to be revealed.[12, 32, 42]. It should be noted that eczema reported in our patients is not necessarily caused by house dust mite allergy, for it is difficult to identify the exact cause of this type of eczema. It is unclear how systemic IgA2 may protect against eczema, while this association was not found for HDM-specific serum-IgA1. Probably local tissue factors like a proliferation inducing ligand (APRIL) and B cell activating factor (BAFF or BLyS) that are expressed in the mucosa and skin are involved in causing eczema. APRIL enhances the production of IgA2, rather than IgA1, and its expression is reduced in the skin of patients with atopic eczema [43, 44]. Similarly, infants colonized with toxic Staphylococcus aureus produced increased levels of APRIL (4 months of age) and IgA and were less frequently sensitized to food (18 months of age) and less frequently developed eczema (18 months of age) [31]. Thus it is possible that eczema patients have a decreased ability to produce allergen-specific IgA2 in skin, which may be reflected by decreased levels of allergen-specific IgA2 in serum. To functionally differentiate between production of IgA1 and IgA2, different mechanisms of induction of the two subclasses are required. Indeed, different mechanisms for induction of IgA1 and IgA2 seem to exist (Chapter 6 and [44, 45]). In contrast to IgA1, the induction of IgA2 production may be more efficient under the influence of innate factors like

APRIL and BAFF produced by mucosal dendritic cells, epithelial cells and probably also in the skin by keratinocytes[44, 46]. The same mechanism could explain why numbers of IgA2-producing B cells but not IgA1-producing B cells in jejunum increase with age in infants, due to prolonged exposure to bacterial ligands [27]. In line with this observation, limited exposure to microbes has been associated with allergic symptoms in sensitized children [47]. Dendritic cells play a central role in mucosal induction of IgA2 production. Allergens delivered by sublingual immunotherapy are taken up by mucosal oral Langerhans-type dendritic cells that respond by increased production of the tolerogenic cytokines TGF-ß and IL-10 [48]. Those cytokines are crucial for both induction of T regulatory cell-mediated oral tolerance and for production of IgA. Tolerogenic DCs are found across mucosal compartments and can be induced by local mucosal factors [49]. DC-mediated induction of allergen-specific IgA2 upon mucosal immunotherapy has not been investigated, but may be an important factor to explain the success of immunotherapy. In this respect, the observation that nasal birch-pollen-specific IgA2 (but not IgA1) was induced after immunotherapy, and that serum-levels of birch-allergen-specific IgA2 were linked to levels of nasal TGF-ß expression levels is promising [13]. In addition, the IgA2 induced by allergen immunotherapy may directly contribute to induction of tolerance as monocytes produce IL-10 upon binding of IgA-allergen complexes [13].

To conclude, allergen-specific IgA2 levels in serum and saliva are associated with reduced symptoms of allergy. As local production of IgA2 probably occurs under the influence of tissue factors, future work should focus on mechanisms to specifically increase allergen-specific IgA2 levels in serum and mucosal sites to protect against development of asthma and eczema.

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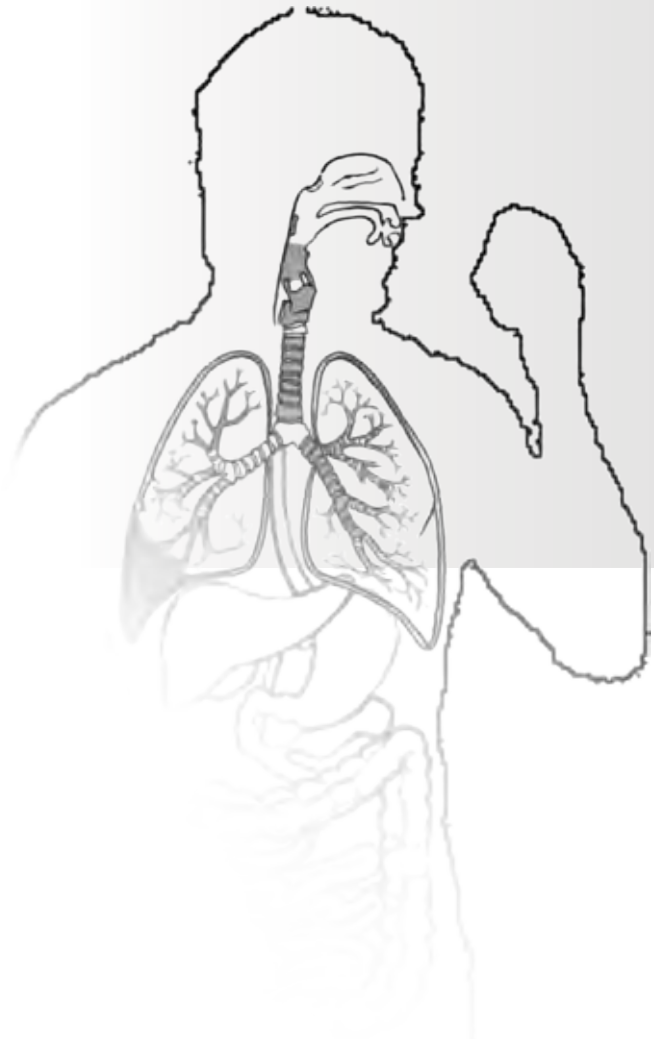


chapter 5

The mucosal factors retinoic acid and TGF-ß1 induced phenotypically and functionally distinct DC types

Gerco den Hartog, Christine van Altena,
Huub F. J. Savelkoul, R. J. Joost van Neerven

ABSTRACT

Non-inflammatory DC subsets are crucial to prevent massive inflammation in the intestines. We wanted to investigate whether the mucosal factors RA and TGF-ß1 can induce phenotypically and functionally distinct DC types.
DCs were differentiated from monocytes in the absence or presence TGF-ß1 and RA. Phenotype as well as responsiveness to bacterial ligands was studied in detail.
Compared to moDC, the expression the co-stimulating molecule CD86 and DC maturation marker CD83 were strongly reduced by RA and TGF-ß1. In addition, both RA and TGF-ß1 induced DC showed strongly decreased responsiveness to stimulation with bacterial ligands LPS and PGN, and produced significantly lower levels of the pro-inflammatory cytokines IL-12 and TNF-α compared to moDCs, whilst IL-10 production was not reduced significantly.
DCs differentiated under the influence of RA uniquely expressed markers related to intestinal homing (CD103 and integrin ß7). In addition, CCR7 that mediates homing to lymph nodes was expressed by DCs differentiated in the presence of RA, but also to a lesser extent by the other DC types. Further, whereas moDCs and TGF-ß1-derived moDCs expressed high levels of CD32, RA-derived DCs lacked CD32 expression but expressed high levels of CD64, suggesting that RA-DC may primarily respond to soluble proteins and moDC and TGF-ß DCs to immune complexes.
The data presented here support the hypothesis that the mucosal factors TGF-ß1 and RA that can also be provided through dietary intake of dairy products, result in functionally and phenotypically distinct DC types with non-inflammatory properties.

INTRODUCTION

A complex network of specialized DC subsets is involved in on the one hand inducing acute inflammatory responses upon invasion by pathogens, and on the other hand in inducing tolerance to harmless dietary and inhaled antigens and commensal microbiota [1,2]. Knowledge on the role of local mucosal and dietary factors that induce these different DC types is still limited.
Intestinal epithelial cells have been shown to be able to induce tolerogenic DCs expressing integrin alpha E (CD103) [3]. Transforming growth factor ß (TGF-ß) and retinoic acid (RA) are major factors for conferring a tolerogenic environment in the mucosa by inducing regulatory T cells (Treg) and IgA-producing plasma cells [4,5]. Intestinal epithelial cells produce TGF-ß1 in steady state and can metabolize dietary vitamin A to retinoic acid. TGF-ß present in breast milk, has been associated with development of gut homeostasis and prevention of allergy development in infants [6]. Murine but not human studies have indicated a role for the hematopoietic Fms-related tyrosine kinase 3 ligand (Flt3l) for induction of CD103 DCs [7,8]. Other reports have demonstrated that RA is able to induce CD103 expression on human monocyte derived DCs [3] or mouse bone marrow cells [9], but data on responsiveness to bacterial ligands is lacking. Both DCs and $CD4^+$ T cells can display tolerogenic properties, which is reflected by expression of CD103 by DCs and forkhead box P3 (FoxP3) by T regs [1,3,5].
DC subsets exert different functional roles in the intestines, as evidenced by altered composition of the DC network in relation to disease. Increased numbers of $CD11b^+$ DCs and decreased numbers of $CD103^+$ DCs were observed after sensitization with peanut allergens in a mouse peanut allergy model [10]. DCs are also involved in the mechanism underlying inflammatory bowel disease [11], The capacity of $CD103^+$ DCs to induce peripheral tolerance by inducing $FoxP3^+$ $CD4^+$ Tregs has received much attention [1,5]. Next to changes in

numbers of CD103$^+$ DCs, expression of CD103 Treg inducing capacity was lost in a mouse model of colitis [12]. Intestinal epithelial cells (IEC) are also important in the maintenance of intestinal homeostasis as IEC of patients suffering from colitis produce lower levels of Thymic stromal lymphopoietin (TSLP), TGF-ß1 and aldehyde dehydrogenase (ALDH). ALDH is involved in synthesis of RA, and reduced expression of ALDG was concluded to be explanatory for the altered RA-dependent induction of Tregs in those patients [3,13].

Tolerogenic CD103$^+$ DCs are discriminated from CX3CR1$^+$ DCs for their different migratory capacity (homing to mesenteric lymph node) and location in the intestines [14] CX3CR1$^+$ DCs locate close to the epithelium, whereas CD103$^+$ DCs are more frequent deeper in the lamina proria. CX3CR1$^+$ DC are abundantly found in the gut and sample soluble antigen and bacteria and have been shown to be able to protrude extensions through the epithelium into the lumen [15]. Complete activation and differentiation of T cells in contact with antigen presenting DCs is determined by expression levels of costimulatory molecules like CD80 and CD86, and the production of cytokines (TNF-α, IL-10, IL-12) and other soluble factors (e.g. RA). Next to induction of Tregs, TGF-ß and the metabolite of Vitamin A, RA, have been shown to play a role in the induction of IgA in vitro and mouse models, which is a major contributor to the first line of mucosal defence [4,16,17]. Vitamin A deficiency is associated with pathological signs in epithelia, which could be restored after supplementation with vitamin A [18], but it is not known if the restoration of pathology was mediated by CD103$^+$ DCs.

As data on the direct effects of RA and TGF-ß1 on DC phenotype and responses to bacterial ligands is strikingly limited, the current study aimed to identify how these mucosal factors affect DC phenotype and activation upon stimulation with bacterial ligand. Conventional monocyte derived DCs (moDC) were compared to moDCs that were differentiated under the additional influence of TGF-ß1 or RA. The expression of DC subtype specific cell surface markers, homing receptors, and IgG receptors was evaluated, as were their functional responses to stimulation by TLR ligands.

MATERIALS AND METHODS

PBMC and monocyte isolation

Peripheral blood mononuclear cells (PBMCs) obtained from buffy coats of healthy blood donors (Sanquin Blood Bank Nijmegen, The Netherlands) were diluted 1:1 in IMDM (Gibco-BRL, Paisley, Scotland) and isolated by gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) for 5 minutes at 200g and subsequently for 15 min. at 500g (without brake at 20^{o}C). The PBMCs were harvested from the Ficoll layer, gently resuspended in IMDM and washed two times in IMDM.

Freshly isolated PBMCs were labelled with MicroBeads conjugated to mouse IgG2a monoclonal anti human CD14 antibodies (130-050-201, Myltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 15 minutes at 4^{o}C, washed with MACS buffer (0.5% BSA, 2mM EDTA in PBS, pH 7.2), centrifuged (10 min, 200g) and resuspended in MACS buffer. The MACS columns were placed in the quadroMACS (Myltenyi Biotec) and rinsed with MACS buffer. Subsequently, the cell suspension was added, the columns were rinsed three times with MACS buffer and removed from the quadroMACS; labelled cells were collected in a new tube by rinsing with MACS buffer and the supplied plunger.

Purity of the CD14+ cell population was between 90 and 95%, as determined by flow cytometric analysis (FACS Canto II BD Biosciences, San Jose, CA, USA) by labeling the cells with mouse IgG2a anti human CD14 APC or an isotype control (clone M5E2, 555399 or 555576, BD Biosciences).

DC generation and stimulation

Isolated monocytes were cultured in RPMI (Lonza, Basel, Switserland) containing 10% standardized FCS (A15-551, PAA laboratories) and 1% pen/

strep (Gibco, 15140) at a concentration of 1*106 cells/ml. 50 ng/ml recombinant human GM-CSF (Peprotech, 300-03) and IL-4 (Peprotech, 200-04) were added and refreshed every second or third day. On day 7, expression of cell surface markers was analyzed using flow cytometry (FACS Canto II, BD Biosciences). Alternatively, DC subsets were generated by adding 10-5 M all-trans retinoic acid (Sigma Aldrich) or 10 ng/ml recombinant human TGF-ß1 (Peprotech) in addition to Il-4 and GM-CSF. Like IL-4 and GM-CSF, RA and TGF-ß1 were refreshed every second or third day. Also TSLP (50 ng/ml, Peprotech) was tested as factor for CD103 induction.

After differentiation DCs were stimulated with 10 ng/ml recombinant human IL-1ß (Peprotech) or 2 µg/ml of LPS (S. typhimurium, L7261, Sigma, St Louis, USA), PGN (S. aureus, tlrl-pgnsa, InvivoGen, Toulouse, France) or flagellin (S. typhimurium, tlrl-stfla, InvivoGen). Cell free supernatants were collected and stored at -80oC until analysis. DCs were collected and cell surface marker expression was analyzed.

Cell Surface marker analysis

DCs were stained with antibodies against CD14 (APC-H7, 560270, BD Biosciences), CD103 (PE, 550260, BD Biosciences), CX3CR1 (PerCP-eFluor710, 46-6099, eBioscience), HLA-DR (APC-eFluor780, 47-9956-41, eBioscience), CD11b (PE-Cy7, 25-0118, eBioscience), CD11c (V450, 560370, BD Biosciences), CD83 (FITC, 11-0839-42, eBioscience), CD86 (V450, 560357, BD Biosciences), ß7 chain (APC, 551082, BD Biosciences), CCR7 (FITC, 561271, BD Biosciences), CD209 (PerCP-Cy5.5, 558263, BD Biosciences), CD16 (PE, 332779, BD Biosciences), CD32 (GTX74628, GeneTex. Irvine, USA), CD64 (305015, BioLegend), FcRn (sc-271745, Santa Cruz, California, USA). For FcRn, CD32 and CD64 a secondary antibody labeled with FITC was used (goat anti mouse IgG/IgM, 555988, BD Biosciences). Of each sample a control sample was analyzed, stained with control antibodies (same concentration as positive staining was used) of the same host species and isotype, conjugated with the same fluorochrome.

Cells were incubated at room temperature for 20 minutes in the dark. Subsequently, cells were centrifuged, supernatant discarded, 200 µl FACS Buffer (BD Biosciences) added, and analyzed for surface marker expression by flow cytometric analysis (10 000 cells were analyzed using BD Canto II us-

Table 1. *Basal expression of cell surface molecules*

	moDC	RAmoDC	TGFmoDC
CD14	-	-	-
HLA-DR	++	++	++
CD1a	+	-	+
CD103	-	+	-
CX3CR1	-	-	-
CD11b	++	+	+
CD11c	++	++	+
CD86	+	+/-	+/-
CD83	+	+/-	+/-
FcRn	-	-	-
CD16	-	+/-	-
CD32	+	-	++
CD64	+/-	++	+

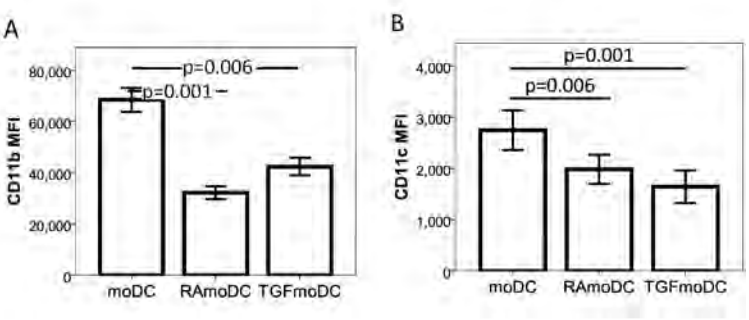


Figure 1. *Basal expression of CD11b and CD11c differs between DC subsets. Error bars indicate standard error. Significant differences are indicated. Significant differences are indicated in the diagrams.*

ing DIVA software, BD). Data was batch processed and percentages and delta mean fluorescent intensity (MFI) values (surface marker staining – isotype control staining) were obtained.

Human cytokine measurements

Human cytokine concentrations (IL-10, IL-12 and TNF-α, 558274, 558283 and 558273 respectively, BD Biosciences) in cell culture supernatants were determined using the cytometric bead array flex sets (BD Biosciences) and incubations were performed in the recommended Protein Master Buffer Kit (BD Biosciences). Briefly, 50 µl supernatant was incubated with capture beads diluted in 50 µl Capture Bead Diluent for one hour at room temperature in the dark. Next, PE Detection Reagent was diluted in 50 µl Detection Reagent Diluent, added and incubated for two hours at room temperature in the dark. For the standard curve, the provided lyophilized recombinant cytokines were used and assayed together with the samples. After incubation the samples were centrifuged, supernatant discarded and 200 µl Wash Buffer was added. The samples were analyzed by flow cytometric analysis (FACS Canto II, BD Biosciences).

Statistical analysis

Statistical tests were performed in IBM SPSS Statistics v19. The independent samples t-test was used to test mean differences between DC types or treatments. Depending on equality of variances between test conditions, the p-value of equal or unequal variances assumed was reported.

RESULTS

Effects of RA and TGF-ß on DC differentiation

IL-4 and GM-CSF are generally used to generate DCs from monocytes. moDC are often used as model DC in studies on intestinal immunomodulation. As the intestines contain several DC subsets, of which tolerogenic subsets are crucial for maintaining homeostasis, we developed two additional DC subsets using the relevant mucosal factors RA and TGF-ß1. These factors were added to the differentiating DCs in addition to IL-4 and GM-CSF. All three DC subsets expressed HLA-DR and lacked CX3CR1 and CD14 expression (Table 1). Conventionally differentiated moDCs and TGF-ß1-derived DCs (TGFmoDCs) expressed CD1a, but RA-derived DCs (RAmoDCs) did not express CD1a (Table 1).

All three DC types expressed CD11b and CD11c. Analysis of the MFI of CD11b revealed that TGF-ß1-derived DCs and even more so the RA-derived DCs expressed reduced levels of CD11b compared to moDC (p=0.006 and p=0.001 respectively, Figure 1A). CD11c expression was also

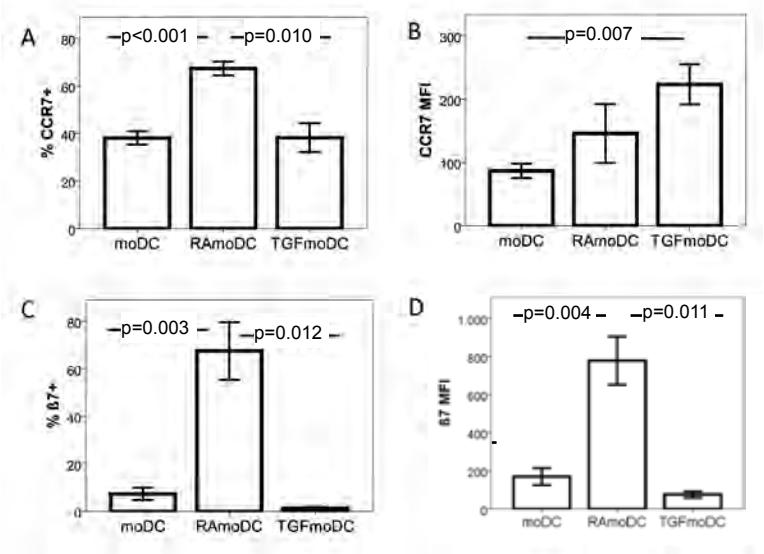


Figure 2. *Basal expression of MLN homing receptor CCR7 and ß7 chain of α4ß7 complex is different between DC subsets (isotype corrected). Error bars indicate standard error. Significant differences are indicated in the diagrams.*

Table 1. *Basal expression of cell surface molecules*

	moDC	RAmoDC	TGFmoDC
CD14	-	-	-
HLA-DR	++	++	++
CD1a	+	-	+
CD103	-	+	-
CX3CR1	-	-	-
CD11b	++	+	+
CD11c	++	++	+
CD86	+	+/-	+/-
CD83	+	+/-	+/-
FcRn	-	-	-
CD16	-	+/-	-
CD32	+	-	++
CD64	+/-	++	+

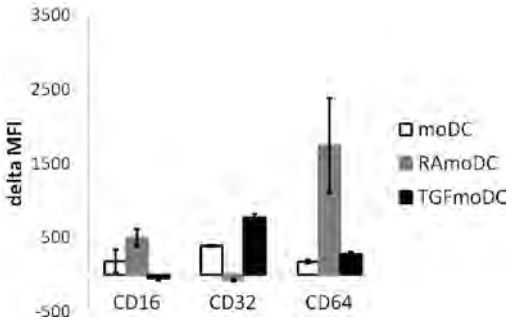


Figure 3. *DC subtype specific expression of high affinity (E.g. CD64) and low affinity complex-binding (CD32) FcγR. Isotype controlled MFI values are shown. Error bars indicate standard error.*

reduced (p=0.001 respectively p=0.006, Figure 1B). This was more prominent for TGF-derived moDCs than for RA-derived moDCs, resulting in a significantly reduced CD11b/CD11c ratio for RA-derived moDCs compared to moDCs (p=0.020, data not shown).

As in vivo functionality of CD103$^+$ DCs depends on co-expression of (α4)ß7 to adhere to E-cadherin and migrate to the intestine and on CCR7 for homing to the MLN, we assessed expression of those cell surface markers on the different DC types. The percentage of CCR7-expressing cells doubled under the influence of RA but not under the influence of TGF-ß1 (Figure 2A). The MFI level of CCR7 was significantly higher on TGF-derived moDC compared to moDCs, but not compared to RA-derived moDCs. Addition of TGF-ß1, however, did not result in increased percentage of cells CCR7$^+$ moDCs (Figure 2B). Apart from expression of CD103, the percentage (but not MFI) of DCs expressing the integrin ß7 was significantly higher for RA compared to moDC and TGF-ß1 differentiated DCs (Figure 2C and 2D).

The basal expression data of the three DC subsets are summarized in Table 1.

Differential expression of Fcγ receptors

Fc gamma Receptors (FcγR) have different affinities for IgG. Low affinity FcγR (CD16) and high affinity FcγR (CD64) can bind to monomeric IgG whereas low affinity FcγR (CD32) binds to immune complexes only [19]. CD32 was expressed by moDCs and TGF-derived moDCs, but not on RA-derived moDCs (Figure 3). CD64 was expressed by all DC types, but its expression was highest on RA-derived moDCs. FcγR expression pattern is similar for moDCs and TGF-derived moDCs, although expression of CD32 and CD64 seems to be higher on TGF-derived moDCs. RA-derived moDC have a different FcγR expression profile by expressing low levels of CD16 and high levels of CD64, but no CD32. Expression of the neonatal receptor (FcRn) could not be shown on any of the DC types.

Bacterial ligands reduce expression level of CD103 on RA-derived DCs

CD103 expression was induced by RA only and not by TGF-ß1 (Figure 4A). The integrin CD103 binds to E cadherin expressed on epithelial cells when in complex with integrin ß7. CD103$^+$ DCs are believed to migrate to the MLN upon activation, for which reduction of CD103 expression levels may be essential, next to upregulation of CCR7 which is responsible for homing to the MLN. RA-derived DCs were stimulated with several bacterial ligands and changes in CD103 expression level

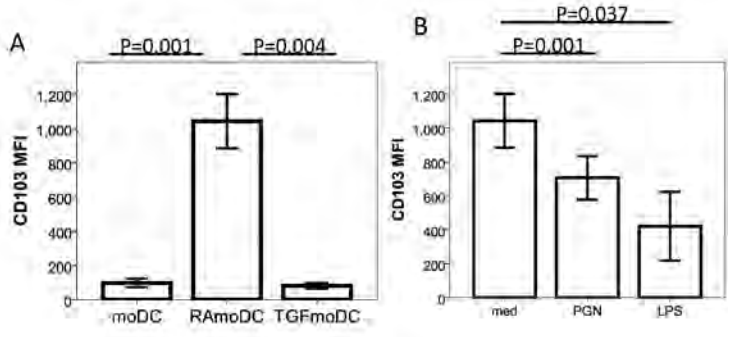


Figure 4. *CD103 is expressed on RA-derived moDCs (A) and its expression is reduced after stimulation with bacterial ligands (B). Test of significance is performed on raw MFI data (left panel) or on normalized MFI data (right panel). Error bars indicate standard error of 4 donors tested.*

were measured after 48 hours. Addition of bacterial PGN (TLR 2/6 ligand) and LPS (TLR4 ligand) significantly reduced expression of CD103 (Figure 4B).

DC subsets vary in expression of CD86 and CD83

Expression of co-stimulatory molecules is essential for complete activation of T cells. The percentage CD86-expressing cells was reduced in RA- and TGF-derived moDCs relative to moDC (p=0.013 respectively p=0.007), whereas the MFI of CD86 was only reduced in RA-derived moDCs (not significant, Figure 5). All CD83-expressing cells also expressed CD86 and comprise roughly half of the $CD86^+$ cells. RA- and TGF-ß1-derived moDCs showed a reduced percentage of $CD83^+$ cells compared to moDCs (p=0.002 respectively 0.004), whereas the level of expression (MFI) was the same.

Although the percentage of basal expression of CD86 on TGF-ß1 and RA-derived moDCs was reduced, addition of bacterial ligands (48 hours) resulted in a pronounced upregulation of CD86 expression on all DC types with most significant changes induced by exposure to LPS (Figure 6 and Table 2). However, after stimulation with the TLR ligands PGN and LPS the MFI of CD86 of RA- and TGF-derived moDC was still significantly lower compared to conventional moDCs, especially for RA-derived moDCs (Figure 6), indicating a state of non-responsiveness. Also, the percentage $CD83^+CD86^+$ cells upon activation with bacterial ligands, was significantly reduced when DCs were

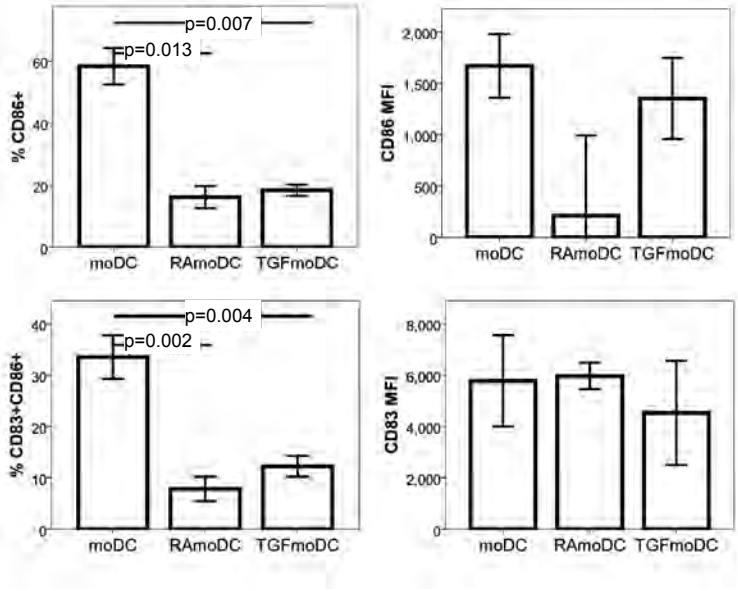
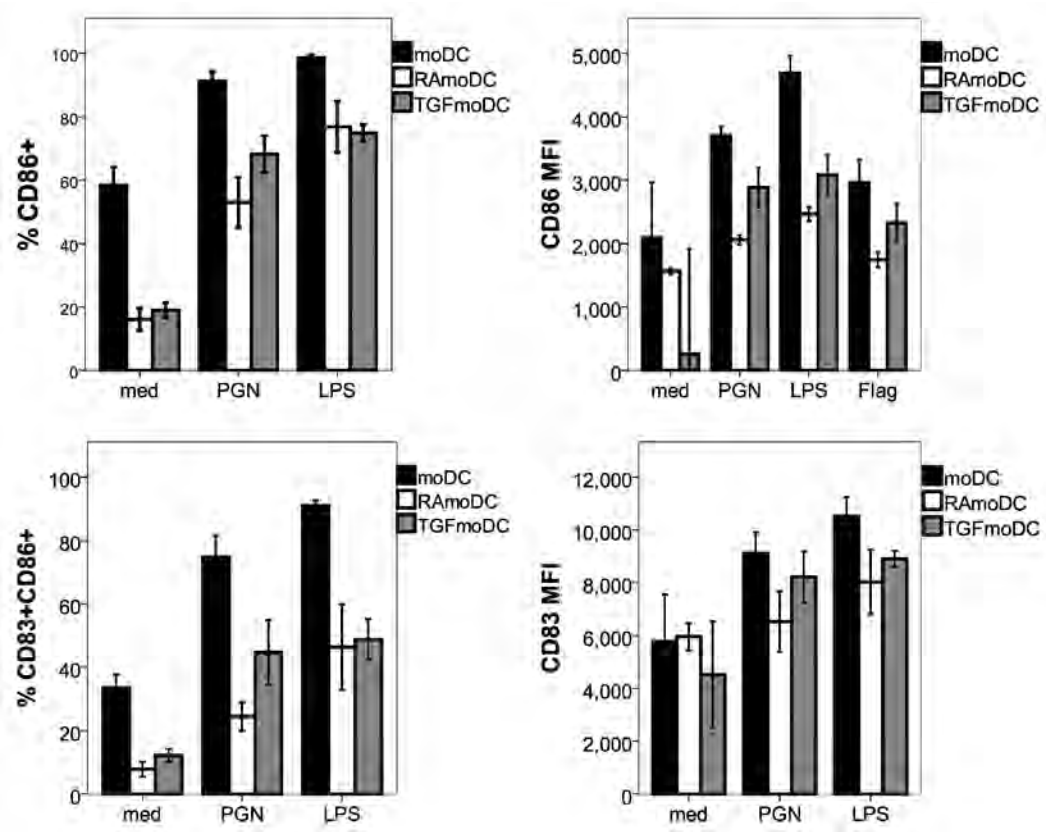


Figure 5. *Basal expression of costimulatory molecule CD86 and maturation marker CD83. All cells expressing CD83 co-expressed CD86 (not shown). Data are corrected for isotype staining. Error bars show standard error of data obtained from 4 different donors. Significant differences are indicated.*



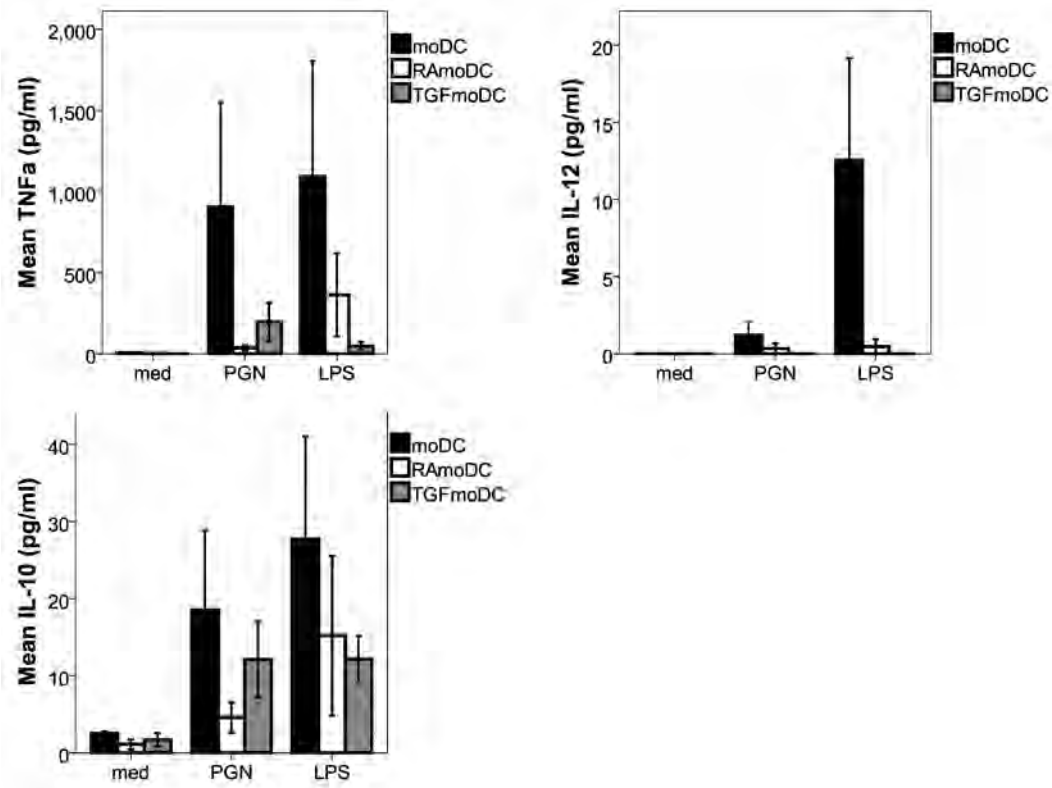
P-values of T-test between DC subsets after activation with bacterial ligands												
	moDC versus RAmoDC				moDC versus TGFmoDC				RAmoDC versus TGFmoDC			
	CD86		CD83		CD86		CD83		CD86		CD83	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
PGN	0.004	0.002	0.001	0.113	0.012	0.049	0.049	0.503	0.172	0.158	0.117	0.301
LPS	0.072	0.053	0.017	0.13	0.000	0.087	0.005	0.112	0.826	0.194	0.876	0.511

Figure 6. *Percentage and expression level of CD83 and CD86 is different between DC subsets after stimulation with bacterial ligands for 48 hours (isotype corrected). P-values are indicated at the bottom of the figure. Error bars indicate standard error; of 4 donors tested. Similar results were obtained after stimulation for 24 hours in independent experiments.*

differentiated in the presence of TGF-ß1 and RA. However, no differences in MFI of CD83 were observed.

Production of the proinflammatory cytokine TNF-α and IL-12 is reduced in RA and TGF-ß1 – derived DCs

After stimulation with the bacterial ligands LPS and PG for 48 hours, cell culture supernatants were collected and cytokine production levels (TNF-α, IL-10, IL-12) were determined. Production levels of all cytokines were decreased in cultures with DCs differentiated in the presence of RA and TGF-ß1 (Figure 7). RA and TGF-ß1-derived moDCs, showed significantly reduced production of the pro-inflammatory cytokines TNF-α and IL-12 compared to moDC (RA: p=0.017 and p=0.021 respectively; TGF-ß1: p=0.013 and p=0.017 respectively). TGF-derived moDC did not produce detectable IL-12, and strongly reduced amounts of



P-values of T-test between DC subsets after activation with bacterial ligands (relative cytokine levels)									
	moDC versus RAmoDC			moDC versus TGFmoDC			RAmoDC versus TGFmoDC		
	TNF-α	*IL-12*	*IL-10*	*TNF-α*	*IL-12*	*IL-10*	*TNF-α*	*IL-12*	*IL-10*
PGN	0.085	0.340	0.054	0.168	0.315	0.497	0.006	0.391	0.049
LPS	0.018	<0.001	0.100	0.020	0.002	0.124	0.040	0.391	0.602

Figure 7. *Cytokine production (y-axes, pg/ml) after stimulation with bacterial ligands LPS and PGN for 48 hours. Error bars show standard error and significant differences are indicated at the bottom of the figure.*

IL-12 were produced by RA-derived moDCs when PGN or LPS was added. IL-10 production by RA- and TGF-derived DCs had a tendency to reduction upon stimulation with PGN, but this did not reach significance.

DISCUSSION

In this report we describe two DC subsets that can be induced by the mucosal ligands RA and TGF-ß1. RA- and TGF-ß1-derived DCs were compared to the commonly used moDCs that are differentiated with IL-4 and GM-CSF only. Addition of RA gave rise to a CD103 and integrin ß7-expressing subset that also expressed CCR7 and resembles non-inflammatory DCs observed in vivo. DC differentiated under the influence of IL-4 and GM-CSF in the presence or absence of TGF-ß did not express CD103 and integrin ß7. Both differentiation in the presence of RA or TGF-ß1 resulted in reduced responsiveness to bacterial ligands.

All three regimens used to differentiate peripheral monocytes seem to result in differentiation of immature DC types as expression of CD14 was lost

Table 2. *Modulation of cell surface marker expression and cytokine production after stimulation with PGN or LPS.*

	moDC	RAmoDC	TGFmoDC
CD103	-	↓	-
CD83	↑↑	↑	↑
CD86	↑↑	↑	↑
IL-10	↑↑	↑	↑↑
IL-12	↑	-	-
TNF-α	↑↑	↑	↑

and HLA-DR, CD11b and CD11c up-regulated. Both $CD103^-$ and $CD103^+$ DCs expressed CD209, but $CD103^+$ DCs lacked CD1a. The in vitro differentiated $CD103^+$ DCs expressed CD11b, but at lower levels compared to $CD103^-$ moDCs. The cytokine TSLP did not induce expression of CD103. CX3CR1 expression could also not be induced by differentiation of DCs in the presence of IL-1ß. Though progress has been made, until now, the precise factors differentially inducing $CD11c^+CD103^+$ and $CD11^{low}CD103^-$ DCs have not been revealed [20]. Though functional consequences of different expression levels of CD11b and CD11c by DCs are not understood well, CD11b may correlate with more inflammatory subsets, whereas CD11c might be more expressed on tolerogenic $CD103^+$ subsets in vivo [21]. None of the moDCs generated, nor basally neither after addition of bacterial ligands, expressed CX3CR1.

Compared to moDCs, differentiation using additional RA and TGF-ß1 resulted in reduced expression of CD86 and CD83 and reduced production of cytokines, indicating that these cells may have tolerogenic properties, which was confirmed by their reduced activation by TLR ligands.

$CD103^+$DCs are believed to migrate to the lymph node upon activation in a CCR7-dependent manner [22]. $CD103^+$ DCs present in the lamina propria and MLN express CCR7 [3,20], which also was found in a significant proportion of RA-derived moDCs. Expression of ALDH does not depend on TLR-signaling [23], which is in line with our finding that expression of CD103 was downregulated after stimulation with bacterial ligands. It can be envisaged that CCR7-dependent migration is more efficient when expression of CD103 is reduced upon activation. Another effect of the downregulation of CD103 expression upon stimulation with bacterial ligands may be that $CD103^+$ DCs lose their non-inflammatory properties [12].

Another interesting difference observed between the three DC types was the level of expression of Fcγ receptors on the cell membrane. Addition of TGF-ß1 during differentiation resulted in a similar pattern as on moDC ($CD32^{hi}$, $CD16^{lo}$, $CD32^{lo}$) but higher levels of both low affinity CD32 and high affinity FcγR CD64. RA however, resulted in a lack of CD32 and relatively high expression of CD64. This may implicate that moDCs and TGF-derived moDCs have a higher potency to bind to immune-complexes including bacteria, whereas RA-derived moDCs may be specialized in binding soluble antigens (e.g. dietary proteins). Immune complex binding (e.g. bacteria) by moDCs may preferentially result in activation of T-cells, whereas those complexes bound by non-inflammatory TGF-derived DCs may result in tolerance to those bacteria. It has been established that ligation of CD64 by antigen-IgG complexes could result in activation of T cells by DCs [24]. As RA-derived DCs show non-inflammatory characteristics, ligation of CD64 on RA-derived DC ($CD103^+$) may result in induction of oral tolerance as those cells in vivo do migrate to the MLN and induce Tregs. Currently, data describing Fc gamma receptor expression of DC subsets is limited.

Like $CD103^+$ DCs found in vivo, the RA-differentiated DCs described here could induce increased percentages of $FoxP3^+$ in allogeneic $CD4^+$ cells under the influence of suboptimal doses of anti-CD3 and anti-CD28 monoclonal antibodies (data not shown). This increased expression was not observed when T cells were co-cultured with conventional moDCs, and could be abrogated by blocking TGF-ß and RA simultaneously (data not shown). The different levels of cytokines produced by the DC types may result in differential skewing of T helper cells. Absence of production of Th1 inducing IL-12 by TGF-derived DCs is beneficial for the

development for Th2 cells, also because IL-10 expression was largely maintained [25,26]. Recently, DCs have been identified as important players in induction of mucosally produced IgA. $CD103^+$ DCs are a candidate IgA-inducing DC subset [9] as those cells are able to produce the major IgA switch factor TGF-ß, and RA which induces IgA plasma cell homing to the intestines [3,4]. For synthesis of RA, humans depend on dietary intake of RA precursors. Those precursors are present in several dietary ingredients, including a number of vegetables like carrot (ß carotene) and cow's milk. Induction of peripheral antigen specific $FoxP3^+$ Tregs may indirectly depend on dietary intake of RA. Also TGF-ß is present in physiological relevant amounts (several ng/ml) in cow's milk. The bio-active form of TGF-ß1 and TGF-ß2 is identical between cow and human, and TGF-ß bioactivity is induced by acidic conditions in the stomach than inhibited [27]. Also bovine IL-10 is cross-reactive with the human IL-10R, though IL-10 is mainly present in colostrum and lacking in the majority of commercially available dairy products [28]. Presence of IL-10 during terminal differentiation into immature DCs gives rise to DCs with increase capacity to induce type 1 regulatory T cells [29,30]. Thus, several mucosal and dietary factors may contribute to a non-inflammatory intestinal milieu.

In conclusion, the RA- and TGF-ß1- differentiated DCs induce distinct mucosal DC subsets, including $CD103^+$ DC. The $CD103^+$ DCs resemble the $CD103^+$ DCs described in in vivo studies, and can advance widely used in vitro models as DC type with a different functional response to bacterial ligands. Importantly, the expression of homing markers and Fcγ receptors differed between the DC types, suggesting different functional roles in intestinal immunity. Induction of these non-inflammatory DC types may very well be modulated by the dietary intake of vitamin A and TGF-ß.

Authorship

GdH designed the study, performed the experiments and wrote the manuscript, CvA performed experiments and edited the manuscript, HFJS and RJJvN designed the study and edited the manuscript

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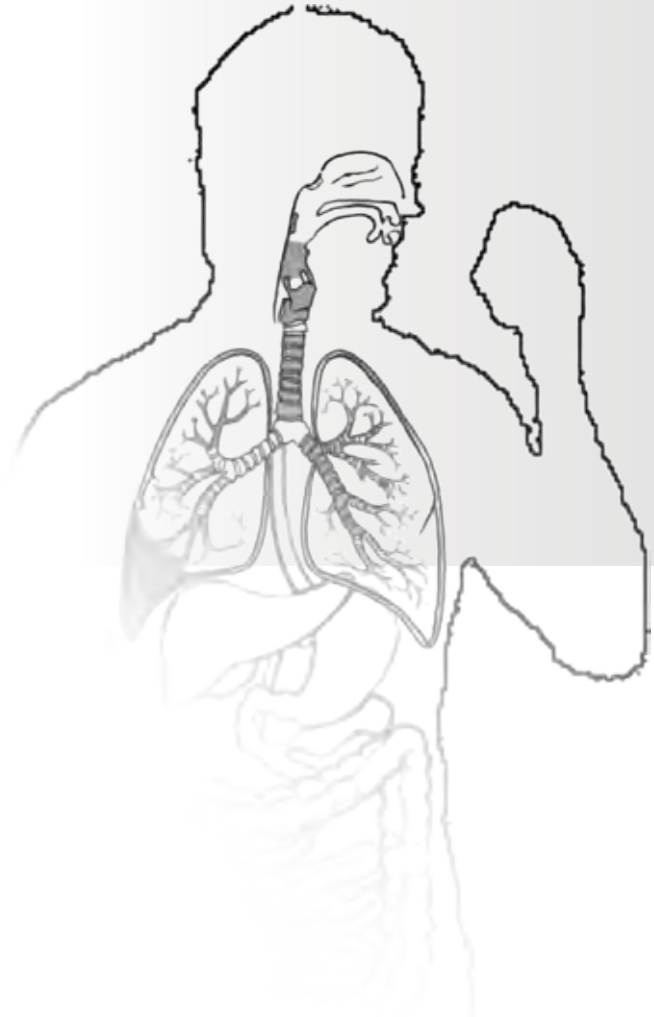

chapter 6

Differential regulation of IgA2 production by factors associated with eczema

Gerco den Hartog, Tom Berghof,
R. J. Joost van Neerven, Huub F. J. Savelkoul

ABSTRACT

IgA forms a first line of defence against pathogens at mucosal surfaces and may also be involved in protection against allergy. The mucosal factors APRIL and BAFF, supplemented with TGF-ß1, IL-10 and retinoic acid are crucial for induction of IgA. Humans produce two subclasses of IgA, IgA1 and IgA2. We aimed at identifying the role of mucosal factors in the differential regulation of T-cell independent production of IgA1 and IgA2.

B cells were isolated from peripheral blood of healthy donors and stimulated for 7 days with different combinations of cytokines. Cell culture supernatants were analysed for IgA1 and IgA2 levels, and expression of plasma cell (CD38) and homing markers (α4ß1, α4ß7) was analysed by flow cytometry. The highest levels of IgA1 were observed after stimulation with BAFF and IL-10, and the highest levels of IgA2 were observed when APRIL instead of BAFF was added. Addition of TSLP inhibited the production of IgA1 and IgA2. The vitamin A metabolite retinoic acid (RA) increased the expression of the plasma cell marker CD38 and subsequently also increased production of IgA1 and IgA2. When the factors RA and IL-10 were combined IgA-secreting plasma cells that also expressed intestinal-homing integrin ß7 were induced.

Together, these data demonstrate that the differential production of IgA1 and IgA2 is under the influence of APRIL and BAFF. RA and IL-10 can initiate differentiation of peripheral B cells into mucosal-homing IgA plasma cells. Our data provide insight into the regulation of mucosal IgA production and provide a mechanistic explanation for the association observed between reduced levels of IgA2 and APRIL and increased levels of TSLP seen in allergic eczema.

INTRODUCTION

IgA is the dominant immunoglobulin produced in mucosal tissues. The presence of IgA in the mucosa is crucial for the first line of defence against pathogens and regulation of the composition of intestinal microbiota. In contrast to rodents, humans can produce two subclasses of IgA (IgA1 and IgA2). Of the two IgA subclasses, especially IgA2 seems to be associated with protection against allergy (Pilette et al., 2007) [den Hartog et. al., submitted]. It is likely that IgA1 and IgA2 are induced by different mechanisms (Kitani and Strober, 1994). Factors inducing IgA class switch recombination have been identified, but data on regulation of differential production of IgA1 and IgA2 is limited.

IgA production can be initiated in a T helper cell-dependent (TD) and T helper cell-independent (TI) fashion. The most important IgA-inducing factor identified under TD conditions is TGF-ß, which is involved in class switch recombination to both IgA1 and IgA2 (Islam et al., 1991). TGF-ß is produced by a number of cells like T regulatory cells (Tregs), epithelial cells and dendritic cells (DCs), and even by B cells themselves. In the presence of T cells, IL-10 can enhance IgA production, but IgA class-switch recombination (CSR) is dependent on TGF-ß (Zan et al., 1998). Activation of B cells by T-helper cells also induced affinity maturation in addition to class switching (Bemark et al., 2012).

To our current understanding TI induction of IgA largely depends on innate tissue factors and DCs. The TNF-α family members a proliferation inducing ligand (APRIL) and B cell activating factor (BAFF) can substitute CD40 ligation in cases of TI induction of IgA, and are produced by epithelial cells and DCs of both intestinal and respiratory tract (Hardenberg et al., 2007; He et al., 2003; He et al., 2007; Kato et al., 2006; Litinskiy et al., 2002). In the mucosal tissues TGF-ß and IL-10 are produced constitutively, in addition to retinoic acid (RA) that is synthesized from retinal by both epithelial cells and mucosal DCs (Duester, 1996; Guilliams

et al., 2010; Molenaar et al., 2011). IL-10 has been reported to enhance IgA CSR, and to induce CSR to IgA2 in the presence of the TNF-α family member APRIL and in the absence of TGF-ß and Th cells (He et al., 2007). Dietary-derived RA affects accessibility of the IgA locus on the genome and is essential for induction of IgA plasma cell homing to the intestines in mice (Mora and von Andrian, 2009; Tokuyama and Tokuyama, 1999).

IgA production is crucial to confer mucosal protection against pathogens, and appears to be involved in protection against allergy (Kulis et al., 2012; Pilette et al., 2007). Thymic stromal lymphopoietin (TSLP) has been implicated in the induction of Th2 cells and has been shown to be increased in skin of patients with atopic dermatitis. TSLP is primarily produced by epithelial cells and keratinocytes. An association between eczema and decreased levels of serum-IgA and APRIL has also been observed. The aim of this study was to identify the role of the innate cytokines APRIL, BAFF and TSLP, and the mucosal cytokines IL-10 and TGF-ß, and dietary derived RA on IgA1 and IgA2 production and the expression of plasma cell as well as homing markers.

RESULTS

IL-10, but not TGF-ß1, increases IgA1 and IgA2 secretion by peripheral blood B cells

TGF-ß1 has been identified as a major inducer of IgA class switch recombination under the influence of CD40L expressed by Th cells. IL-10 and RA have also been implicated to enhance IgA production. To determine the role of TGF-ß and IL-10 on IgA1 and IgA2 production in the absence of T cells, these cytokines were added to B cells in the presence or absence of RA and incubated for seven days. Compared to medium, addition of IL-10 but not TGF-ß1 increased the production of IgA1 (p=0.050) and IgA2 (p=0.056) by peripheral blood B cells, which was slightly enhanced when RA was also present (p=0.010 and p=0.018, compared to medium) (Figure 1A and 1B). TGF-ß1 and RA alone or added together did not enhance production of IgA1 and IgA2. These data show that IL-10 alone, but not TGF-ß1, could enhance secretion of IgA1 and IgA2.

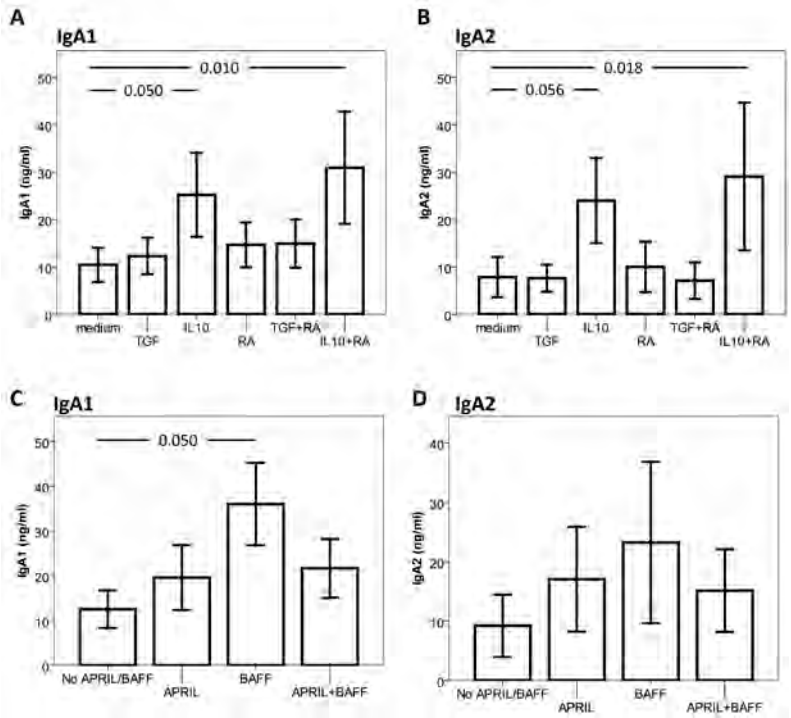

Figure 1. *IgA1 (A and C) and IgA2 (B and D) production by peripheral B cells upon stimulation with TGF-ß1, IL-10 and RA (A and B) or APRIL and BAFF (C and D) after incubation for 7 days. Error bars indicate standard error and p-values of (borderline) significant differences are shown. Data shown is of 5 to 8 healthy donors.*

BAFF and APRIL differentially induce IgA1 and IgA2 production

APRIL and BAFF are produced in mucosal tissues and skin, and have been identified as factors inducing IgA production, class switching, as well as plasma cell survival. In order to study the cooperation between BAFF and APRIL with TGF-ß1 and IL-10 on differential regulation of IgA1 and IgA2 production, these cytokines were added to peripheral B cells in different combinations.

Addition of BAFF alone significantly increased levels of IgA1 (p=0.050), but not of IgA2, in supernatant of peripheral B cells after 7 days of culture (Figure 1C and 1D). The presence of APRIL abrogated the increased production induced by BAFF. No significant increase of IgA2 production was observed after addition of APRIL or BAFF, or a combination thereof.

Addition of TGF-ß1 enhanced the production of IgA2 in the presence of APRIL (p=0.036), but not in the presence of BAFF (Figure 2). When also BAFF was added, the increased production of IgA2 in the presence of APRIL and TGF-ß1 was inhibited. This synergistic effect of TGF-ß1 with APRIL or BAFF was not observed for IgA1. In contrast to IgA2, IgA1 levels increased in the presence of BAFF alone, whether TGF-ß1 was present or not (Figure 2).

When the combination of IL-10 and BAFF was added to freshly isolated B cells, a strongly increased production of IgA1 was observed (p<0.001). This increased production of IgA1 was reduced when APRIL also was added, but IgA1 production was still significantly increased (p=0.027) compared to medium (Figure 2C). In contrast to IgA1, production of IgA2 was slightly increased when IL-10 was added together with APRIL (p=0.061), but not with BAFF.

Together, these data demonstrate that IgA1 production is optimal in the presence of BAFF and IL-10, whereas highest levels of IgA2 were observed in the presence of APRIL and TGF-ß1or IL-10.

TSLP inhibits secretion of IgA1

As TSLP levels are increased and IgA levels are decreased in patients with eczema, the role of TSLP on IgA production was studied. The addition of TSLP to B cells strongly inhibited the production of IgA (Figure 3). The inhibition of IgA1 production was observed independent of presence or ab-

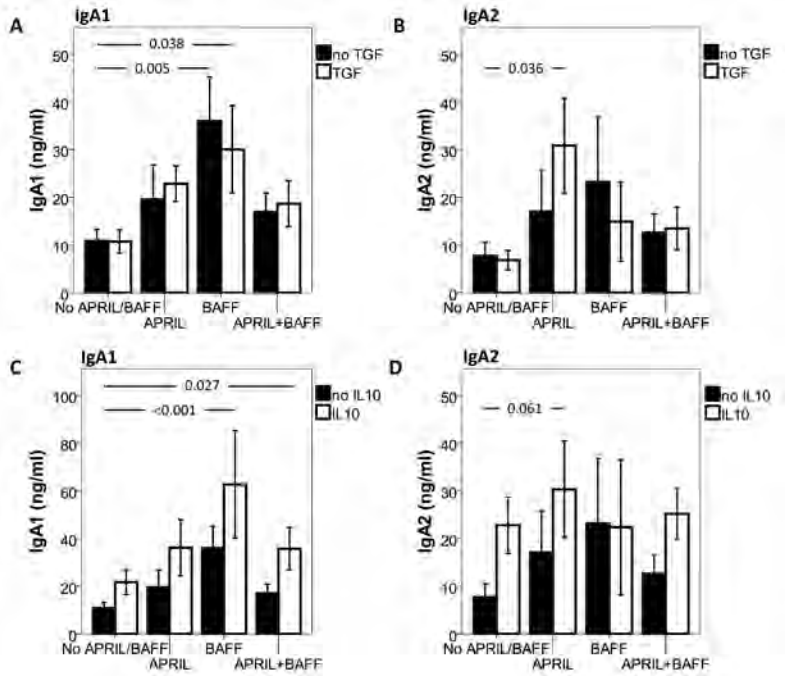

Figure 2. *IgA1 (A and C) and IgA2 (B and D) production upon stimulation with APRIL and BAFF and TGF-ß1 (A and B) or IL-10 (C and D) after incubation for 7 days. Error bars indicate standard error and p-values of (borderline) significant differences are shown. Data shown is of 5 to 8 healthy donors.*

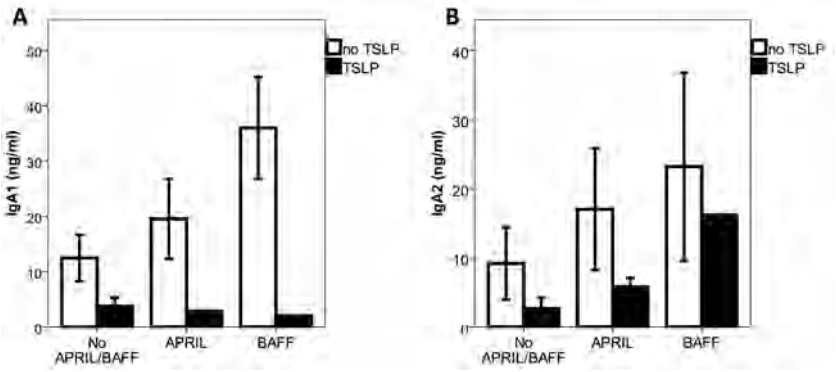

Figure 3. *The inhibition effect of TSLP on IgA1 (A) and IgA2 (B) production upon stimulation of B cells with APRIL and BAFF after incubation for 7 days. Error bars indicate standard error and p-values of (significant) differences are shown. Data shown is of 2 to 4 healthy donors.*

sence of APRIL and BAFF (Figure 3A). Inhibition of IgA2 production was observed in the absence of APRIL and BAFF, the presence or APRIL alone, and the presence of APRIL and BAFF, but not in the presence of BAFF alone (Figure 3B). These data show that the Th2 skewing factor TSLP inhibits IgA1 and IgA2 production, which might affect the protective role of mucosal IgA against allergy, especially in eczema where TSLP is increased.

RA induces plasma cell differentiation of IgA-B cells

IgA$^+$ B cells need to differentiate into plasma cells to secrete high levels of immunoglobulins, in order to confer mucosal protection against pathogens or allergens. Therefore the effect of mucosal factors on the induction of expression of the plasma cell marker CD38 on IgA1$^+$ as well as IgA2$^+$ B cells was studied. Expression of CD38 was significantly enhanced by RA (p=0.001). Addition of TGF-ß1 partly inhibited RA-induced expression of CD38, whilst CD38 expression was maintained or even increased when IL-10 was added in addition to RA (Figure 4A). Addition of RA to B cells resulted in induction of CD38 expression of the majority of IgA1 and IgA2 membrane-positive B cells, indicating differentiation of IgA1$^+$ as well as IgA2$^+$ plasma cells by RA (Figure 4B).

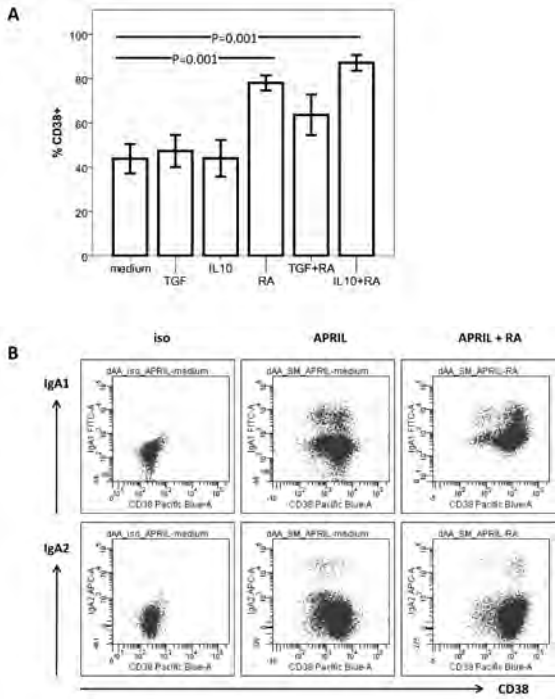

Figure 4. *Modulation of plasma cell marker CD38 expression after stimulation with TGF-ß, IL-10 and RA after incubation for 7 days (A). Error bars indicate standard error and p-values of differences are shown. Data shown is of 5 till 8 healthy donors. The majority of IgA1 and IgA2 positive B cells express CD38 after stimulation with RA in the presence of APRIL (B).*

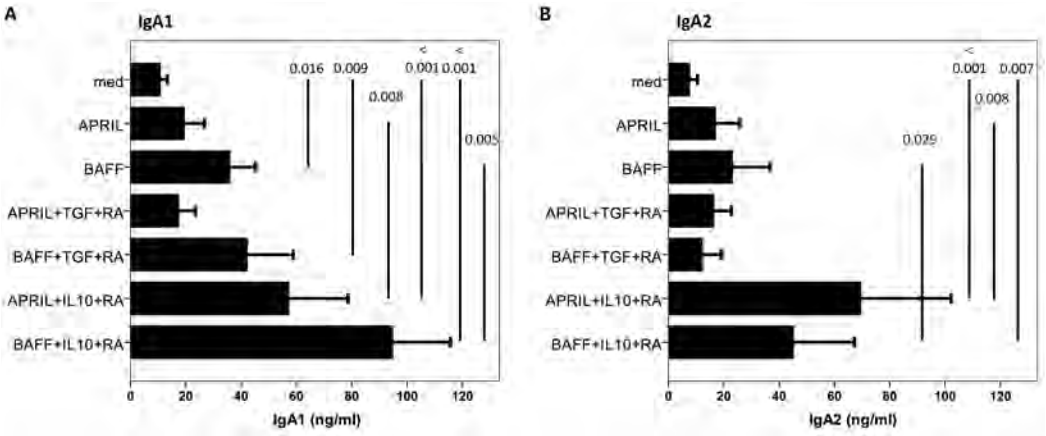

Figure 5. *Effect of RA on IgA1 (A) and IgA2 (B) production upon stimulation with APRIL or BAFF and TGF-ß1 or IL-10 after incubation for 7 days. Error bars indicate standard error and p-values of (significant) differences are shown on top of the graph. Data shown is of 5 to 8 healthy donors.*

RA enhances secretion of IgA1 and IgA2 in the presence of IL-10

As RA induced the expression of plasma cell marker CD38, experiments were performed to demonstrate if actual production levels of IgA1 and IgA2 were also enhanced by RA. Addition of RA to peripheral blood B cells in the presence of IL-10 resulted in increased production of both IgA1 and IgA2 (Figure 1B). This synergistic effect of RA was not observed when TGF-ß1 was added instead of IL-10. The combination of IL-10 and RA enhanced production observed after stimulation with APRIL or BAFF alone (Figure 5). The synergistic effect of IL-10 and RA with APRIL or BAFF alone was inhibited when both APRIL and BAFF were added (data not shown). The highest levels of IgA1 production were observed when RA and IL-10 were added to B cells together with BAFF. IgA2 production however, was maximal when RA and IL-10 were added together with APRIL. These data indicate that induced expression of CD38 by B cells results in IgA-secreting plasma cells in the presence of IL-10, but not TGF-ß1.

Induction of mucosal-homing markers on IgA1 and IgA2 plasma cells by RA and IL-10

In addition to induction of IgA-secreting plasma cells, homing properties of B cells are required for effective mucosal protection by IgA. All cultured peripheral B cells expressed integrin ß1, regardless of the absence (data not shown) or presence of any of the factors tested (Figure 6A). Expression of integrin ß7 however, was increased after stimulation with APRIL or BAFF alone, but not when APRIL and BAFF were added together (Figure 6B). Integrin ß7 expression was induced when B cells were cultured in the presence of IL-10 and RA, but not in the presence of TGF-ß1 and RA, or any of those factors alone (Figure 6C). The majority of B cells expressing integrin ß7 also expressed CD38 after stimulation with APRIL, RA and IL-10 (Figure 6D). Expression of both integrin ß7 and CD38 on B cells was further increased by IL-10 and RA, suggesting the induction of intestinal-homing plasma cells. Also IgA1 and IgA2 positive B cells expressed CD38 and integrin ß7. Together our data indicate that IL-10 and RA are involved in induction of intestinal-homing $IgA1^+$ and $IgA2^+$ plasma cells, whereas under these T-cell independent conditions no profound role for TGF-ß1 was observed.

DISCUSSION

Although it has been known for decades that IgA1 and IgA2 production levels vary between different mucosal tissues and compartments, knowledge on

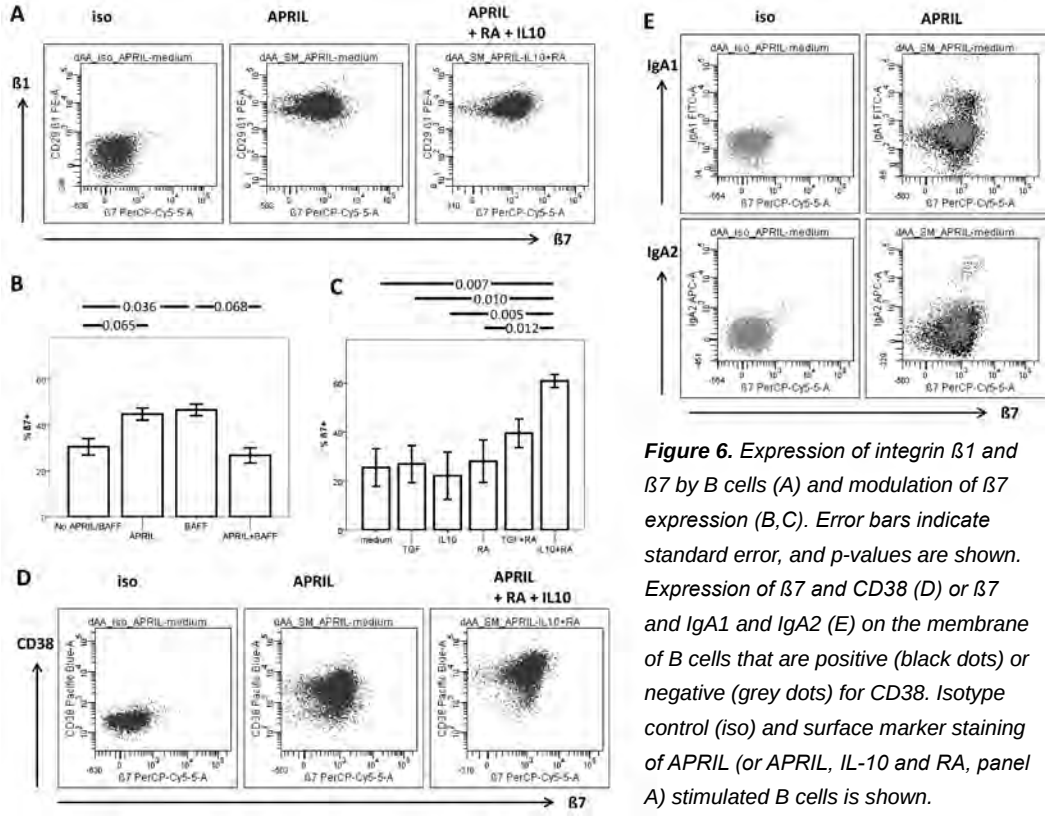

Figure 6. *Expression of integrin ß1 and ß7 by B cells (A) and modulation of ß7 expression (B,C). Error bars indicate standard error, and p-values are shown. Expression of ß7 and CD38 (D) or ß7 and IgA1 and IgA2 (E) on the membrane of B cells that are positive (black dots) or negative (grey dots) for CD38. Isotype control (iso) and surface marker staining of APRIL (or APRIL, IL-10 and RA, panel A) stimulated B cells is shown.*

differential regulation of production of IgA1 and IgA2 in humans is still limited (He et al., 2007; Kitani and Strober, 1994). In this study the combination of BAFF and IL-10 promoted IgA1 production, whereas APRIL and IL-10 promoted IgA2 production. Upon further addition of RA the plasma cell marker CD38 was induced and secretion of IgA1 and IgA2 was increased. The combination of IL-10 and RA induced expression of receptors for homing to the intestines by IgA-producing B cells. A significant amount of IgA production in the intestines is induced upon triggering by bacterial ligands, and occurs independently of Th cells (Bemark et al., 2012). The advantage of TI IgA induction over TD IgA induction may the time required to initiate production as no T cells are involved. IgA plasma cells induced in the absence of T cells, but in the presence of APRIL and BAFF may stay local in the tissue (Figure 6) and be relatively short-lived and unable to induce systemic antigen-experienced B cells. However, factors like APRIL and BAFF have also been identified as crucial for survival of long-lived IgA plasma cells (Benson et al., 2008; O'Connor et al., 2004).

That factors differentially regulating IgA1 and IgA2 may exist has been known for a long time, based on the observation of different distribution of $IgA1^+$ and $IgA2^+$ B cells through the body (Crago et al., 1984). In addition to TGF-ß, IL-10 has been implicated before as inducer of IgA2 production when stimulated with anti-CD40 monoclonal antibodies or CD40 ligand, or when B cells were stimulated with APRIL and IL-10 (He et al., 2007; Kitani and Strober, 1994). In line with this, we also observed highest levels of TI IgA2 production under the influence of APRIL and IL-10, whereas TI IgA1 production was highest in the presence of BAFF and IL-10. Addition of BAFF alone significantly increased IgA1 production, which was further enhanced in the presence of IL-10, but not TGF-ß1. To increase production of IgA2, the presence of APRIL and TGF-ß1 or IL-10 was required.

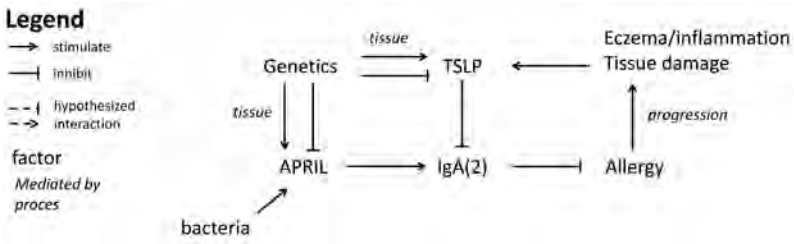

Figure 7. *Hypothetical model explaining the association observed between allergy and allergic eczema with TSLP and APRIL.*

When APRIL and BAFF were added together, the production of IgA1 in the presence of BAFF and the production of IgA2 in the presence of APRIL and other factors were inhibited.

Depending on the location of the B cell and the differentiation stage, different receptors for BAFF and APRIL are expressed on B cells, of which some bind to both APRIL and BAFF (TACI), while others bind with higher affinity to APRIL (BCMA) or BAFF (BAFF-R) alone (Bossen and Schneider, 2006; Darce et al., 2007). Future studies should confirm if membrane expression or intra cellular signalling of those receptors is influenced by IL-10 and RA, and to what extent IgA plasma cell differentiation is affected by those receptors. The data presented here identify factors enhancing IgA1 and IgA2 production by a mixed population of freshly isolated peripheral B cells. Therefore, the IgA1 and IgA2 production observed originates from a combination of de-novo induced $IgA1^+$ and $IgA2^+$ B cells, and B cells that were already committed to IgA1 or IgA2 production before the start of the cell culture experiments. Increased production of IgA1 by BAFF alone might well be caused by IgA plasmablasts that are sensitive to stimulation with BAFF, whereas IgA2 induction more likely depended on de-novo CSR under the influence of APRIL. As our aim was not to identify factors for IgA CSR, a heterogeneous population of B cells was used in order to resemble more closely IgA production upon activation of heterogeneous B cell populations in vivo. Future studies should identify which process (e.g. CSR) and what B cell development stage contributes most to IgA1 and IgA2 production.

In order to confer IgA-mediated protection at mucosal surfaces, regulation of expression of homing molecules is required to guide the cells to appropriate sites in the body. Expression of integrin α4ß7 by mouse B cells was shown to be abrogated in the presence of a RA inhibitor (LE540), and the presence of IgA^+ B cells in the gut is dependent on dietary vitamin A (Mora et al., 2006). Based on our data and a literature report describing induction of CD38 plasma cells under the influence of IL-10 and RA under T cell dependent conditions, similar mechanisms seem to exist in humans (Dullaers et al., 2009; Morikawa and Nonaka, 2005). Our data indicate that neither RA alone nor IL-10 alone, but the synergism of IL-10 and RA induced integrin α4ß7 expression. Integrin α4ß7 binds to MAdCAM-1, which is expressed in the intestines but seems to be lacking in the respiratory tract (Xu et al., 2003). However, homing of lymphocytes to respiratory tissue is mediated by, amongst others, integrin α4ß1, which was expressed on all B cells in our cultures (Xu et al., 2003). To our current understanding of plasma cell homing pathways, intestinally-induced B cells ($\alpha4ß1^+\alpha4ß7^+$) may migrate to a variety of other mucosal sites, including the respiratory tract, whereas B cells activated in the respiratory tract ($\alpha4ß1^+$) preferably migrate back to the VCAM-1 expressing respiratory tract rather than the intestines (expressing MadCAM-1) (Rott et al., 2000; Xu et al., 2003). As a result, antigenic challenges in the gut could result in systemic dissemination of antigen-specific B cells conferring protection against subsequent encounter of those antigens.

Recently, Pilette et. al. described that upon specific allergen immunotherapy allergen-specific IgA2 B cells were induced in the nasal mucosa, but not IgA1 B cells (Pilette et al., 2007). Immunotherapy has been shown to increase the numbers of aller-

gen specific Tregs (Jutel et al., 2003). Until, now there is no evidence showing that Tregs preferentially induce IgA2 and not IgA1. This induction of IgA2 upon allergen specific immunotherapy may well be mediated by local tissue factors like APRIL and IL-10. In addition to correlation with absence of allergy, serum IgA2 levels were associated with the presence of eczema in house dust mite allergic patients (den Hartog et. al., submitted). A double blinded randomized dose-response study where house dust mite allergic patients received immunotherapy, showed a reduction of eczema after immunotherapy (Werfel et al., 2006). Together these findings may need further study to reveal if allergen-specific serum IgA2 levels are causally related to the absence of eczema in sensitized individuals. A possible underlying mechanism (Figure 7) could be the reduced production of APRIL in patients suffering from eczema, in combination with increased expression of TSLP (Chen et al., 2011; Lee et al., 2010; Lundell et al., 2009). Here we have shown that APRIL is involved in induction of IgA2 production, whereas TSLP may inhibit IgA2 production. APRIL and TSLP are constitutively produced in the mucosa and skin and therefore induction or inhibition of IgA2 production may occur both in the intestine and skin (Demehri et al., 2009; Soumelis et al., 2002). TSLP has been implicated in both intestinal Th2 skewing and progression of eczema into asthma (Blázquez et al., 2010; Demehri et al., 2009; Soumelis et al., 2002). The fact that numerous B cells are present in the intestine is widely known. However, B cells expressing α4ß1 can also migrate to the skin, at least under inflammatory conditions (Geherin et al., 2012). Consequently, factors produced in the skin may affect B cell IgA production, also because induction of CSR (not necessarily CSR itself) is being reported in different tissues that are not organized lymphoid tissues like Peyers patches or lymph nodes (Cameron et al., 2003; Chvatchko et al., 1996; Payne et al., 2011). An alternative mechanism underlying the association between IgA levels and absence of eczema could be induction of IgA production in early life. Indeed, colonization with toxic Staphylococcus aureus has been associated with increased levels of APRIL and IgA in serum, and reduced numbers of infants with eczema later in life (Lundell et al., 2009). In line with this, an association has been found between bacterial exposure, secretory IgA levels in saliva and reduced clinical symptoms in infants (Böttcher et al., 2002; Fagerås et al., 2011). In addition to these environmental factors, genetic factors like polymorphisms in IL-10 and TGF-ß promoter regions have been associated with increased incidence of eczema (Hobbs et al., 1998).

In summary, this study shows that mucosal and T cell independent IgA1 and IgA2 production is optimal under the influence of BAFF and APRIL respectively, which may explain the different levels of IgA1 and IgA2 observed in different tissues. In addition, the data presented here may help to explain the observed association between allergic eczema and IgA2 and APRIL induced by mucosal and skin factors.

MATERIALS AND METHODS

PBMC and B cell isolation

Peripheral blood mononuclear cells (PBMCs) obtained from buffy coats of healthy blood donors that provided informed consent (Sanquin Blood Bank Nijmegen, The Netherlands) were diluted 1:1 in IMDM (Gibco-BRL, Paisley, Scotland) and isolated by gradient centrifugation on Ficoll-Plaque PLUS (Amersham Biosciences, Uppsala, Sweden) for 5 minutes at 200g and subsequently for 15 min. at 500g (without brake, 20°C). The PBMCs were harvested from the Ficoll layer, resuspended and washed two times in IMDM.

Freshly isolated PBMCs were incubated with CD19 MicroBeads (130-050-201, Myltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C, washed with MACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH=7.2), centrifuged (10 min, 200g) and resuspended in MACS buffer. The MACS columns were placed in the quadroMACS (Myltenyi Biotec) and rinsed with MACS buffer. Subsequently, the cell suspension was added, the columns were rinsed three times with MACS buff-

er and removed from the quadroMACS; labelled cells were collected in a new tube by rinsing with MACS buffer and the supplied plunger.
Purity of the $CD19^+$ cell population was between 90 and 95%, as determined by labelling the cells with CD19-antibodies or isotype control (561121 or 560787, BD Biosciences), followed by flow cytometric analysis (FACS Canto II BD Biosciences, San Jose, CA, USA).

B cell cultures
B cells were cultured at a concentration of 0.5-1 million cells/ml in 48 wells plates in RPMI supplemented with 10% FBS and 1% pen/strep. Stimuli were added and incubated with the cells for 7 days at 37oC in a humidified environment and 5% CO2. Stimuli were used as follows: All-trans RA 10-5M (Sigma Aldrich, St. Louis, MO, USA), 10 ng/ml TGF-ß1, 20 ng/ml IL-10, 125 ng/ml APRIL and BAFF (all from Peprotech, Rocky Hill, NJ, USA). Cell culture supernatants were collected and stored (-20oC) until use. Cells were harvested, centrifuged and stained for flow cytometric analysis.

IgA1 and IgA2 ELISA
ELISA plates (Greiner Bio One, Alphen a/d Rijn, The Netherlands) were coated with 0.2 or 1.0 µg/ml Polyclonal goat anti human IgA (2050-01, Southern Biotec). Plates were washed (0.05% tween in PBS) once and blocked with biotin free casein (UCBD, SDT reagents, Baesweiler, Germany). After blocking plates were washed three times and undiluted cell culture supernatants or 50 ng/ml IgA1 or IgA2 (16-16-090701-1M or 16-16-090701-2M, Athens Research, Athens, Greece) was added in 1:2 block and wash buffer respectively. The IgA1 and IgA2 antibodies were serially diluted to obtain a standard curve. Plates were washed and 1/5000 or 1/2000 biotinylated anti IgA1 or IgA2 was added (Southern Biotec). Plates were washed 6 times and 1/10 000 streptavidin poly HRP 80 (SDT reagents) was added and covered. 100 µl TMB (SDT reagents) was added. Plates were developed until wells developed clear colours, without obtaining too much back ground signal, stopped with acid and measured at 450 nm and 650 nm as reference. Calibration curve was calculated using 4-parameter logistic fit. Incubations were performed for 1 hour at room temperature on a shaker.

Flow cytometric analysis
B cells were stained with CD19 (V500, BD Biosciences, Franklin Lakes, NJ, USA), CD38 (BV421, BD Biosciences), IgA1 (FITC, Southern Biotec, Birmingham, AL, USA), CD29 (ß1) (PE, BD Biosciences), ß7 (PerCP-Cy5.5, BioLegend, San Diego, CA, USA), CD27 (PE-Cy7, BD Biosciences), IgA2 (APC, Miltenyi Biotec, Germany), or the respective isotype controls: Mouse IgG1 conjugated to V500, PE, BV421, PE-Cy7 (BD Biosciences), mouse IgG1 FITC (0102-02, Southern Biotec),, Rat IgG2a PerCP-Cy5.5 (400532, BioLegend),), or mouse IgG1 APC (130-092-214, Miltenyi Biotec). At least 10 000 events within the cells gate were collected using BD FACS Canto II (BD Biosciences). Data were batch processed and values reported corrected for background staining (FACS Diva software).

Statistical analysis
Data were entered in IBM Statistics version 19. Data were square root transformed to improve distribution of the data. Differences were tested using the two independent samples T-test or generalized linear models.

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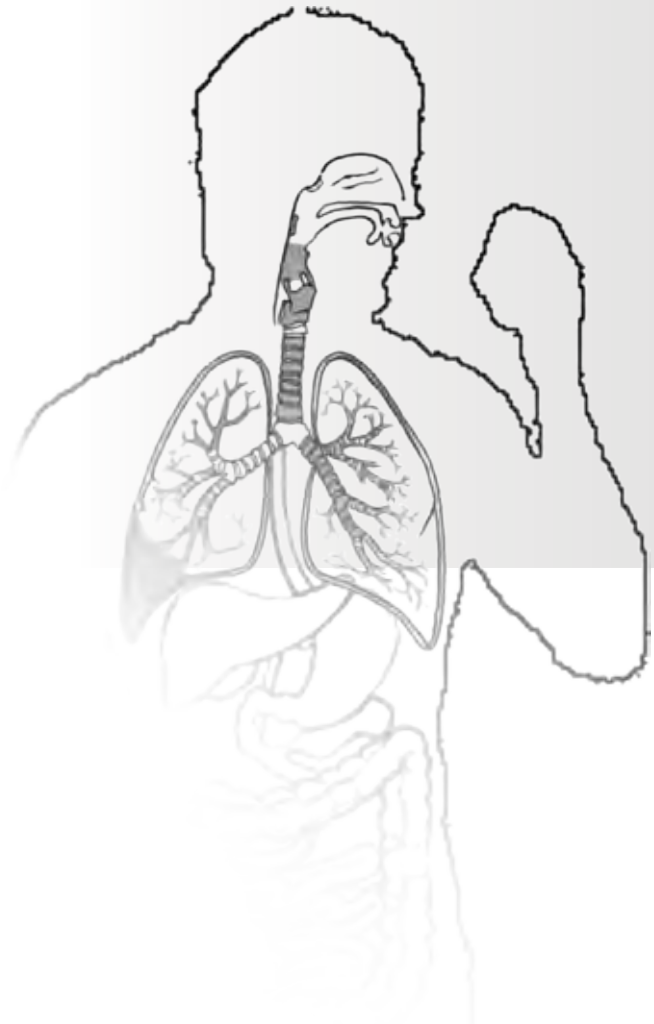

chapter 7

The level of IgA but not the Ig-repertoire is related to intestinal microbial composition during ontogeny

Gerco den Hartog, Richard P. M. A. Crooijmans,
Ger de Vries-Reilingh, Ariane M. Wehrmaker, Nicolaas A. Bos,
Huub F. J. Savelkoul, Henk K. Parmentier, Aart Lammers

ABSTRACT

In this study the interplay between intestinal microbiota and functional maturation of the antibody response during avian ontogeny was investigated. The IgM, IgG and IgA CDR3 spectratypes successively changed over time. However, IgA VH pseudogene usage remained the same over time. Flow cytometric analysis of the fecal microbiota revealed that in the first weeks of life maximally 30 percent of fecal bacteria were coated with IgM, and from day 35 onwards up to 70% of fecal microbiota were coated by IgA. No coating with IgG was observed. Microbiota composition was transiently changed (day 14-42) and related to IgA expression levels but not to deviations of CDR3 spectratypes from the normal distribution. The results obtained indicate that endogenous humoral immunity plays a limited role in the first four weeks post-hatching. We hypothesize that modification of the naive IgA CDR3 repertoire is not required to recognize the majority of intestinal bacteria.

INTRODUCTION

Immunoglobulin M and A in the gut are essential for the first line of defense against infections in vertebrates(1, 2). Initially, neonates receive protective maternal antibodies either via egg yolk (aves) or via the placenta and milk (mammals). Avian maternal antibody levels will decline and reach negligible levels within three weeks post-hatch. In aves maternal derived antibodies are mainly of the IgG class whilst novel findings indicate that maternal-derived IgA might be present in the intestinal tract of hatchlings (3).

Primary activation and maturation of the intestinal humoral immune system is at least partly dependent on (commensal) intestinal microbiota, as shown by a poorly developed immune system of animals grown in germ-free conditions(1, 2, 4-9). Naive B cells express IgM and initial intestinal body defense depends exclusively on IgM, which like IgA can be transported across the gut epithelium into the lumen by the polymeric immunoglobulin receptor (pIgR)(10, 11). This is demonstrated by pIgR-/-mice, which lack IgM and IgA in the intestinal lumen. Lack of secretory IgA at mucosal surfaces results in increased mucosal leakiness as shown by the presence of serum proteins in mucosal secretions of pIgR-/- mice, and in increased vulnerability to mucosal infections as compared to normal genotypes(12-14).

In mammals, intestinal microbiota are believed to be recognized by T cell independent and poly-specific antibodies, as well as T cell-dependent high affinity IgA(15), (16, 17). These poly-specific antibodies can belong to either IgM or IgA classes. Data on the role of the antigen-binding repertoire maturation in intestinal immunity is limited. Maturation of the antibody repertoire into functional binding antibodies may be required to confer protection to the host against pathogens and influence microbial composition in the intestinal lumen. This antibody repertoire is largely determined by the complementarity determining region 3 (CDR3). The CDR3 length distribution of antibodies of unstimulated or highly heterogeneous populations of B cells can be approximated by a normal distribution (18, 19). Thus deviations from the normal distribution could indicate activation of a selective subset of B cells resulting in increased expression of antibodies with a certain CDR3 length. Especially plasma cells are highly productive and hence important determinants of the intestinal IgA CDR3 repertoire. CDR3 length could affect the shape of the antigen recognition region and consequently its binding specificity (20-23). The CDR3 diversification process in chickens is different from that described in mammals: in chickens, the CDR3 encoding domain consists of a single VH-gene, combined with a D (used from a set of 15 D genes) and one J gene(24). After recombination of the heavy chain gene, the variable domain is subsequently diversified by somatic gene conversion of variable pseudogene segments into the 3' end of the heavy chain gene(25, 26). CDR3-diver-

sification in combination with antibody production levels determines the ability to coat microbes by binding to surface molecules.

Currently, data describing the maturation of the intestinal IgA-CDR3 repertoire in relation to microbial composition during ontogeny is limited. In the current study, we investigated if the microbial composition is related to the CDR3 repertoire of the various immunoglobulin isotypes. Additionally, the functionality of IgM, IgG or IgA in the intestines during ontogeny was investigated by analyzing the percentage of bacteria coated with immunoglobulins. Finally, we assessed age related changes in microbiota composition and their association with local cytokine production and the CDR3 repertoire diversity.

MATERIALS AND METHODS

Animals

Commercially obtained eggs of Lohman Brown chickens were incubated at 41°C and 65% humidity and allowed to hatch in climate respiration cells. Hatchlings were housed in groups of five per cage. 100 mg ileum tissue was collected at days 4, 7, 21, 35, 49 and 70 after hatch from 10 chickens at each time point, based on experience from previous experiments. The performed experiments were reviewed and approved by the Animal Experimentation Committee of Wageningen University, The Netherlands, according to Dutch law.

RNA isolation and cDNA synthesis

Total RNA extraction from ileum tissue samples was performed using TRIzol® reagent (Lifetechnologies, Breda, The Netherlands) according to the manufacturer's recommendations. RNA concentrations were determined by spectrophotometric analysis and equalized by dilution in DEPC water. RNA was checked by agarose gel electrophoresis for integrity and treated with DNase I for 15 min at RT (Invitrogen, Breda, The Netherlands, 18068-015) and inactivated using EDTA (65°C for 10 min). 300 ng random hexamers, 1 µl 10 mM dNTP mix, 4 µl 5× first strand buffer, 2 µl 0.1 M DTT and 10 U RNase inhibitor (Invitrogen, 15518-012) were added and incubated for 10 min at 37 °C. 200 U Superscript RNase H– reverse transcriptase (RT; Invitrogen, 18053-017) was added and incubated for 50 min at 37 °C.

Spectratyping

The universal forward primer (CACCATCTCGAGGGACAAC, FAM-labeled) was located at the VH gene segment (genbank M30319.1), 97 bp upstream of the CDR3 region and could amplify the complete CDR3-repertoire. Pseudogenes 57-12 and 57-3 contain substitutions in the area covered by the forward primer, and might depending on the part used from the pseudogene, not be amplified in our PCR. The reverse primers (GGTGCATGGTGACGAAAAG (X01613.1) for IgM, CAGAGGGAGGAGCTGGTAGA (S40610.1) for IgA and GGGTCCCCGAAACTCTGC (X07174.1) for IgG were located downstream of the CDR3 region at the isotype specific constant part of the heavy chain.

PCR was performed with 1 µl cDNA, 0.4 µM forward and reverse primer, 6 µl ABGene Master Mix (Applied Biosystems, Foster City, U.S.A) and 4 µl de-ionized water. The PCR program consisted of a preheating step (10 min, 95°C), 36 cycles at 58 °C and 10 min extension. One µl PCR-product (20 ng/µl) was diluted in 9 ul formamide (Applied Biosystems) with GeneScan™ 600LIZ® Size Standard and analyzed on a DNA Analyzer 3730 (Applied Biosystems). A dilution experiment showed that in samples with high expression of Ig, the cDNA sample could be diluted 10 000 times to result in similar spectratype profiles. Data files were imported in ABI PRISM® GeneMapper v4.0 (Applied BioSystems) and batch processed to extract product size and relative frequency data which was further processed in MS Excel (Microsoft corp., U.S.A.). A cut off value was used to eliminate background signal and minor PCR artifacts. Peaks of spectratype profiles needed to be separated by three nucleotides, yield sufficient signal to discriminate multiple peaks. Each spectratype profile was standard-

ized by dividing the area under a single peak by the total area of all peaks of that spectratype diagram. The average CDR3 lengths were calculated: $\bar{x} = averageCDR3size(x) = \sum_{i=1}^{n} \frac{z_i}{Z} x$, where and z_i is frequency of product size x_i. For each sample, CDR3 length was used as $\bar{x}$ and 's' was estimated using the following formula: $s = \sqrt{\frac{Z_1}{Z}(x_1 - \bar{x})^2 + \frac{Z_2}{Z}(x_2 - \bar{x})^2 + \ldots + \frac{Z_n}{Z}(x_n - \bar{x})^2}$. The obtained and 's' values were inserted in the formula describing the normal distribution (18). The variability between the standardized spectratype data and the normal distribution was calculated: sum of squares of X = SS = (Figure S1). Ig expression was very low in a number of samples, which resulted in too low frequencies of CDR3 regions (eg IgG day 70). Those samples were not further analyzed.

Sequencing

Immunoglobulin sequences were PCR amplified using the same procedure as described for spectratyping, but different reverse primers were used for IgA and IgG (AGGAGGGTCACTTTGGAGGT respectively CTCTTCCCTTCCCTCCAATC). 3 µl PCR product was used for ligation in pGEM-T Easy Vector (Promega, Madison, USA, Quick protocol, 3). After transformation and incubation overnight at 37°C 40 – 50 positive clones were collected and a colony-PCR was performed. 1 µl colony suspension, 12 µl 2x ABgene Master Mix, 0.5 µl forward and reverse primers (10 µM, T7 forward and Sp6 reverse) and 11 µl MQ (5 min at 95°C, 30 cycles of 1 min at 55°C, 10 min extension), amplicons were checked on 1% agarose gel and purified using SephacrylTM S-400 (GE Healthcare, 17-0609-01). On the purified product a dual sequence reaction (same program as colony PCR) was performed (using either T7 or Sp6, 1.6 µM), purified again and analyzed on a DNA Analyser 3730 (Applied Biosystems) using 1µl sequence reaction product and 9 µl formamide.

Sequence analysis

A number of VH pseudogenes have been published (26). The same position of the start of the CDR3 region was used as proposed by Reynaud et. al.(26). CDR3 sequences were blasted with the following settings: nucleotide blast, database, other: nucleotide collection (nr/nt), Organism: gallus (taxid: 9030), entrez query: pseudogene NO mRNA NO cDNA, discontinuous mega blast. The three scores with the lowest E-value describing a germ line pseudogene were exported and used for further analysis in Excel). To select the best fitting pseudogene, query coverage was multiplied by the sequence identity and the pseudogene with the highest score was selected. The dendrograms of the germ line pseudogenes and obtained CDR3 sequences were made with MEGA 4, using the neighbor joining p-distance model.

Microbial Community Profiling (MCPC)

Mid sections of ileum were opened with a sterile scalpel, rinsed in cold PBS and sampled by moving rigid nylon brushes (Servoprax GmbH, Wesel, Germany) up and down over a length of 3 cm. Brushes were stored at -20 °C until further use. Bacterial DNA was isolated by putting the nylon brushes in 6 M guanidine thyocyanate, 0.6% Tween-20 (v/v), 10 mMEDTA, 50 mM Tris-HCl, pH 6.5 and 2 g of zirconia beads (<0.1 mM, Biospec Instruments, Bartlesville, OK, USA). The bacteria were physically disrupted by shaking for 4 minutes in a MiniBeadbeater-96™ (Biospec Instruments), followed by heating for 5 minutes at 90oC and centrifugation (1 min, 10.000 g). 200 µl of the supernatant was used for DNA isolation according to Carter and Milton (1993)(27), with minor modifications (omission of the final acetone wash step). Terminal restriction fragment polymorphism (T-RFLP) analysis was essentially performed as described by Liu et al. (1997)(28). The 16S rRNA genes were amplified by PCR using fluorescently labeled primers 8f (6-FAM) and 926r (NED). The PCR reaction was performed in 15 µl containing

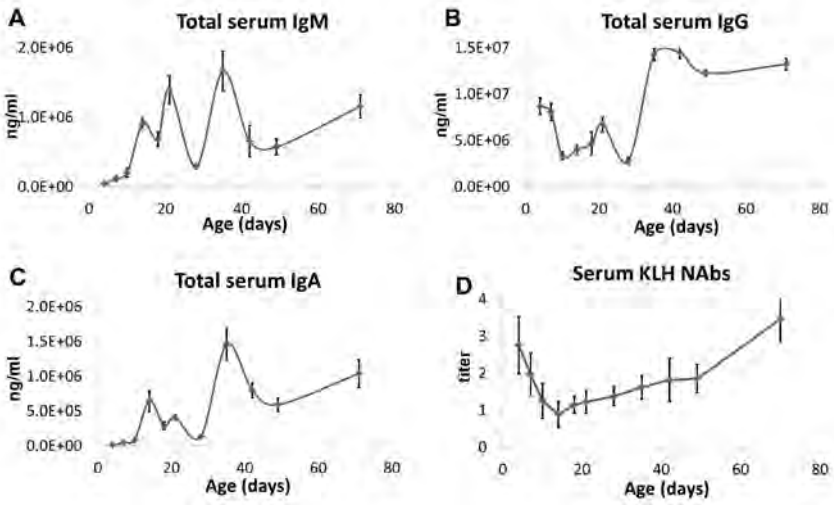

Figure 1. *Total and vaccine-specific serum levels of antibodies in chickens during the first 70 days following egg hatching. No autologous antibodies are produced as IgM (initially produced isotype) is absent the first days after hatch (A). IgG antibodies that are initially present are maternally derived (B). Also circulating IgA is self-produced. Natural antibodies (Nabs) against Keyhole limpet hemocyanin (KLH) (D) initially originate from the mother and are subsequently endogenously produced. Data is shown of ten animals per time point. Error bars show standard error.*

1x PCR buffer (Applied Biosystems), 62.5 µM, deoxynucleoside triphosphate (Applied Biosystems), 1.5 mM MgCl2, 0.5 µM of each primer, 0.5 U Taq DNA polymerase (Promega) and 1 µl of DNA sample. The PCR program consisted of 5 min at 94 °C, followed by 35 cycles (45s at 56°C), and a final extension at 72°C for 5 min. The PCR amplicons were digested using MspI and HinpII (New England Biolabs, Ipswich, MA, USA). The digested reaction products were size separated on 36 cm capillary electrophoresis POP4 gel matrix (ABI 3100, Applied Biosystems). The size standard marker MapMarker 30–1000, ROX labeled (Bioventures, Murfreesboro, TN, USA) was added to each sample for accurate sizing. Fragment length analysis was performed using GeneScan software (Applied Biosystems).

Flow cytometry of coated microbiota

0.5 g feces and 4.5 ml filtered PBS was homogenized and centrifuged for 2 min at 2000 g. The supernatant (1 ml) was collected, centrifuged for 10 min at 9000 g to generate the bacterial pellet. The pellet was washed twice in PBS, fixed in 4% para-formaldehyde and stored at 4°C overnight. The cells were recovered by centrifugation for 10 min at 9000 g. The pellet was washed twice with PBS, resuspended in 1 ml PBS-ethanol (1:1) and stored at -20°C until further use.

Bacteria were stained with monoclonal FITC conjugated mouse IgG1 anti chicken IgG (Southern Biotec), IgM or IgA. Monoclonal mouse IgG1 anti chicken MCAM was used as isotype control. Just before cytometric analysis (BD FACS Canto II) samples were loaded with 1 µl 1 mg/ml propidium iodide (Sigma) to identify the microbiota. Mother daughter gates were applied for forward plus sideward scatter, subsequently for PI staining (PerCP-Cy5.5 channel) and finally for Ig coating (FITC channel).

qRT-PCR

5 µl cDNA and forward and reverse primer (500 nm each) were added to 12.5 µl SYBR® Green PCR Master Mix (Applied BioSystems) and filled up with demineralized water to a final volume of 25 µl. The primer sequences used for qRT-PCR are listed in Table S3. All primer sets allowed DNA

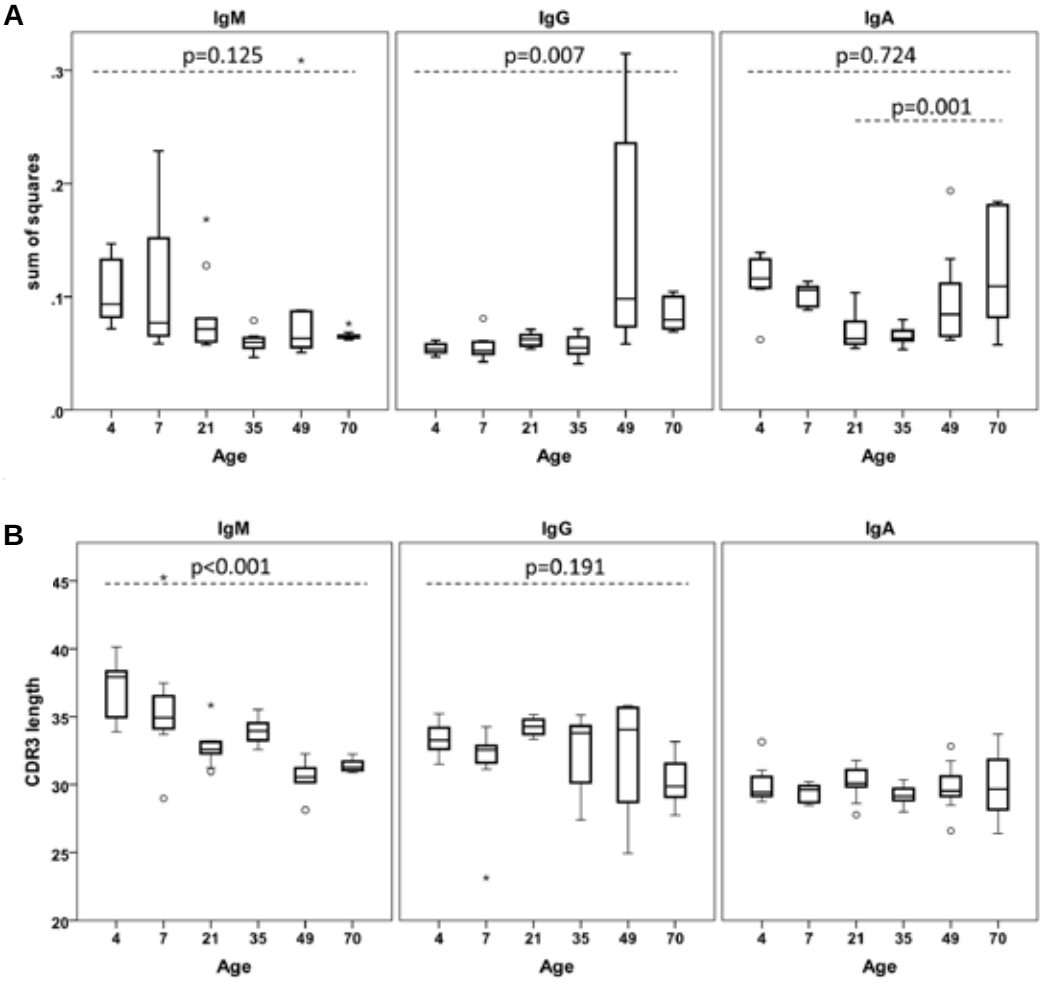

Figure 2. *Changes in spectratype-based deviation from the normal distribution (sum of squares) (A) and CDR3 length (B) with age, by isotype. A) Sum of squares of normalized spectratypes show changes of spectratype profiles upon antigenic stimulation. Box plots show median (horizontal bar), the interquartile range (box), 1st and 4th percentiles (line bars), and outliers (o) or extreme outliers (*). CDR3 length is indicated by the number of nucleotides. P-values result from running a generalized linear model; dashed lines indicate time points included in the statistical model.*

amplification efficiencies between 94% and 100%. RQ-PCR (10 min 95°C, 40 cycles of 15s 94°C, 30s 59°C and 36s 72°C, followed by 15s at 95°C, 1 min at 59°C) was carried out on an Applied BioSystems 7500 real-time PCR machine (Applied BioSystems, The Netherlands). After each run, melting curves were collected by detecting the fluorescence from 60 to 90 °C at 1 °C intervals. Relative expression was expressed as 2−ΔCt. Primers used are indicated in Table S1. Gene expression analysis was performed on ileum tissue samples and from the same animals as the microbiota profiling analysis.

Statistical analysis

Data were entered in PASW Statistics 19 (SPSS Inc., Chicago, USA). General Linear Model (multivariate) was used to test the CDR3 length and SS change over time. An independent samples double sided T-Test was used to test the overall CDR3 length differences between isotypes.

To study age-related changes in microbiota composition and to relate microbiota composition to mRNA expression levels of several immune related genes, principal component analysis (PCA) respectively redundancy analysis (RDA) (29) was performed using CANOCO® software (version 4.5, developed by Cajo J.F. ter Braak and Petr S Milauer) (30). Square root transformed MCPC data (Peak areas) were used as response variables. Log-transformed values of immune related gene expression data (previously published(31)) were used as environmental variables (29). Cage was used as a covariate in CANOCO. RDA was performed focusing on inter-sample correlation, and an unrestricted Monte Carlo Permutation Test (499 permutations) was applied to decide whether gene expression had a significant effect on the microbiota composition. Diagrams were plotted as biplots using CanoDraw for Windows.

RESULTS

The CDR3 length distribution changes during ontogeny

During different stage in ontogeny maternal-derived and endogenously produced antibodies can be detected in serum, reflecting humoral immunity of the neonate. Analysis of antibody levels in serum showed that maternally derived antibodies mainly consists of IgG. Titers of natural antibodies against keyhole limpet hemocyanin (KLH) disappeared within three weeks post hatch and with age gradually increased again. Strikingly, kinetics of autologous antibody serum levels are similar to intestinal expression profiles of immunoglobulin isotypes (Figures 1 and S1).

CDR3 spectratype profiles can be approximated by a normal distribution, which can be deduced from the normalized length distribution shown in Figures S1 and S2. Polyclonal expansion of specific B cells results in deviations from the normal distribution, whilst at a polyclonal background. The deviation of the spectratype profile from the normal distribution (sum of squares, SS) of the different isotypes tested was calculated using normalized spectratype data (Figures S1 and S2). The SS for IgM spectratypes was highest at days 4 and 7 after hatch, reached a nadir at day 35 (Figure 2A). The SS for IgG was low until day 35 but increased after day 49. The SS for IgA was average at days 4 and 7, low at days 21 and 35, increased at day 49 and was maximal at day 70. With aging, the SS numerically decreased for IgM, but changes were not significantly different ($p=0.125$). SS increased for IgG ($p=0.007$) and IgA (not significant for all ages, days 21 till 70, $p=0.001$). Age dependent changes in spectratype profiles are also shown in a principal component analysis (PCA) (Figure S3), which indicates deviating CDR3 profiles of individual chickens by placing the individual animal away from the center of the plot. Spectratyping IgA CDR3 sequences for animals aged 4 and 7 days succeeded, but originates from relatively low numbers of plasma cells and hence copies of Ig genes.

As CDR3 length could affect the shape of the antigen recognition region and the binding specificity, this length was calculated from our spectratype data. The data shown in Figure 2B reflects the average isotype-specific CDR3 length produced in situ. The average CDR3 length of IgM decreased by 6 nucleotides during the experimental period from approximately 37 to 31 nucleotides ($p<0.001$). The CDR3 length of IgG did not change with age (decrease by 33 to 30 nucleotides). IgA CDR3 length was approximately 30 nucleotides, which did not change with age.

Regardless of age, IgM and IgG had a significantly longer mean CDR3 region compared to IgA ($p<0.001$ respectively $p<0.001$). IgM CDR3 was longer than IgG CDR3 length in the period from day 4 until day 21 ($p=0.009$).

IgA CDR3 sequences do not reveal major changes during ontogeny

Since age-related differences in peak distribution of IgA spectratypes (Figures 2A and S3) were found, we studied whether IgA CDR3 regions were different at the sequence level between immunological immature (7 days of age) and mature chickens (70 days of age).

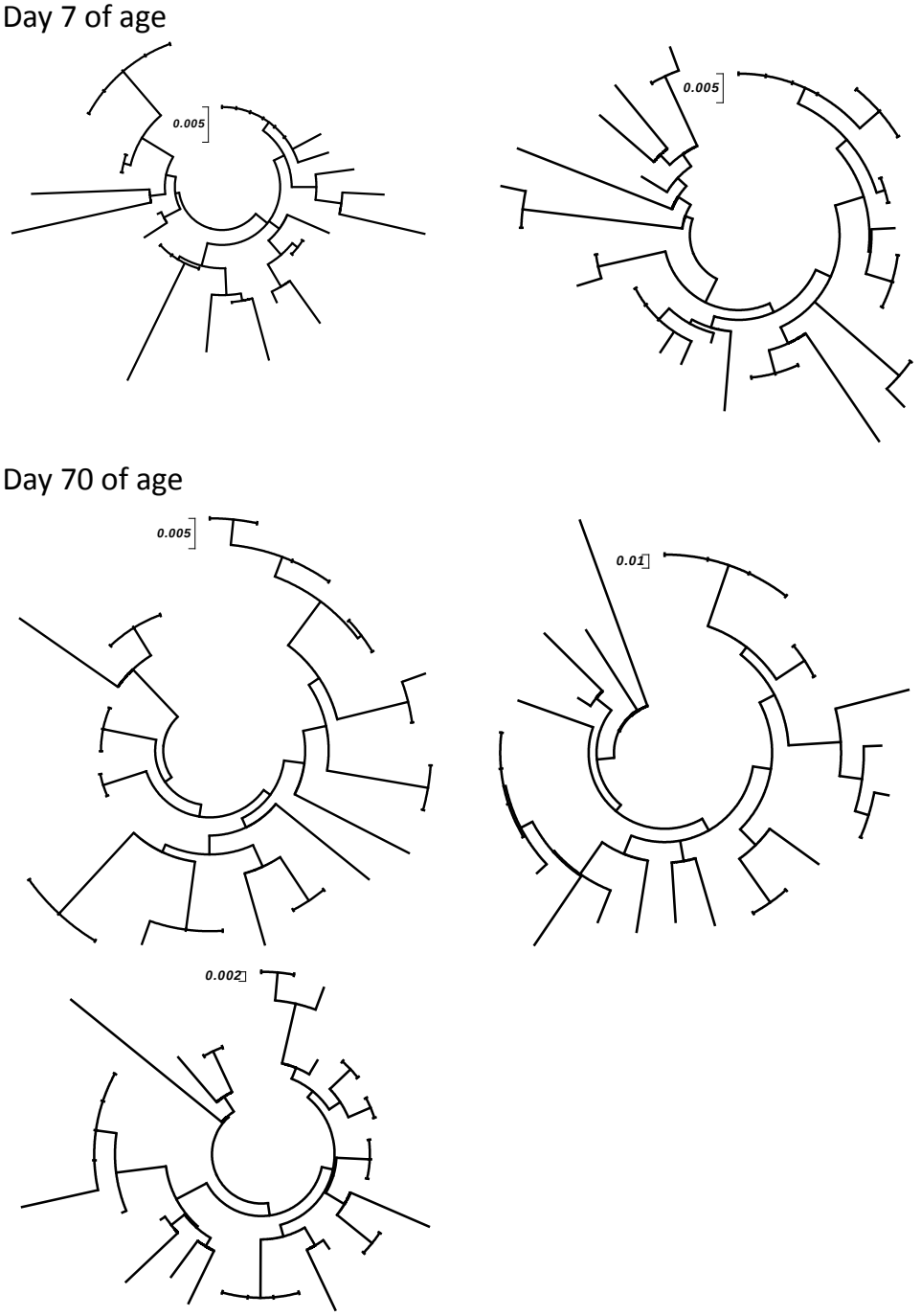

Figure 3. *Dendrograms of IgA CDR3 sequences. The NJ joining p-distance model (consensus 500 bootstraps) was used. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates have been collapsed. Animals aged 7 days have numbers Dendrograms of IgA CDR3 sequences are shown of two animals aged 7 days (top) and three animals aged 70 days (bottom). All dendrograms show more divergent CDR3 sequences and identical CDR3 sequences, no apparent age differences were observed.*

164 IgA-CDR3 sequences were obtained, which were variable in size and nucleotide composition. Sequences with the same size also differed in their nucleotide composition. Of each chicken, 60% to 89% of the sequences obtained were unique whilst the sequences remaining were identical to any of the other sequences. In the dendrograms (Figure 3), several clades containing highly similar IgA CDR3 sequences are visible in the dendrogram. CDR3 sequences of the same size ended up in both the same as well as in different clades of the dendrogram, indicating that both similar as well as divergent CDR3 sequences of the same size were obtained.

Although the improved draft genome sequence is available (build 8), the complete annotation of the VH pseudogene sequences of the Ig locus appeared not to be present. Only eighteen out of the estimated 70-90 pseudogenes have been identified and are available (26). To compare pseudogene sequence composition, a dendrogram was constructed also including the VH1 gene (Figure 4A). The VH1 gene is clearly distinct from the pseudogenes. Pseudogenes VH15-9, VH57- 5, VH57-12 and VH57-15 did not group with other pseudogenes. Pseudogenes VH15-5, VH57-3, VH57-10 and VH57-16; VH57-4 and VH57-11; VH57-1 and VH57-13 clustered closely together.

To assess if pseudogene usage was selective, CDR3 sequences obtained were blasted against germline nucleotide sequences to identify which pseudogene could have been used. The pseudogene with the highest coverage and sequence similarity was selected, identifying which of the currently known

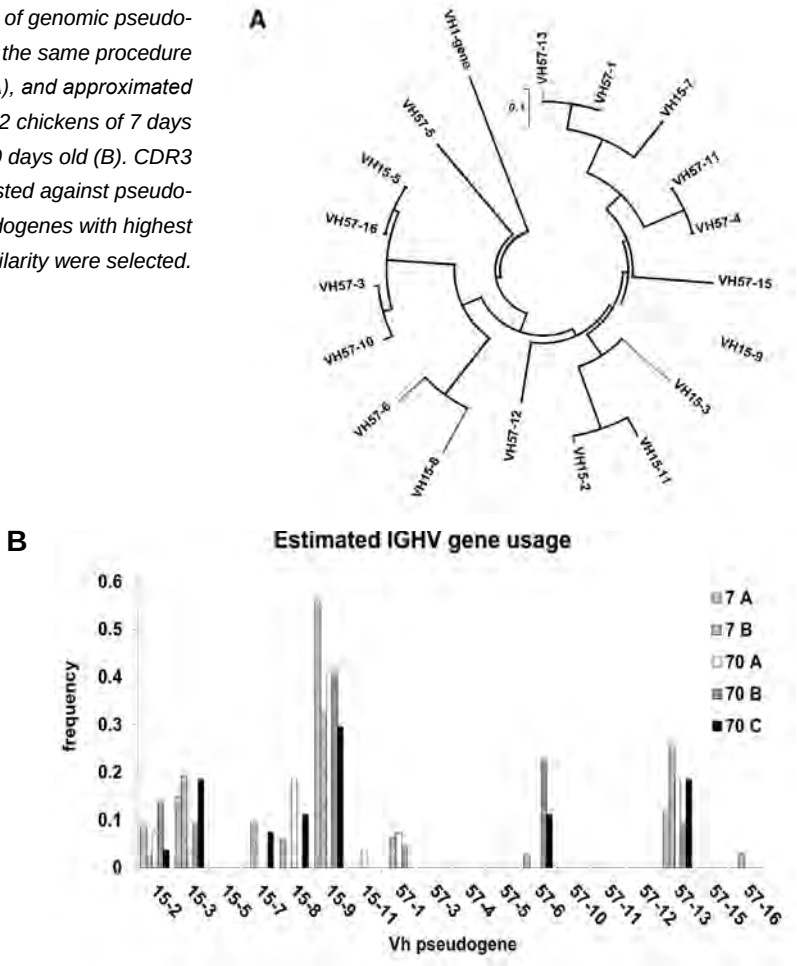

Figure 4. *Dendrogram of genomic pseudogene sequences using the same procedure used for Figure 3 (A), and approximated pseudogene usage by 2 chickens of 7 days and three chickens of 70 days old (B). CDR3 sequences were blasted against pseudogenes published. Pseudogenes with highest coverage and similarity were selected.*

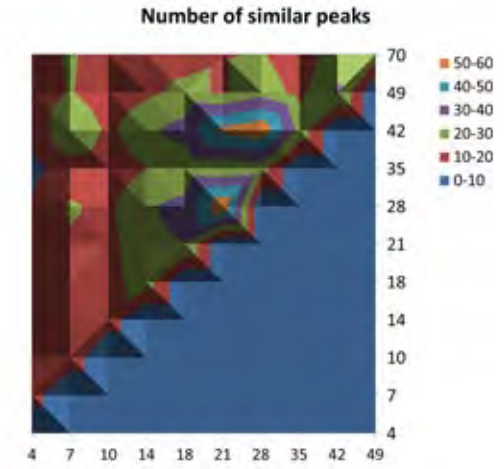

Figure 5. *Number of similar 16S peaks between age of at least 7 chickens per age group. Higher numbers as indicated by color in the legend indicate more similarity between profiles characterizing microbiota composition. Data should be read where ages intersect. Maximum 60 out of 580 ribotypes were present in 7 animals or more between different age groups, showing that microbiota composition is highly variable between animals and time points.*

pseudogenes most closely resembled the obtained cDNA CDR3 sequences (Figure 4B). Pseudogene VH15-9 occurred most frequently (approximately 40% of the cases), followed by VH57-13, VH15-3, VH57-6, VH15-8, VH15-2, VH15-7 and VH57-1. Pseudogenes VH15-5, VH57-3 – 5, VH57-10 – 12 and VH57-15 did not appear in our results. This indicates restricted use of pseudogenes. No differences were found between animals at the age 7 or 70 days. As not all pseudogenes have been identified, no accurate counts of point mutations could be obtained.

Microbial composition is temporarily changed

To study the interplay between the gut microbiota and expression of immune related genes, bacterial 16S rRNA genes were analyzed by PCR-amplification and subsequent enzymatic digestion (T-RFLP) resulting in ribotypes identifying microbial composition differences between animals. Because chickens had to be sacrificed to perform this analysis at each time point ten different chickens were analyzed.

The ileal microbiota composition was highly vari-

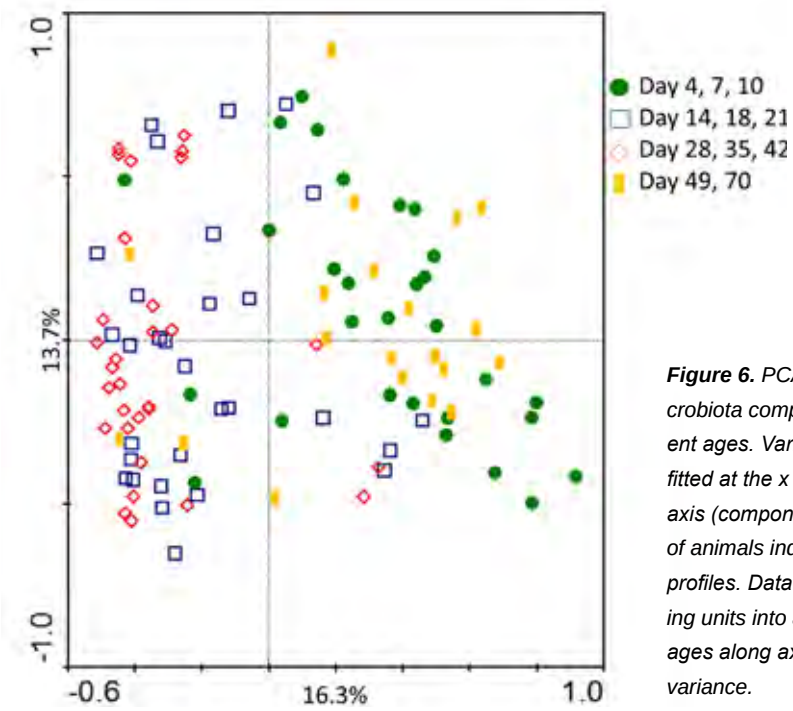

Figure 6. *PCA analysis of intestinal microbiota composition in ileum at different ages. Variance explained by PCA is fitted at the x axis (component 1) and y axis (component 2). Separate clustering of animals indicate different microbiota profiles. Data were derived taking housing units into account (cage). Percentages along axes indicate explained variance.*

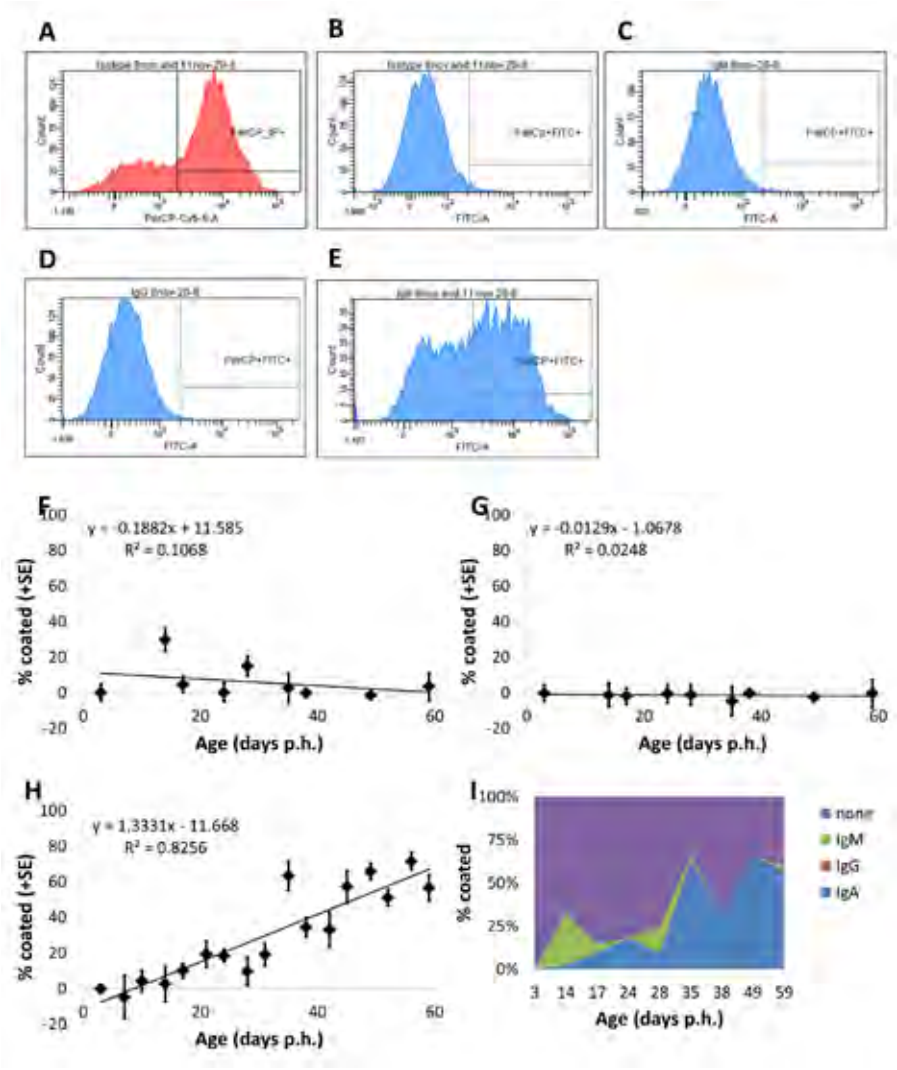

Figure 7. *Antibody coating of fecal microbiota. Example of FACS staining showing staining for PI (A) and daughter gatings for isotype control (B), IgM (C), IgG (D) and IgA (E). Panels F-H show the percentage of microbiota coated by IgM (F), IgG (G) and IgA (H); line bars indicate standard errors derived from data from 9 chickens (average). Panel I shows cumulative percentage of coated bacteria.*

able over time. Presence or absence of products of a specific size was used to directly compare similarity between age groups (Figure 5). T-RFLP of chickens at day 4 had very low numbers of similar peaks (10-20 similar for 7 or more chickens per age group, out of a total of 580 ribotypes) compared to other ages. Chickens at the age of 7 days showed relatively high similarity with chickens of the ages 28, 42, 49 and 70 days. Chickens aged 10 and 35 days had low similarity with chickens at other ages, suggesting shifts in microbial composition. Chickens of day 21, 28 and 42, and chickens of day 42 and 49 had more similar T-RFLP profiles.

Next to these differences, the microbial compositions transitionally changed during the period from 14 until 42 days of age as analyzed by principal component analysis (PCA, Figure 6). PCA analysis allows evaluation of complex data

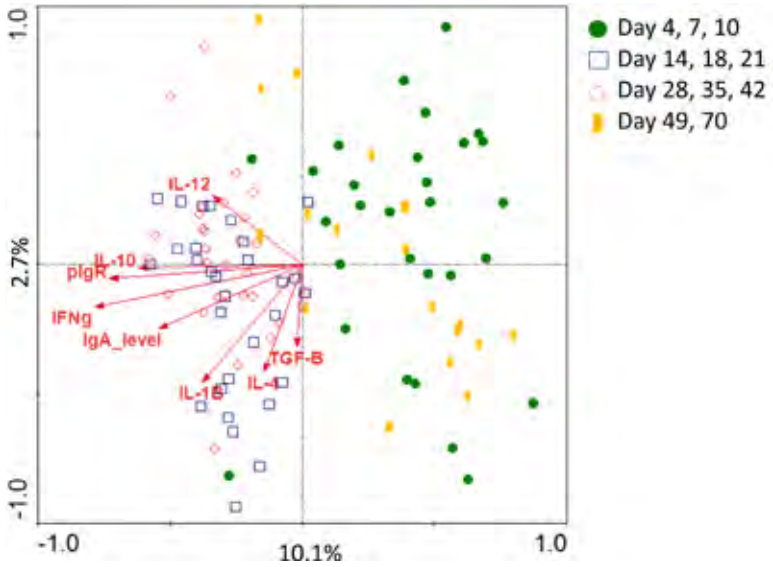

Figure 8. *RDA analysis of microbial composition and immune gene expression, taking housing units into account. Gene expression depicted are significantly ($p<0.05$) related to microbiota composition, except for IL-4 and IL-12 ($p=0.06$). Expression of IgM, IgG, IL-2 and iNOS is not shown ($p>0.10$). Percentages along axes indicate variance in microbiota composition explained by immune gene expression. Direction of arrow indicate correlation in animals having a specific microbiota profile. Arrows opposite to each other indicate negative correlation, perpendicular arrows indicate that variables are not correlated.*

sets by statistically explaining as much variance in the data set as possible in the first and subsequent components (displayed on x and y axes respectively, Figure 6). Data of individual animals is plotted in relation to those components. Most variance in microbiota composition is explained along the x axis and second most on the y-axis. As microbiota composition can be affected by housing conditions, analysis accounted for cage. Animals aged 4 to 10 days cluster together with animals at the age of 49 and 70 days at the right part of the graph, whereas animals of intermediate age (14 till 42 days) mainly cluster at the left part. This indicates a temporarily distinct composition of the ileum microbiota in animals aged 14 to 42 days.

Microbial coating with host Ig shows maturation of intestinal humoral immunity

The IgA immune response can be considered to be mature when it is able to exert its function by for instance binding to the intestinal microbiota, which depends on IgA production levels and antigen-repertoire. The fecal microbiota was stained with either FITC conjugated anti-chicken IgM, IgG, IgA or isotype control and analyzed by flow cytometry (Figure 7). No IgG was detected on fecal microbiota. Percentage of IgM stained bacteria decreased slightly with age (not significant). Bacterial coating by IgM was increased at day 14 compared to day 3 ($p=0.052$) and 17 ($p=0.012$). The percentage of IgA stained microbiota increased with age, up to 55-70 percent on average ($p<0.001$). Bacteria at days 3 and 7 were only coated with IgM and from day 35 onwards bacteria were mainly coated with IgA. FACS data from days 17 till 28 showed fluctuations of IgM and IgA coating.

Cytokine mRNA expression levels correlate with ileum microbiota

As the microbial composition changed during the experimental period and expression levels of immune related genes were temporarily elevated from 10-42 days compared to the days before and after this period (previously published (31)), the ques-

tion arose whether the variability in the composition of the ileum microbiota could be explained by the expression levels of these immune-related genes (Figure 8). Eighteen percent of the total variability in the microbial profile can be related to the immune gene expression pattern as determined by the Monte Carlo permutation test. Only the immune genes that significantly explained part of the variability in the 16S rRNA gene RFLP profile are shown in the plot. The directions of arrows indicate to what microbial communities gene expression levels are associated and the length of the arrow the amount of association. Positive relations were found between the intestinal microbial composition and expression levels of IL-10 ($p=0.004$), IFN-γ ($p=0.002$), IL-1ß ($p=0.03$), TGF-ß ($p=0.002$), pIgR ($p=0.02$) and IgA ($p=0.002$) and a tendency with IL-4 ($p=0.06$) and IL-12 ($p=0.06$). No relation was found between expression levels of IgM, IgG, IL-2 or iNOS ($p>0.10$) and the ileum microbiota composition. In general, immune genes ordinated to the left part of the diagram together with microbiota composition of animals aged 14 until 42 days (Figure 8). Immune gene expression did not associate positively with microbiota composition of animals early or later in our experiment, but only with the transiently changed microbiota composition. Composition of the microbial community did not correlate with the degree of deviation from the normal distribution of CDR3 repertoire profiles.

DISCUSSION

Our data show that 5-7 weeks are required for intestinal humoral immunity in chicken to mature into a functional microbiota-binding IgA response. The observation that coating of bacteria with IgA was observed at earlier ages than increased SS of IgA CDR3 spectratypes and that no profound changes in CDR3 sequences were observed, indicate that IgA production levels but not changes in CDR3 repertoires are required to coat the intestinal microbiota.

In mucosal IgA production, we can distinguish between antibody production itself and the variation of the IgA antigen recognition repertoire. In the early period (day 4 and 7), only IgM spectratypes showed clonally expanded CDR3 regions, while the spectratypes for IgG (day 49) and IgA (day 49 and 70) had clonally expanded CDR3 regions later in ontogeny. Clonally expanded CDR3 regions could be caused by a combination of proliferation and activation of existing B cells and differentiation or influx of B cell subsets. However, changes in CDR3 spectratype profiles reflect the CDR3 length distribution of all B cells in the tissue sample analyzed, and do not reveal which B cell subset is a major contributor to the CDR3 repertoire. Because of their approximately 1000-fold higher Ig-production rate compared to naïve B cells, plasma cells are the main contributors to spectratype profiles (32). The CDR3 length of IgM decreased with increasing age and the IgA and IgG CDR3 length did not change with age. At day 70 the three isotypes had similar CDR3 lengths. The CDR3 length affects the three dimensional shape of the antigen binding sites (33). Longer CDR3 domains may result in poly-specific antibodies, and recently it was published that pneumococcal vaccination resulted in decreased CDR3 lengths (23, 34). Different pseudogenes were used to generate the IgA CDR3 repertoire, with a preference for pseudogene VH15-9. Interestingly, there was no evidence for differential pseudogene usage between individual chickens or between chickens of different age (7 or 70 weeks post hatch). The unchanged pseudogene usage suggests that age of the animals and intestinal microbioata composition, and probably environmental factors in general, may have limited effect on pseudogene usage. These results should be confirmed when more pseudogenes have been identified and could be combined with analysis of mutations in the CDR3 region. Restricted variability in gene usage in intestinal CDR3 repertoires has also been published for rodents (35). VH-pseudogenes used by intestinal B cells may be selected for recognition of microbial surface molecules. However, the IgA CDR3 repertoire does not only depend on pseudogene usage, but also on somatic mutations.

Though not observed from our spectratype data and pseudogene usage, the CDR3 repertoire might be slightly different between animals aged 7 or 70 days. We conclude that the IgA CDR3 repertoire modification does not seem to depend on differential pseudogene usage at different ages.

Intestinal IgA regulates the composition of the intestinal microbiota. Therefore we hypothesized that regulation of the microbiota by IgA may depend on the antigen specificity of IgA and therefore maybe related to the IgA CDR3 repertoire. However, we found no relation between the CDR3 repertoire and the microbial composition, whereas the IgA expression level was related to the microbial composition. Functional coating of fecal microbiota with IgA was significant from five weeks post hatch and later. Ileum IgA expression level data correlated with pIgR expression data and were consistent with functional IgA coating of fecal microbiota data. We found that functional coating of microbiota with IgA, preceded changes in CDR3 repertoire as determined by spectratyping. It should be noted, however, that the CDR3 repertoire analysis was performed on mRNA from ileum, while bacterial coating was analyzed by flow cytometry on stool samples. In addition, bacteria binding the J-chain could contribute to numbers of bacteria found to be positive for secretory Ig. That bacterial coating preceded CDR3 repertoire changes suggests that coating of intestinal microbiota is independent of functional maturation of the ileal IgA CDR3 repertoire and that the initial IgM and IgA repertoire is already able to recognize large numbers of intestinal bacteria. This hypothesis corroborates the observation by Stoel et.al and Harris et.al. (35, 36), that the majority of IgA controlling the commensal microbiota in the intestines undergoes limited changes in the CDR3 region. We did not study if IgA binds to specific species or groups of bacteria. It is still possible that, dependent on the CDR3 repertoire, different species at different time points were coated.

Because functional IgM levels are relatively low and maternal-derived IgA in the gut is limited just after hatch, more adherence of bacteria to the epithelium and translocation of bacteria may occur in avians where to our current understanding less maternal IgA or IgM is available compared to mammals. Lack of IgA results in increased translocation of bacteria, which coincides with increased expression of IgG (11, 12, 37). During this period, de microbiota composition is temporally changed and therefore IgG specificities may be biased towards transiently dominant and translocating bacteria. Increased IgA levels later in life is believed to prevent translocation of bacteria, and IgA present in sufficient amounts inhibits IgM mediated classical complement activation and dose-dependently inhibits IgG mediated complement activation(38, 39). Though maternally derived antibodies can be readily detected systemically, no IgG coating of fecal microbiota was observed. This implies a limited role for maternal IgG in recognizing intestinal microbiota and that the role of IgG might be more restricted to protection against translocated bacteria. It would be interesting to identify to what extent IgG specific immunity is initiated to translocating bacteria in this transitional period.

The transiently increased expression of innate and T helper cell cytokines likely reflects this increased activation of the host's immune system. As in the same period the microbial community underwent temporally changes, cytokine expression data was hypothesized to relate to the composition of the intestinal microbiota. Indeed the analysis of the epithelia-adherent ileal microbiota composition was correlated with increased immune gene expression. Our data suggest that the period from 2-6 weeks post hatch with features of low inflammation (31) is the main immune-inducing window. Immune gene expression was decreased in chickens of 7 weeks and older (31). This reduction in immune activation may indicate that homeostasis was established between 7 to 10 weeks post hatch. It is interesting to note that especially at day 35 a change is observed in microbiota composition and IgA production. A role for IgA in immune homeostasis has been observed before (14). The present study, however, shows that initiation of IgA production during ontogeny significantly contributes

to microbiota composition and establishment of homeostasis.

We conclude that chickens have only minor levels of functional immunoglobulins of all three isotypes in the gut lumen in the first weeks post-hatch. From the second week post-hatch, the microbiota composition changed, which correlated with enhanced immune gene expression and subsequent return to a microbial community resembling the initial community. The changes and correlations observed between microbial community and host cytokine and IgA gene expression suggest a two-way interplay between host immune system and microbiome: initially microbiota initiate immune activation, while later in life IgA is binding large amounts of microbiota. IgA production seems to be initiated prior to maturation of the CDR3 repertoire and these initially formed IgA molecules are able to bind to microbes. Therefore, our data indicate that levels of IgA expression rather than IgA CDR3 repertoire are important in controlling the intestinal microbiota.

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footnotes

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Table S1. *Primers used for RT-qPCR*

Gene	Genbank no	Primer	Product size (bp)
28S	DQ018756	F: GGCGAAGCCAGAGGAAACT	62
		R: GACGACCGATTTGCACGTC	
IL-1	AJ245728	F: CAGCAGCCTCAGCGAAGAG	86
		R: CTGTGGTGTGCTCAGAATCCA	
IL-2	AF033563	F: TTCAAAATATCGAAAAGAACCTCAAG	51
		R: CGGTGTGATTTAGACCCGTAAGAC	
IL-4	AJ621249	F: GTGCCCACGCTGTGCTTAC	82
		R: AGGAAACCTCTCCCTGGATGTC	
IL-10	AJ621614	F: CGCTGTCACCGCTTCTTCA	88
		R: TCCCGTTCTCATCCATCTTCTC	
IFN-γ	Y07922	F: GTGAAGAAGGTGAAAGATATCATGGA	71
		R: GCTTTGCGCTGGATTCTCA	
TGF-β4	M31160	F: ACCTCGACACCGACTACTGCTT	86
		R: ATCCTTGCGGAAGTCGATGT	
IgA	S40610	F: GTCACCGTCACCTGGACTACA	192
		R: ACCGATGGTCTCCTTCACATC	
IgG	X07174.1	F: ATCACGTCAAGGGATGCCCG	118
		R: ACCAGGCACCTCAGTTTGG	
IgM	X01613.1	F: GCATCAGCGTCACCGAAAGC	98
		R: TCCGCACTCCATCCTCTTGC	
PIgR	AY233381	F: GGATCCGACGTGCAGATCCAGCTCCTTCGT	247
		R: TCACCATCATCGACTTCCCAGAGCAGG	
INOS	U46504	F: TGGGTGGAAGCCGAAATA	241
		R: GTACCAGCCGTTGAAAGGAC	

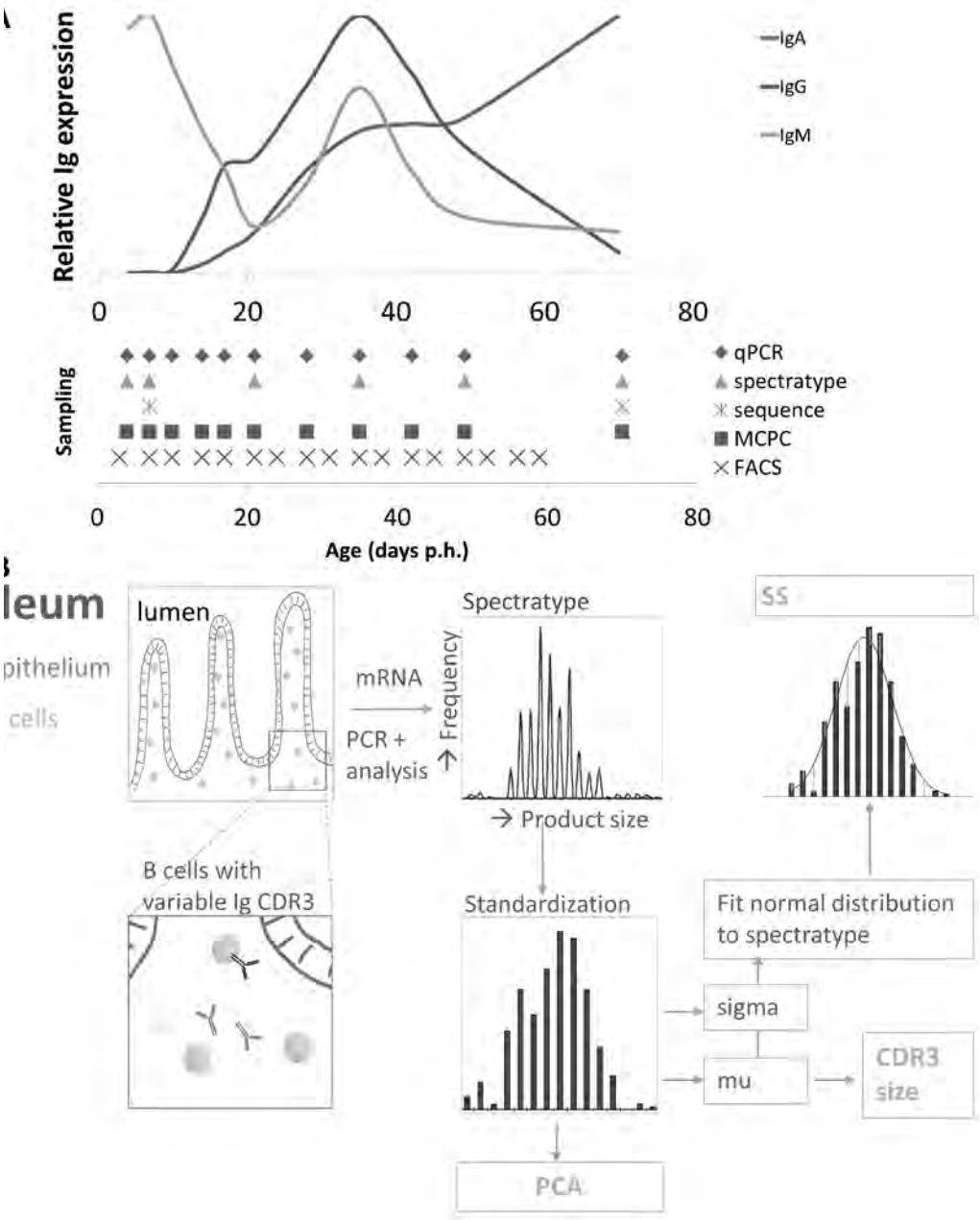


Figure S1. *Panel A shows a cartoon of Ig expression kinetics in ileum based on previous findings (Lammers 2010) and the samples points of Ig expression, spectratyping, sequencing (IgA), microbial community profiling (MCPC) and flow cytometric analysis of fecal bacteria (FACS). Panel B shows the experimental procedure of spectratyping and data analysis.*

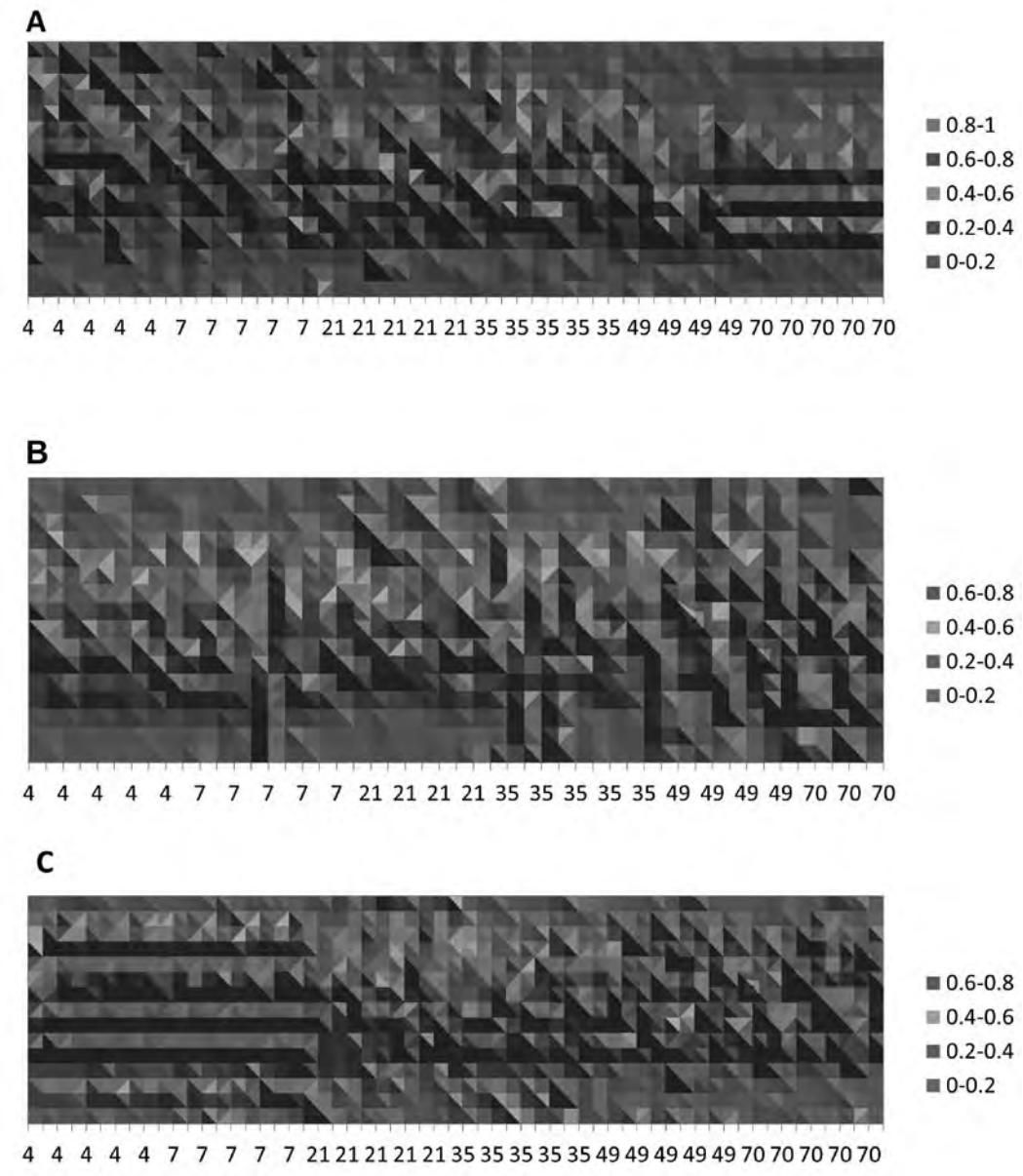


Figure S2. *Heat maps of standardized spectratype profiles of IgM (A), IgG (B) and IgA (C), of all chickens tested. X axes show age (days post hatch), y axes peak number (product size) and the color coding shows the relative amount a peak contributes to the total repertoire. Use of e.g. green or purple colors indicates clonal expansions.*

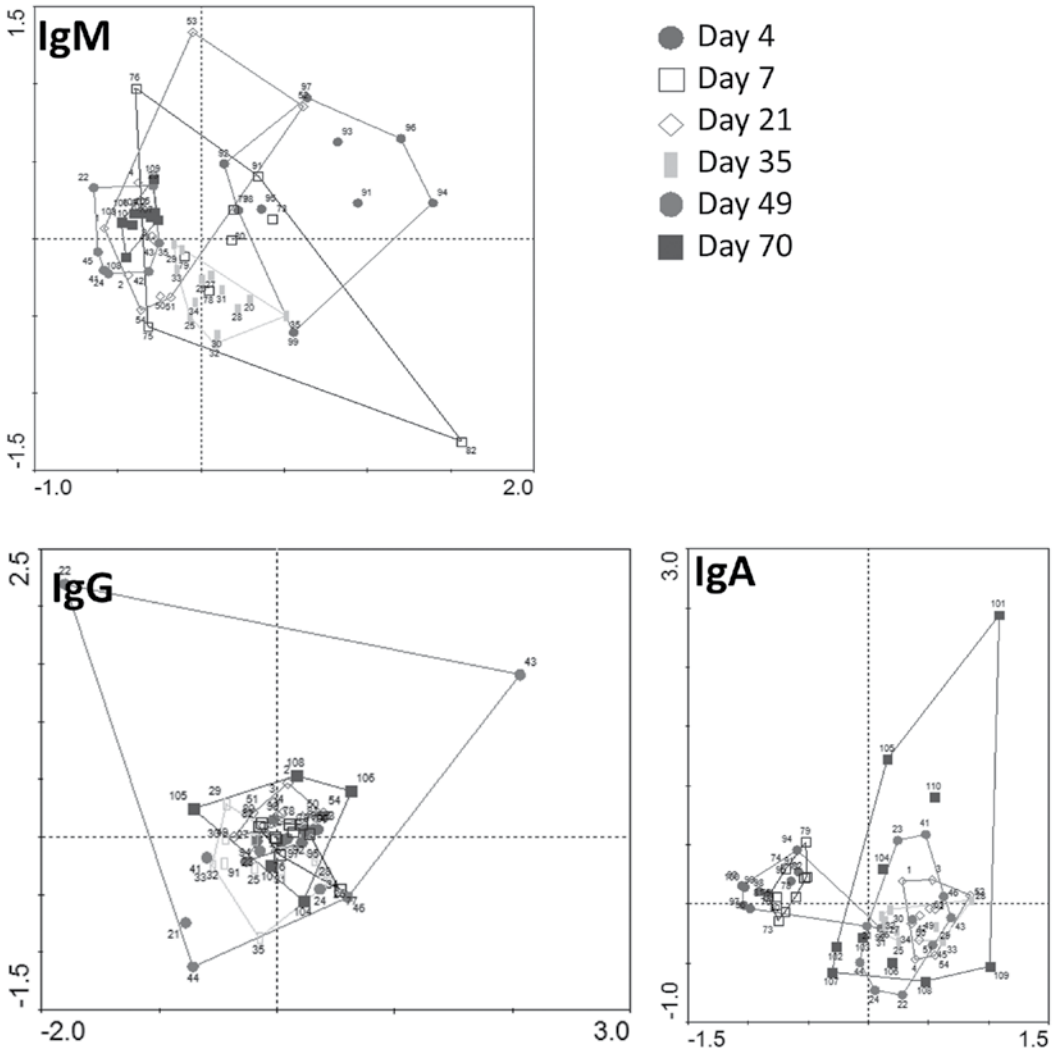
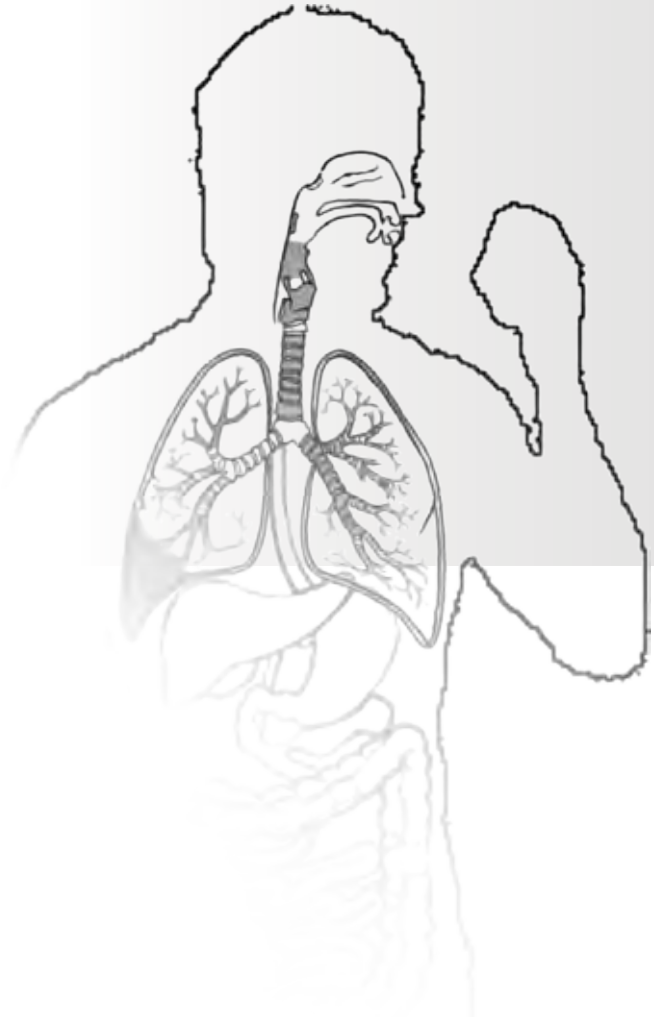


Figure S3. *IgM, IgG and IgA CDR3 repertories changed successively with age. PCA analysis of CDR3 repertoires was performed of chickens of different ages. Each individual animal is depicted and an envelope drawn through most extreme individuals. The more a spectratype deviates from normality the more the individual ends up away from the center of the graph, where the axes intersect.*



chapter 8

Modulation of human immune responses by bovine interleukin-10

Gerco den Hartog, Huub F. J. Savelkoul, Ruud Schoemaker,
Edwin Tijhaar, Adrie H. Westphal, Talitha de Ruiter,
Elise van de Weg-Schrijver, R. J. Joost van Neerven

ABSTRACT

Cytokines can be functionally active across species barriers. Bovine IL-10 has an amino acid sequence identity with human IL-10 of 76.8%. Therefore, the aim of this study was to evaluate whether bovine IL-10 has immunomodulatory activities on human monocytes and dendritic cells.
Peripheral blood monocytes were isolated from healthy donors, and used directly or allowed to differentiate to dendritic cells under the influence of IL-4 and GM-CSF. Recombinant bovine IL-10 inhibited TLR induced activation of monocytes, and dose-dependently inhibited LPS-induced activation of monocyte-derived DCs comparable to human IL-10. By using blocking antibodies to either bovine IL-10 or the human IL-10 receptor it was demonstrated that inhibition of monocyte activation by bovine IL-10 was dependent on binding of bovine IL-10 to the human IL-10R.
These data demonstrate that bovine IL-10 potently inhibits the activation of human myeloid cells in response to TLR activation. Bovine IL-10 present in dairy products may thus potentially contribute to the prevention of necrotizing enterocolitis and allergy, enhance mucosal tolerance induction and decrease intestinal inflammation and may therefore be applicable in infant foods and in immunomodulatory diets.

INTRODUCTION

Dietary components are capable of modulating intestinal immune responses [1,2]. Dairy products, including cow's milk, are widely consumed in Western societies and contain a wide range of immunoprotective factors such as immunoglobulins, lactoferrin, anti-microbial enzymes and cytokines. Bovine IL-10 was found to have an amino acid sequence identity of 76.8% with human IL-10, indicating that bovine IL-10 may exert functional effects on human immune cells [3,4,5,6]. Therefore, bovine IL-10 present in dairy and dairy related products could potentially have immunomodulatory activity in the human consumer. Functional cross species activity of cytokines has been reported for chicken IFN-γ and turkey IL-2 [5,6], and both porcine IL-2 and human IL-2 were reported to enhance proliferation of human, bovine, porcine and murine cells in vitro [3]. Also, human IL-10 is functionally active on a mouse mast cell line, but mouse IL-10 was not functionally active on a human B cell line [7]. Together these findings indicate that cytokines can be functionally active across species. The potential cross-species bioactivity of IL-10 depends mostly on the sequence identity of the IL-10 receptor (IL-10R) binding sites [4] and three dimensional structure of the proteins involved.

Biologically active IL-10 binds to the IL-10R, which is expressed on monocytes, macrophages, dendritic cells (DCs), NK cells, T cells and B cells. IL-10 is bound as a homodimer at two sites by both the IL-10R1 dimer and the IL-10R2 dimer, resulting in four IL-10/IL-10R interaction sites [8,9,10]. The IL-10R1 dimer binds the IL-10 molecule with high affinity; subsequently, this complex is recognized by the low affinity IL-10R2 dimer. IL-10 bound to IL-10R1 activates phosphorylation of Jak1 and Tyk2, which leads to Signal Transducer and Activator of Transcription 3 activation [11]. Signal Transducer and Activator of Transcription 3 translocates to the nucleus and activates Suppressor of Cytokine Signalling-3 [11,12], resulting in suppression of MyD88 - NFκB activated TLR-inducible cytokines like IL-1β, IL-6 and TNF-α [13,14,15,16,17]. These cytokines are selectively inhibited by IL-10 in a dose-dependent manner [18,19].

IL-10 is a potent cytokine and essential in controlling excessive immune responses to infections, thereby reducing immunopathology [20]. T cell dependent and T cell independent IgA class switching and production can be initiated by IL-

10 in secondary immune organs and in the lamina propria [21,22,23]. IL-10 is involved in tolerance induction and immune regulation in both the innate and adaptive immune system. IL-10 can also inhibit homing of DC's to the draining lymph node [24], and IL-10 treated DCs can induce tolerance [25]. The maturation and activation induced expression of CD40, CD80 and CD86 by macrophages and DCs can be inhibited by IL-10 [26,27], affecting the ability to stimulate T cells. Indeed, IL-10-exposed APCs fail to induce IFN-γ production by Th1 cells [28,29].

IL-10 can directly regulate T cell responses and has been shown to be related to successful allergen immunotherapy [30,31,32]. IL-10 excreted by transfected Lactococcus lactis in the lumen of the intestine of mice can induce IL-10 production by cells of the Peyers patch and prevent allergic sensitization to food [33]. Next to this, in a neonatal rat model, decreased necrotising enterocolitis (NEC) correlated with increased in situ IL-10 production [34]. These findings show the potential significance of the presence of IL-10 in the intestine.

In this report, we investigated whether bovine IL-10 could exert functional activity on human monocytes and dendritic cells. Bioactive bovine IL-10 could potentially be used for the prevention of inflammatory diseases as NEC and allergy in infant nutrion, or in immunomodulating diets for patients suffering from intestinal inflammatory disorders. We show that bovine IL-10 is recognized by the human IL-10 receptor and dose-dependently inhibits cytokine production and surface marker expression during LPS induced DC maturation.

MATERIALS & METHODS

IL-10 sequence analysis

IL-10 sequences were obtained from the online databases of NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/). Existing signal peptide data or signalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) were used to identify IL-10 signal peptides, which were removed before performing the sequence alignment. Sequence alignment was done in BioEdit (version 7.0.9.0) using ClustalW Multiple Alignment with default settings. Subsequently, the amino acid sequence identity was calculated using the sequence identity option in BioEdit. Accession numbers of the IL-10 sequences are: Human, UniProt, P22301; Bovine, UniProt, P43480, Epstein-Barr virus (EBV), UniProt, P03180; Rat, NCBI, EDM09836; Sheep, NCBI, CAA82546; Mouse, NCBI, AAI37845; Pig, NCBI, CAL29498 and Papiine herpesvirus 1 (PapHerp), NCBI, AAF23949.

Three-dimensional modeling of bovine IL-10

The dimeric structure of human IL-10 (PDB entry: 1j7v, resolution 2.9 Å) was used as a template to model the dimeric bovine IL-10 protein using the program MODELLER (version 9v8 [35,36]), which incorporates the CVFF force field [37]. Stereochemical quality of the homology models was assessed using the program PROCHECK [38]. Protein folding quality was verified using the program PROSAII [39], which independently evaluates the compatibility of each residue to its environment.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were diluted 1:1 in IMDM (Gibco-BRL, Paisley, Scotland) and isolated by gradient centrifugation on Ficoll-Plaque PLUS (Amersham Biosciences, Uppsala, Sweden) for 5 minutes at 200g and subsequently for 15 min. at 500g (without brake at 20 °C). The PBMCs were harvested from the Ficoll layer, gently resuspended in IMDM and washed two or three times in IMDM.

Monocyte isolation and stimulation

Monocytes from freshly isolated PBMCs were labeled with MicroBeads conjugated to mouse IgG2a monoclonal anti human CD14 antibodies (130-050-201, Myltenyi Biotec, Germany), and isolated using the quadroMACS (Myltenyi Biotec) according to the manufacturers descriptions. Briefly, cells were incubated with MicroBeads for 15 minutes at 4°C, washed with MACS buffer, centrifuged and

resuspended in MACS buffer. The MACS columns were placed in the quadroMACS and rinsed. Subsequently, the cell suspension was added, rinsed and the columns removed from the quadroMACS; labeled cells were collected in a new tube by rinsing with MACS buffer and the supplied plunger. Purity of the $CD14^+$ cell population was between 90 and 95%, as determined by flow cytometric analysis (FACS Canto II BD Biosciences, San Jose, CA, USA) by labeling the cells with mouse IgG2a anti human CD14 APC or an isotype control (clone M5E2, 555399 or 555576, BD Biosciences). Myltenyi Biotec indicated that clone M5E2 was not used on the microbeads used for isolation of the cells. After MACS sorting monocytes were resuspended in IMDM + 1% Yssels medium [40] + 1% penicillin and streptomycin (Gibco).

Recombinant bovine IL-10

The recombinant bovine IL-10 was produced in house. Therefore the bovine IL-10 cDNA sequence encoding the mature part of the protein was cloned in pET15bGW essentially as described previously for rhinoceros IFN-γ [41]. Vector pET15bGW encodes for an N-terminal tag containing 6 histidine residues (his6-tag) under control of a T7 promoter. This tag enables purification of recombinant protein by immobilized metal affinity chromatography (IMAC). Protein production, purification by IMAC endotoxin removal and refolding was essentially performed as described previously for carp CXCL8 chemokines [42]. The sequence of recombinant bovine IL-10 used in the cell culture experiments is identical to the bovine IL-10 sequence showed in Figure 1 (NCBI database), as was confirmed by sequencing the IL-10 coding insert of the vector (Baseclear, Leiden, The Netherlands).

IL-10R inhibition

Monocytes were stimulated with 1 µg/ml LPS (S. enterica, Sigma Aldrich, St Louis, USA), 2 µg/ml peptidoglycan (S. aureus, Fluka, Buchs Switzerland) and 2 µg/ml flagellin (S. typhimurium, InvivoGen, Toulouse, France). Human (200-10, Peprotech, London) or bovine IL-10 was used at a concentration of 10 ng/ml. To block the biological activity of recombinant bovine IL-10, 50 µg/ml total chicken IgY isolated from egg yolks of chicken immunized with recombinant bovine IL-10 was used. Antibodies isolated from egg yolks of non-immunized chicken (50 µg/ml) were used as a negative control. To block the human IL-10R, 5 µg/ml rat IgG2a anti human IL-10R (CDw210) (Clone 3F9, 556011, BD Biosciences) and an isotype control (554687, BD Biosciences) were used. Antibodies were added to the cells and incubated at 37°C and 5% CO2 for 30 minutes prior to addition of other stimuli. Monocytes were stimulated with different TLR ligands with or without bovine IL-10 for 24 hours at 37°C and 5% CO_2. LPS and a widely used dose [12,19] of 10 ng/ml human or bovine IL-10 were added and incubated for 24 hours at 37°C and 5% CO2. Cell culture supernatants were collected and stored at -80°C for later cytokine analyses.

DC generation and stimulation

Monocytes for DC generation were purified from freshly isolated PBMCs by density centrifugation using Percoll (GE Healthcarespec. grav. 1.130 ± 0.005 g/ml, <25 mOsm/kg H20). Standard isotone Percoll was prepared by adding 1 part PBS to 9 parts Percoll and used to make three layers of different densities of Percoll (2.5 ml 60%, 5.0 ml 45% and 2.5 ml 34.2% in HBSS in a 15 ml tube). Cells (in HBSS + 10% FBS) were gently added on top of the Percoll layers (max 40*106 cells) and centrifuged at 18-22°C for 45 minutes at 1750g without brake. Monocytes were collected and transferred to a new tube, centrifuged again (20 minutes at 1000g) and washed two times with 1% FBS in PBS (Gibco, 16000-044). Isolated monocytes were cultured in FBS free CellGroD medium (CellGenix, 2005) with pen/strep (Gibco, 15140) at a concentration of 1*106 cells/ml. 40 ng/ml recombinant human GM-CSF (Peprotech, 300-03) and 20 ng/ml recombinant human IL-4 (Peprotech, 200-04) were added and refreshed every second day. On day 6, expression of surface markers was analyzed using flow cytometry (FACS Calibur, BD Bio-

sciences).
On day 6, DCs were stimulated with 50 ng/ml (or 1000 ng/ml, data not shown) LPS (S. enterica, Sigma Aldrich, St Louis, USA) for 48 hours in the absence or presence of IL-10. Cell free supernatants were collected and stored at -80ºC until analysis. DCs were collected and cell surface marker expression was analyzed.

Cell Surface marker analysis
DCs were stained with FITC or PE labeled mouse IgG1 antibodies anti human CD40 (FITC), CD80 (PE), CD83 (FITC,), CD86 (FITC), CD1a (PE), MR (CD206, PE) or HLA-DR (PE) (555588, 557117, 556910, 555657, 555807, 555954, 555812, BD Biosciences) or isotype controls (PE 555749, FITC 555748, BD Biosciences), according to the manufacturers descriptions. Cells were incubated at room temperature for 30 minutes in the dark. Subsequently, cells were centrifuged, supernatant discarded, 200 µl FACS Buffer (BD Biosciences) added, and analyzed for surface marker expression by flow cytometric analysis.

Human cytokine measurements
Human cytokine concentrations in cell culture supernatants were determined using the cytometric bead array flex sets (BD Biosciences) and incubations were performed in the recommended Protein Master Buffer Kit (BD Biosciences). Briefly, 50 µl supernatant was incubated with capture beads in 50 µl Capture Bead Diluent for one hour at room temperature. Next, PE Detection Reagent in 50 µl Detection Reagent Diluent was added and incubated for two hours at room temperature in the dark. For the standard curve, the provided lyophilized recombinant cytokines were used and assayed together with the samples. After incubation the samples were centrifuged, supernatant discarded and 200 µl Wash Buffer was added. The samples were analyzed by flow cytometric analysis (FACS Canto II, BD Biosciences).

Bovine IL-10 ELISA
A bovine IL-10 specific capture ELISA was developed in house using an anti-bovine IL-10 specific monoclonal (MCA2110, AbD Serotec, Oxford, UK) as a capture antibody and biotinylated anti-bovine IL-10 IgY as a detecting antibody. The anti-IL-10 IgY had been affinity purified against in house produced recombinant bovine IL-10 coupled to CNBr-activated Sepharose 4B from eggs of chickens that had been immunized repeatedly with recombinant bovine IL-10.

Statistical analysis
Statistical analysis was performed with PASW Statistics SPSS version 17.0.3. Cytokine production levels upon stimulation with bacterial ligands vary greatly between donors. Therefore we tested the inhibitory capacity within the donor. We performed a generalized linear model after logarithmic transformation of the cytokine data: cytokine production level = donor (stimulus * inhibitor). Stimulus (LPS, PGN or Flagellin) and inhibitor (whether IL-10 was added or not) were included as fixed factors (this will compare groups) and the interaction term (*) tests the effect of the inhibitor in relation to the stimulus used.
Statistical analysis of the dose-dependent modulation of DC surface marker expression levels by bovine IL-10 was performed using a one by one correlation model: surface marker expression level or cytokine concentration = bovine IL-10 concentration.
The bioactivity between bovine and human IL-10 was compared using a univariate general linear model: cytokine production = IL-10 source (bovine or human) * IL-10 concentration. Low p-values would indicate a difference between human and bovine IL-10.

RESULTS

Identification of human IL-10R binding sites in bovine IL-10
In order to make a more detailed comparison possible of potential interactions between bovine IL-10 and human IL-10R, IL-10 sequences of different species were aligned and the IL-10R binding sites

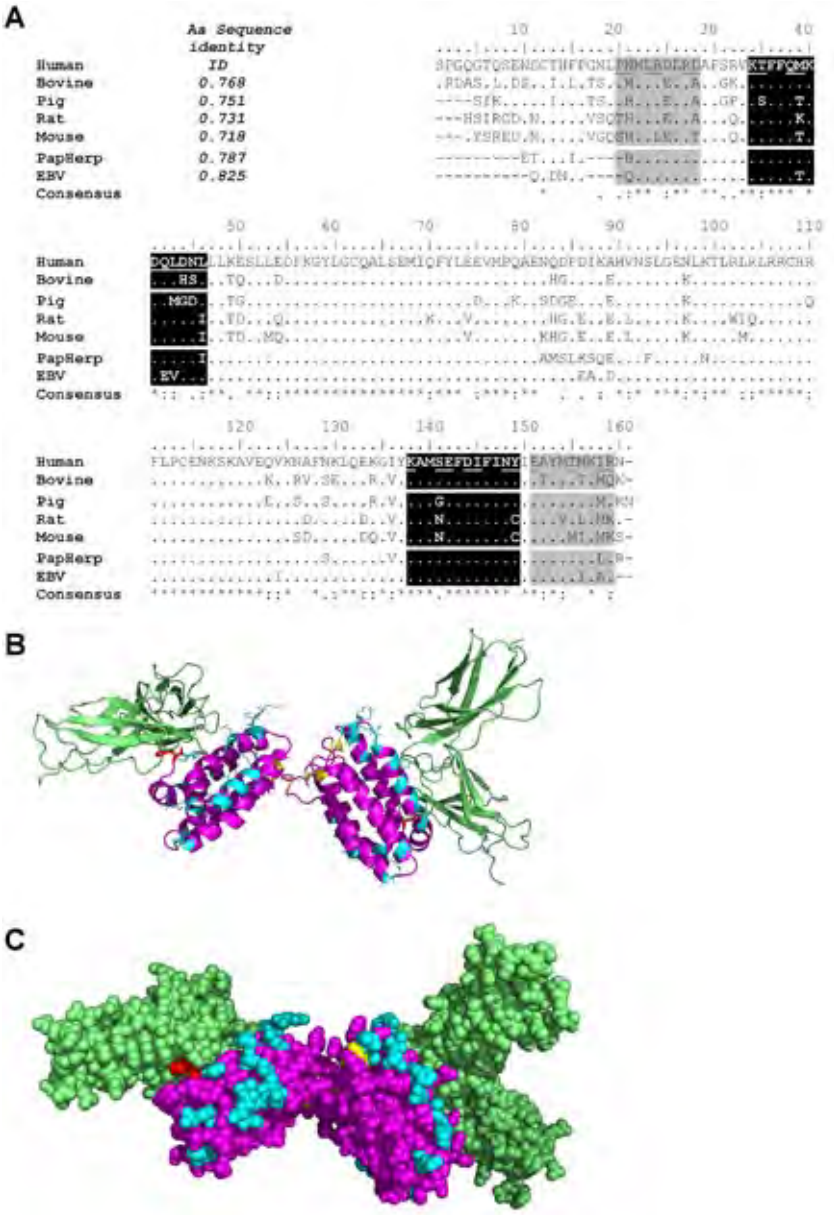


Figure 1. *Comparison of human and bovine IL-10 in the human IL-10R. A: ClustalW sequence alignment of IL-10 from different species. IL-10 sequences were retrieved from the NCBI and Uniprot databases and analyzed for signal peptides (SignalP 3.0) which were removed from the sequences before performing the alignment (18 amino acids for human and bovine IL-10). At the top left the overall sequence identity is shown. In grey (Ib) and black (Ia) background shading the IL-10 receptor 1 binding sites are indicated as published by Josephson [8]. The underlined residues indicate > 5 Å² surface area in IL-10RA site I. At the bottom of each row the consensus (*) sequence is shown; "." and ":" indicated homologous amino acids. B: Bovine IL-10 was modeled using human IL-10 in the human IL-10/IL-10R complex as template. The human IL-10R is shown in green and amino acid substitution between human and bovine IL-10 are depicted in cyan. Cysteine residues are colored yellow, the amino acid colored red (indicated with an arrow for one of the two IL-10 chains) is His 44, which is and amino acid substitution in close contact with the human Il-10R. C: The same model as in B, but displayed using spheres.*

Table 1. *Summary of bovine IL-10 amino acid substitutions in human IL-10R binding sites.*

Binding site (residues)	**Ib** (20-28)	**Ia** (34-46)	**Ia** (138-149)	**Ib** (151-159)	**Total**
No. residues	9	13	12	9	43
Substitutions	3	2	0	4	9 (21 %)
- *Homologous*	2	2	-	3	7 (78 %)
Substitutions >5 Å^2	2	2	-	3	7
- *Homologous*	1	2	-	2	5 (71 %)

Data are obtained from a ClustalW Multiple alignment as shown in Figure 1A. Of the amino acid substitutions and the substitution burying >5 Å2 in the human IL-10R [9] the number of homologous amino acid substitutions is indicated.

indicated (Figure 1). IL-10 sequences were obtained from the NCBI and Uniprot online databases, and SignalP 3.0 was used to identify signal peptides. Next, signal peptides were removed from the sequences before performing the ClustalW Multiple Alignment. Data on the IL-10R binding sites (obtained from Josephson [8]), were in line with the receptor binding sites reported by Reineke [9]. The IL-10R binding sites in IL-10, as published by Josephson, are indicated in Figure 1A, with black (Ia) and grey (Ib) background colors. The interactions between IL-10 and the IL-10R are largely determined by residues of IL-10 in close contact with the receptor. Therefore, residues burying a surface area of more than 5 Å^2 into human IL-10R1 [8] are underlined in Figure 1A, allowing evaluation of potential consequences of sequence differences between human and bovine IL-10 in the human IL-10R binding sites.

Human and bovine IL-10 were found to have an amino acid sequence identity of 76.8% (Figure 1A). Twenty-nine percent (11 out of 38) of the amino acid substitutions are present in the first 20 amino acids of the IL-10 sequence. The IL-10R binding site Ia (amino acid residues 138-149) is identical for human and bovine IL-10. Divided over the three other IL-10R binding sites, bovine IL-10 contained nine amino acid substitutions compared to human IL-10 (summarized in Table 1). Seven of the nine different residues bury more than 5 Å^2 in the IL-10R binding site and therefore are expected to contribute to the binding of IL-10 to the IL-10R. Three amino acid substitutions had a different polarity, and different hydrophobicity indexes. These three non-homologous substitutions occurred in the Ib IL-10R binding sites and two of them buried more than 5 Å^2 in the IL-10R binding site. Six of the nine amino acid substitutions had similar polarity and none had opposite (positive versus negative) polarity.

To support the linear peptide analysis, a 3D model of bovine IL-10 bound to the human IL-10R was built, based on the available crystal structure of the human IL-10/IL-10R complex (Figure 1B). No structural differences apart from some side chain orientations are observed between the model of bovine IL-10 and the crystal structure of human IL-10. The majority of amino acid substitutions between human and bovine IL-10 are located at the surface of the IL-10 molecule. The IL-10R binding site of IL-10 does not contain any amino acid substitutions, except for one histidine residue (His 44) substitution. Analysis of the 3D model showed that this histidine is located in a pocket of the receptor (Figure 1C) and is not likely to impair IL-10R binding.

Bovine IL-10 exerts functional effects on human monocytes through binding to the IL-10R

In order to investigate the bioactivity of bovine IL-10 on the human immune system, we tested the effect of bovine IL-10 on freshly isolated monocytes from three healthy donors. These freshly isolated monocytes were stimulated with different bacterial ligands: peptidoglycan (PGN), flagellin and lipopolysaccharide (LPS) in the presence or absence of bovine IL-10.

Monocyte cell cultures were 90-95% pure, as determined by flow cytometry for CD14 (data not shown). Addition of recombinant bovine IL-10

significantly inhibited PGN, flagellin and LPS induced IL-1β ($p<0.001$) and TNF-α ($p<0.001$) production by monocytes as tested on three different donors (Figure 2A). We suggest that bovine IL-10 potently inhibits TLR induced activation. The 5-fold inhibition of TNF-α and IL-1β production by monocytes was comparable between bovine and human IL-10 (Figure 2B and Table 2).

Additional experiments were performed to confirm that inhibition of TLR-induced cytokine production by monocytes was specifically caused by bovine IL-10. Therefore, IL-10 blocking antibodies or isotype control antibodies were pre-incubated with bovine IL-10 and LPS, and subsequently added to the monocytes. Addition of IL-10 blocking antibodies in combination with bovine IL-10 completely restored the IL-1β and TNF-α production by LPS stimulated monocytes; which was not observed when the isotype control was used (Figure 2C).

The comparison of the sequence of bovine IL-10 with the IL-10R binding sites on the sequence of human IL-10 (Figure 1), confirmed that bovine IL-10 could potentially bind to the human IL-10R. To investigate whether the functional effect of bovine IL-10 is indeed through the binding of bovine IL-

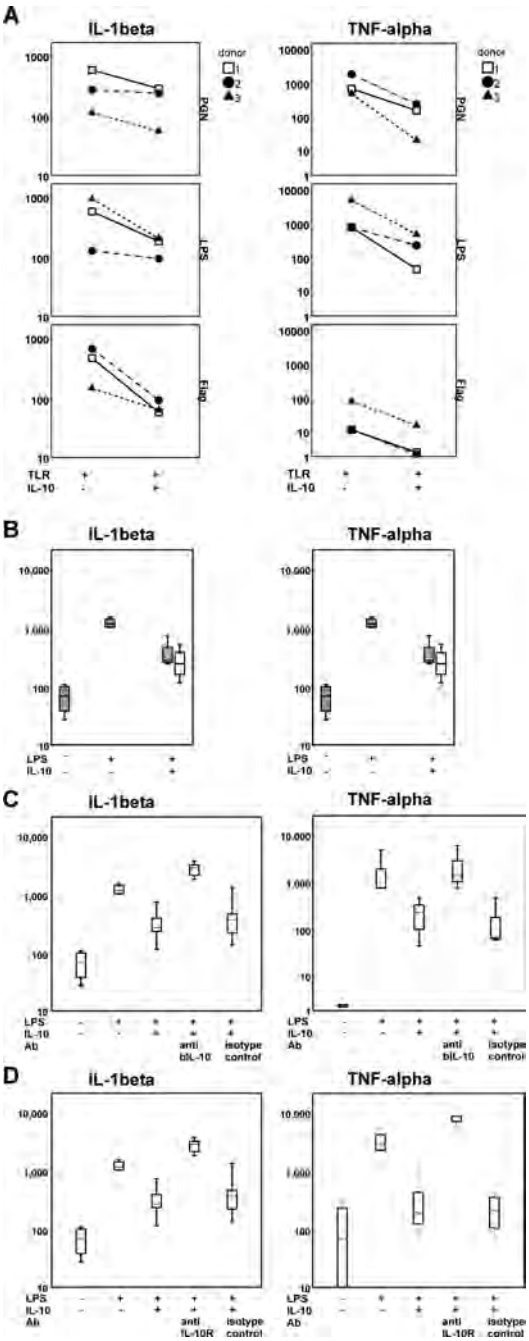


Figure 2. *Bovine IL-10 can regulate TLR ligand induced cytokine production by monocytes by binding the IL-10R. Figures 2B-2D are box plots, showing the median (black horizontal bar), 50% data range (box) and 99% data range (error bars). On the x-axes is the addition indicated (+) of TLR stimuli (panel A), or 10ng/ml LPS (B-D), 10 ng/ml IL-10 (A-D), bovine IL-10 or human IL-10R blocking antibodies (anti bIL-10 or IL-10R) or isotype control (C-D). A: Freshly isolated monocytes were stimulated for 24 hours with different ligands (lipopolysaccharide (LPS); Flagellin (Flag); peptidoglycan (PGN)) with or without recombinant bovine IL-10. IL-1β ($p<0.001$) and TNF-α ($p<0.001$) were significantly inhibited by the three bacterial ligands tested. Data (pg/ml) is shown for three different donors. B: Human monocytes were stimulated with LPS and either human (white box) or bovine (hatched box) IL-10 was added to compare the inhibitory capacity of bovine IL-10 with human IL-10. C: To confirm that the response is specifically inhibited by bovine IL-10 a blocking antibody and an isotope control were pre-incubated with bovine IL-10 and LPS and subsequently added to the monocytes. IL-1β and TNF-α production (pg/ml) is shown of three different donors. D: In order to proof that the bioactivity of bovine IL-10 is mediated through the IL-10R, monocytes were pre-incubated with IL-10R blocking antibodies and subsequently stimulated with LPS and bovine or human IL-10. Data is shown of 3 different donors.*

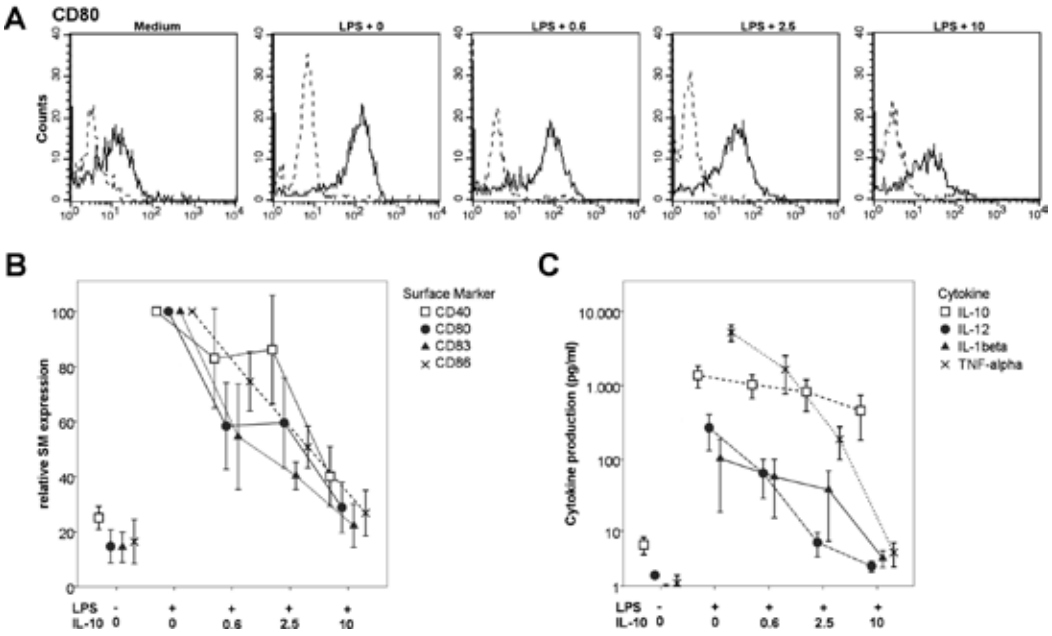


Figure 3. *Bovine IL-10 dose-dependently inhibits DC surface marker expression and cytokine production. A: Typical example of raw data of flow cytometric analysis. Data shown is CD80 expression during LPS induced DC maturation of one donor. The solid line indicates CD80 staining and the dashed line the isotype control. On top of the graphs is indicated whether medium, LPS or LPS plus different doses of IL-10 (ng/ml) were used. B: Bovine IL-10 dose-dependently modulates DC surface marker expression (CD83, p=0.002; CD40, p=0.030; CD80, p=0.018) during LPS induced maturation. Relative values are shown from three different donors. Mean fluorescent intensities were divided by the isotype control and expressed relative to the positive control (LPS, without IL-10), which was set at 100%. C: Recombinant bovine IL-10 dose dependently modulates the production of cytokines by human DC's during LPS induced maturation (IL-12, p=<0.001; TNF-α, p<0.001; IL-1β, p<0.001, IL-10, p<0.001). Raw data is shown from three different donors tested. For each cytokine, a negative control (no LPS, no IL-10) is shown at the left. Data is shown in panels B and C are from three different donors, error bars indicate standard error. On the x-axes the addition of LPS and bovine IL-10 (ng/ml) is indicated.*

10 to the human IL-10R, additional experiments were performed with an human IL-10R blocking antibody (anti human CDw210). PBMC derived monocytes from three different donors were isolated and pre-incubated with the IL-10R blocking antibody or isotype control. Following this incubation, LPS and bovine IL-10 were added. Blocking the IL-10R completely restored the IL-1β and TNF-α production of LPS stimulated monocytes, proving that bovine IL-10 binds to the human IL-10R (Figure 2D). Addition of the isotype control resulted in TNF-α and IL-1β production levels similar to IL-10 inhibited monocytes. The results obtained with the IL-10R blocking antibody showed a similar inhibition as the results from the bovine IL-10 blocking antibody.

These results clearly demonstrate that bovine IL-10 inhibits TLR induced cytokine production by human monocytes, through binding to the human IL-10R.

Bovine IL-10 inhibits LPS-induced DC activation

After testing the bioactivity of bovine IL-10 on human monocytes, experiments with DCs were performed to test if bovine IL-10 is bioactive on other antigen presenting myeloid cells that are potent T helper cell inducers. Freshly isolated monocytes were differentiated into immature DCs under the influence of human IL-4 and GM-CSF. These

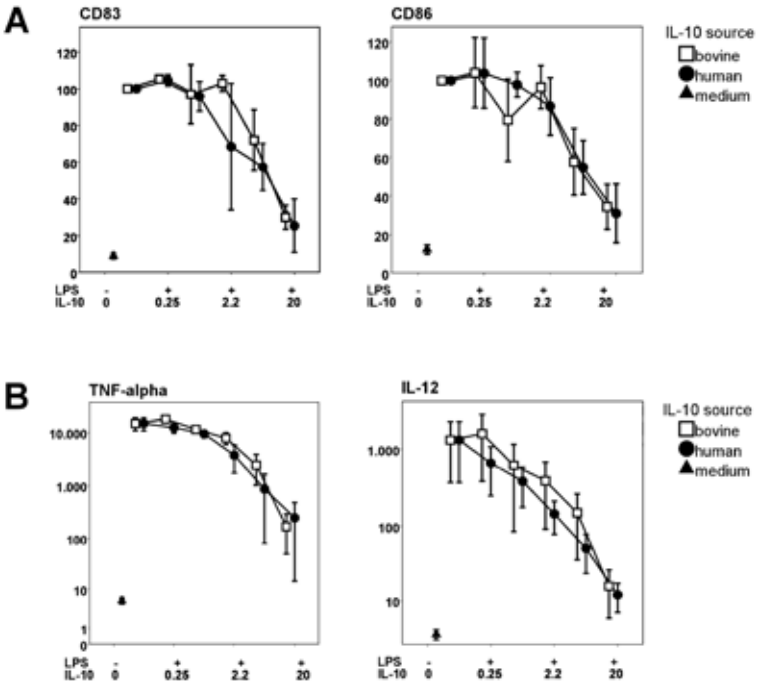


Figure 4. *Dose-dependent inhibition during LPS-induced DC maturation is comparable for human and bovine IL-10. Data shown are from 3 different donors tested, error bars indicate standard error. A: Dose dependent inhibition of CD83 (p=0.753) and CD86 (p=0.936) by human and bovine IL-10. Data were divided by the isotype control and expressed relative to the positive control of only LPS, which was set at 100%. B: Dose dependent inhibition of TNF-α (p=0.916) and IL-12p70 (p=0.962) production by human and bovine IL-10, shown in pg/ml.*

immature DCs from three different donors were maturated with 50 ng/ml LPS in the presence of several concentrations of bovine IL-10. The LPS-induced cytokine production and cell surface marker expression were characterized by flow cytometric analysis.

A typical example of raw flow cytometry data is shown in Figure 3A (CD80). Maturation marker CD83 and activation markers CD40, CD80 and CD86 were up-regulated after stimulation with LPS. Expression of CD83 (p=0.006), CD40 (p=0.030), CD80 (p=0.018) and CD86 (p=0.012) were dose-dependently down-regulated by bovine IL-10 (Figure 3B). Expression of mannose receptor (MR) (p=0.002) was up-regulated with increasing doses of bovine IL-10. The expression of HLA-DR was not influenced by bovine IL-10 or LPS. The expression of CD1a was inhibited after stimulation with LPS, and not affected by bovine IL-10 (data not shown). Addition of bovine IL-10 alone did not affect any of the analyzed surface marker expression levels (data not shown).

Cell culture supernatant of LPS (50ng/ml) stimulated DCs were analyzed for IL-12p70, TNF-α, IL-1β and IL-10 levels. Bovine IL-10 dose-dependently down-regulated the production of IL-12p70 ($p<0.001$), TNF-α ($p<0.001$), IL-1β ($p<0.001$) and IL-10 ($p=0.001$) as shown in Figure 3C. Similar results were obtained for DC's stimulated with 1000 ng/ml LPS (data not shown). On both, DCs and monocytes, 10 ng/ml bovine IL-10 could almost completely inhibit TLR dependent activation.

These data clearly show that bovine IL-10 dose-dependently inhibits LPS induced surface marker expression and cytokine production by DCs.

Bovine and human IL-10 are equally effective in inhibiting LPS-induced DC activation

As bovine IL-10 is able to dose-dependently modulate DC responses, a comparison of the bioactivity of bovine IL-10 and human IL-10 was made, to verify if bovine IL-10 is equally potent as human IL-10 on human dendritic cells. Compared to the previous experiment, a wider range of IL-10 concentrations was used in combination with 10 ng/ml LPS. Expression of CD40, CD80, CD83, CD86, HLA-DR, CD1a, MR and TNF-α and IL-12p70 production was determined for three different do-

Table 2. *Percentage inhibition of DC and monocyte activation of human and bovine IL-10.*

		DC								monocytes			
		IL-12		Tnf-α		CD83		CD86		IL-1β		TNF-α	
		bo	hu	bo	hu	bo	hu	bo	hu	bo	hu	bo	Hu
IL-10 (ng/ml)	0	100	100	100	100	100	100	100	100	100	100	100	100
	0.25	102.9	52.5	143.9	92.1	130.5	128.7	106.3	105.6				
	0.75	30.8	49.0	88.0	74.7	125.5	114.4	83.4	99.2				
	2.2	28.0	16.3	55.2	25.1	125.2	60.6	99.3	83.7				
	6.7	8.9	6.4	13.1	4.6	90.9	72.0	67.9	62.2				
	20	1.5	2.1	0.9	1.3	38.2	26.2	51.0	47.3				
	10									30.3	23.0	14.2	6.1

Average percentage (3 donors, Figure 4) of IL-12 and TNF-α production and CD83 and CD86 expression by DCs and IL-1β and TNF-α production by monocytes (4 donors, Figure 2B) is shown compared to cells only stimulated with LPS (DC's 10 ng/ml,

nors.

Human and bovine IL-10 were both equally potent in inhibiting LPS induced CD83 (linear regression of the difference between human and bovine IL-10: p=0.753 and CD86: p=0.936) expression by LPS stimulated DCs (Figure 4A and Table 2). Also expression of CD40 (p=0.995), CD80 (p=0.971), HLA-DR (p=0.841), CD1a (p=0.873) and MR (p=0.881) was not differentially modulated between human and bovine IL-10 (data not shown). Likewise, the TNF-α (p=0.916) and IL-12p70 (p=0.962) production was equally modulated by human and bovine IL-10 (Figure 4B). From Table 2 it appears that bovine and human IL-10 show similar inhibitory capacity for all parameters tested.

Based on these data, we conclude that bovine and human IL-10 are equally potent in inhibiting LPS induced DC activation.

Bioavailability of bovine IL-10

As applications of bovine IL-10 in humans would depend on the bioavailability we analyzed milk and colostrum samples for IL-10 levels by ELISA and performed an in vitro assay to assess the survival of IL-10 in the upper digestive tract.

In colostrum samples, a range of 150 – 3000 pg/ml of IL-10 was detected in 56 samples from 10 different cows, but in commercially available milk no significant IL-10 levels were detectable (data not shown).

To evaluate the survival of bovine IL-10 in the human upper digestive tract, IL-10 was incubated in an electrolyte solution containing pepsin. IL-10 was dissolved in a protein matrix and incubated for 1 (adults) or 2 (infants) hours at different pH (pH 3 for adults, pH 4 for infants) to resemble respectively the infants and adult stomach. After this incubation 50-60 percent of the IL-10 was still detectable under the conditions of an infant's stomach and 20% for adults (data not shown).

DISCUSSION

Dairy products form an important part of the Western diet, and are a potential source of immunomodulatory ingredients. Bovine milk contains cytokines like IL-10 that can have functional effects on immunological structures in the gut mucosa. IL-10 can exert bioactivity when present in the lumen of the gut [33] and therefore, consumption of IL-10 may potentially modulate innate immune responses in the intestine. The data presented here demonstrate that bovine IL-10 is able to modulate TLR induced cytokine production by human monocytes. Bovine IL-10 binds to the human IL-10R and is able to dose-dependently modulate LPS-induced DC activation, similar to human IL-10. This is in line with the results of a detailed analysis of the cytokine receptor binding sites, and with 3D modeling of the human IL-10R/bovine IL-10 complex.

The recombinant bovine IL-10 used here exerted similar inhibitory capacity and dose-dependency on dendritic cells as human IL-10. The effects noted with human IL-10 are in line with IL-10 studies published before [13,15]. These results demonstrate that the amino acid substitutions between human and bovine IL-10 do not affect the interaction between bovine IL-10 and the human IL-10R. Other amino acid substitutions in IL-10 may how-

ever decrease IL-10 functionality. An extensive genomic analysis of the IL-10 gene and flanking regions of 94 Ulcerative colitis and 94 Crohn disease patients revealed polymorphisms in the IL-10 coding gene, of which one (R159) was in the receptor binding site, as reported by Josephson [8,43]. However, it has not been studied in detail if this polymorphism indeed affects IL-10 – IL-10R interactions in colitis patients.

As far as we know, the IL-10R expression and signal transduction is similar for monocytes and DCs with regard to TLR-dependent activation and cytokine production [11,12,44]. Indeed, our results show that cytokine production by monocytes and DCs could be equally well modulated by bovine IL-10. Involvement of the human IL-10R in the bioactivity of bovine IL-10 implies that all cell types like T cells, B cells and NK cells expressing the IL-10R are probably also modulated by bovine IL-10.

Monocytes and DCs are antigen presenting cells (APC) that are involved in downstream activation of T cells. The modulation by bovine IL-10 of TLR-induced cytokine responses and surface marker expression on DCs will in turn affect their ability to activate CD4+ T helper cells. T helper cells need co-stimulation of CD28 by CD80 (B7-1) or CD86 (B7-2). Bovine IL-10 reduced the upregulation of CD80 and CD86 by human DC's and therefore is likely to reduce T helper cell activation [45]. In addition, bovine IL-10 also modulated IL-12 production, which affects Th1 versus Th2 skewing. As shown in figure 3C, this downregulation of cytokines was more prominent for proinflammatory cytokines (IL-1β, TNF-α, and IL-12), suggesting that bovine IL-10 may dampen Th1 development. The prevention of upregulation of CD40 by bovine IL-10 prevents activation of DCs by CD40L from activated T cells and will reduce the activation of naive T cells by DCs. Moreover, bovine IL-10 can bind to the IL-10Rs expressed on T cells and directly inhibit their activation [45,46,47].

IL-10 and TGF-β both play a role in tolerance induction [48,49] and have complimentary suppressive activity [50]. The presence of intact IL-10 could enhance the success of allergen immunotherapy, as the presence of IL-10 with an antigen at mucosal sites induces tolerance to that allergen [33]. The presence of IL-10 in the lumen resulted in elevated levels of IL-10 producing cells in the Peyers patch [33]. IL-10 in the lumen may be detected by DCs that protrude their dendrites through the epithelial cell layer to sample antigens [41,51] and mouse epithelial cells were proven to express the IL-10R [52]. In individuals tolerant to mucosal antigens, more IL-10 dependent regulatory $CD4^+$ cells are present in the periphery and sublingual allergen immunotherapy induced TGF-β and IL-10 dependent induction of T regulatory cells [50]. $CD4^+$ cells activated in the presence of IL-10 become anergic and can acquire antigen specific regulatory activities [30,53].

IL-10 is essential to develop healthy intestinal immunity [54]. That IL-10 present in the lumen can exert biological activity is shown in a mouse model of autoimmune encephalomyelitis and diabetes where orally administered IL-10 effectively enhanced tolerance induction [33]. In addition oral administration of TGF-β has proven to be functionally active in the intestine when using a mouse model [55]. Likewise, results from our in vitro experiment suggest that bovine IL-10 is able to survive and therefore may be bioactive, particularly in the upper digestive tract of infants. Like in bovine colostrum, IL-10 is present in human breast milk in comparable concentrations ranging from 40 pg/ml till 3 ng/ml [56,57,58]. As infant nutrition is based on resembling breast milk as much as possible, bovine IL-10 could be considered as a replacement for the IL-10 present in human breast milk.

Interestingly, the development of necrotizing enterocilitis (NEC) in premature infants is associated with lower levels of IL-10 in breast milk [59]. In addition, IL-10 knockout mice develop a phenotype resembling NEC [54]. Necrotizing enterocolitis induced in rats can be reversed by human breast milk as well as IL-10 [34,60], and increased cytoplasmic IL-10 levels in epithelial cells in rats correlated with protection to NEC [34]. Moreover, injection of rats with induced NEC with anti TNF-α antibodies reduced incidence and severity

[61]. IL-10 might have similar local effects as IL-10 is a potent regulator of TNF-α production.

From a meta-analysis of the literature, breastfeeding was concluded to be associated with a reduced risk for the development of allergy in the first decade of life [62]. The effect of breastfeeding may increase with duration of breastfeeding [62]. Next to IL-10, TGF-β is present in both human and bovine milk. TGF-β1 and TGF-β2 amino acid sequence identity between cow's and human is 95% respectively 98% after removing the signal peptide, and 100% in their cleaved form (own analysis, data not shown), and therefore are likely to be bioactive in humans. Prolonged application of a low dose of a cytokine could exert functional activity, as has been published for IL-2 [63]. Likewise, application of bovine IL-10 or TFG-β in infant nutrition over a long period may contribute to the prevention of allergy. Administration of bovine IL-10 is mainly expected to be advantageous for individuals with inflammatory diseases, but not for immune-compromised individuals as IL-10 may further inhibit immune activation. Additional administration of IL-10 should be done carefully. This requires further study.

Although several in vivo studies have shown IL-10 bioactivity after oral administration, a potential drawback of applying bovine IL-10 in infant nutrition is that to exert its biological effect in the intestines, bovine IL-10 administered through the diet has to pass the acidity and digestive enzymes of the human upper digestive tract. Another challenge would be to adapt current food processing procedures that currently affect the biological activity of IL-10 [56]. We do not expect that anti bovine IL-10 antibodies will appear when bovine is administered orally, and since bovine IL-10 is bioactive it can inhibit immune cell activation which is a prerequisite for the development of specific antibodies.

We conclude that bovine IL-10 exerts biological activity comparable to human IL-10 on human monocytes and DC's. These findings may have implications for the induction of immune tolerance in the intestinal mucosa in patients with intestinal inflammatory diseases, and for the prevention of NEC and allergy in infants.

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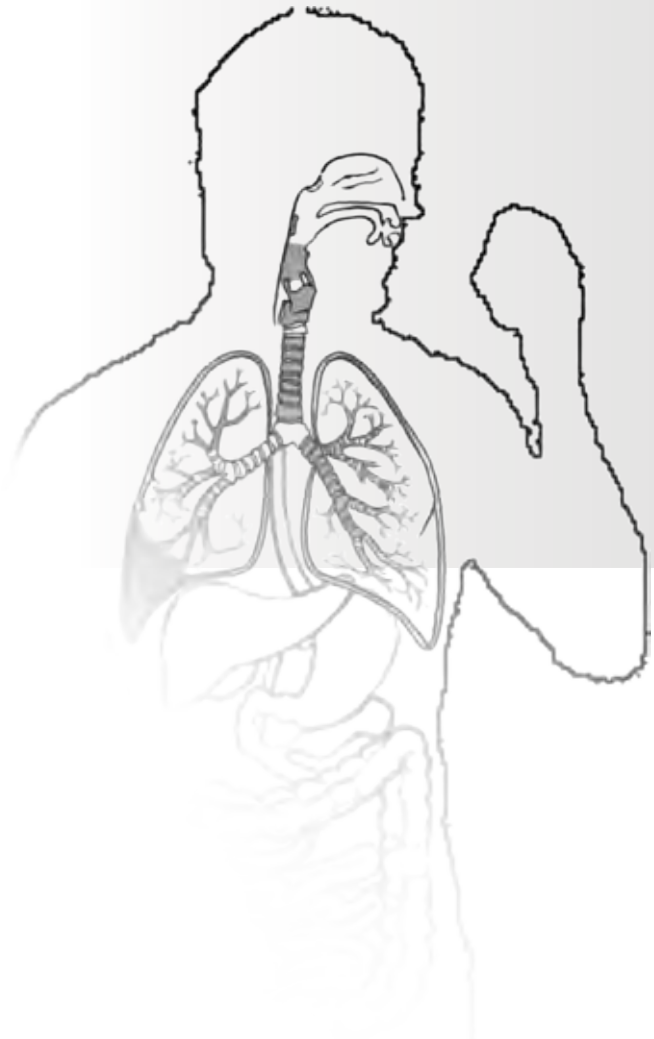

chapter 9

Milk-derived bovine IgG modulates human monocyte and DC responses to LPS by interacting with Fc gamma receptors

Gerco den Hartog, Christine van Altena, Linda Cox,
Huub F. J. Savelkoul, Peter Simons, R. J. Joost van Neerven

GdH and CvA contributed equally to this study

ABSTRACT

Bovine milk from non-immunized cows contains pathogen-specific as well as allergen-specific immunoglobulins that may contribute to mucosal immunity in the gastro-intestinal tract. The aim of this study was to identify if bovine IgG is capable of modulating the activity of human myeloid cells through binding to Fc gamma receptors (FcγR).

To this aim monomeric bovine IgG or IgG in complex with LPS was added to monocytes and dendritic cells, and binding of bovine IgG to human myeloid cells was studied. Bovine IgG was shown to bind to human monocyte-derived dendritic cells, but not to freshly isolated monocytes. Pre-complexing of bovine IgG to LPS increased the binding of IgG to the DCs. This binding could be inhibited by blocking monoclonal antibodies (mAbs) to CD32 (low affinity FcγRII, binds complexes) and by mAbs to CD64 (high affinity FcγRI, binds soluble IgG-antigen). Bovine IgG enhanced IL-10 and TNF-α production by DCs in the presence of LPS, and also increased the expression of activation and maturation markers CD40, CD80, CD86 and CD83. Bovine IgG did not bind to freshly isolated monocytes, and as a result it inhibited the response of monocytes to LPS.

In conclusion, bovine IgG can bind to human IgG receptors and modulate myeloid cell activation by bacterial ligands. Therefore, IgG from bovine milk may contribute to immunity to pathogens and may also play a role in induction of oral tolerance to allergens.

INTRODUCTION

Immunoglobulins with a wide range of antigen specificities are present in breast milk as well as bovine milk [1, 2]. These specific antibodies (Abs) protect neonates against infectious agents that the mother has already been exposed to [3, 4]. Bovine immunoglobulins are able to bind to a wide range of antigens, including pathogenic and non-pathogenic microbes, and inhaled or ingested antigens and allergens [3]. Therefore, it would be useful to evaluate if Abs from cow's milk can enhance antimicrobial immunity in humans, or promote the induction of tolerance to allergens in neonates [5]. Immunoglobulins can have different isotypes, like IgM, IgG and IgA, resulting in different effector functions. Effector functions of antibodies are neutralization, antibody-dependent cellular cytotoxicity, Fc-receptor mediated phagocytosis and induction of downstream activation or inhibition of myeloid cells and B cells. The type of effector function induced by Abs is dependent on the Fc receptor, or complement factor, they can bind to. Human breast milk contains high levels of sIgA, especially in the first weeks postpartum, whereas levels of IgG are about ten-fold lower compared to IgA and IgM [6]. Immunoglobulins in cow's milk are mainly of the IgG class, of which the majority is IgG1 [7, 8]. In bovine milk IgG1 levels are strongly increased around birth.

Even though the pathogens to which cows and humans are exposed may vary, bovine pathogen-specific immunoglobulins can cross-react with conserved structures on human pathogens. Bovine and human pathogens share common pathogen associated molecular patterns like LPS, PGN and double stranded RNA, that enable cross-reactive binding of bovine immunoglobulins with human pathogens. Specific bovine IgG in colostrum has been detected against numerous bacterial and viral pathogens [9, 10].The functionality of those Abs was illustrated for example in a study that could show decreased diarrhoea and frequency after treatment of HIV-infected patients that suffered from recurrent diarrhoea with colostrum of non-immunized cows [9]. Similarly, a bovine colostrum protein concentrate of non-immunized cows that consisted of more than 65% IgG was able to significantly reduce Escherichia coli-associated diarrhoea in infants in a placebo controlled study [11].

Myeloid antigen-presenting cells (APC), like monocytes, macrophages (MΦ) and dendritic cells (DC), express multiple Fc gamma receptors (FcγR) with varying affinities for human IgG [12]. Both activation-inducing and inhibitory IgG receptors have been described, which are differentially regulated by IFN-γ and IL-4 [13]. Upon binding of the Fc chains of Ig-complexes, FcγR triggering induces various effector functions, including phagocytosis, antibody-dependent cell-mediated cytotoxicity, differentiation, cytokine production, and even cross-presentation of antibody-coated antigens [14]. FcγR-mediated uptake of antigens may be especially beneficial by potentiating Th cell activation when only low concentrations of antigen are present [15]. Activation of APC via Toll-like receptors (TLR) or FcγR is a requirement for efficient adaptive immune responses upon infection as APC take up, process and present antigens to T cells resulting in T cell activation and differentiation. Myeloid cells have variable abilities to stimulate T cells. Monocytes can both initiate pro-inflammatory responses, and differentiate further into different DC subsets [16, 17]. DCs are especially efficient antigen presenting cells that are able to activate naïve T cells and induce T helper cell differentiation into different effector Th cells, like Th1 and Th2 [18]. Four types of human IgG Fc receptors have been identified to date, that have low affinity (CD16 and CD32) and high affinity (CD64) to IgG and bind to larger immune complexes and soluble IgG-bound antigen respectively [19, 20]. In addition to classical FcγRs that selectively binds IgG the neonatal FcR (FcRn) has been identified [21]. FcRn is expressed on various cells of the intestinal mucosa, including epithelial cells, and bi-directional transport of human IgG over the epithelial cell wall has been shown to occur [21]. This bidirectional transport both enables antibody-mediated protection at mucosal surfaces and antigen sampling of immune complexes present in the intestinal lumen, and processing for induction of specific immunity or tolerance.

The aim of the current study was to identify if bovine IgG can interact with FcγR expressed on human monocytes and dendritic cells, and if this results in functional activation of these cells.

MATERIALS AND METHODS

Preparation of bovine colostrum

Bovine colostrum (LF200, La Belle, Washington, USA) was dissolved at 10 mg/ml in sterile PBS (Mg2+/Ca2+-free; Lonza), incubate 30 minutes at 37°C and Centrifuged for 10 minutes at 1000g at room temperature. Supernatants were sterile filtered through a 0.45 µm filter and stored at 4oC.

Purification of bovine colostrum-derived immunoglobulins

Commercially available colostrum sample (Colostrum 35% IgG, Reflex Nutrition, UK) was diluted in 0.75 M (NH4)2SO4 loading buffer (two-fold dilution). Additional (NH4)2SO4 was added to obtain a final concentration of 0.75 M (NH4)2SO4. The sample was loaded onto an AFFI-T™ column (Kem-en-Tec) and eluted with 0.05 M Tris-HCl pH 8.9 elution buffer. The sample was diluted with 0.02 M NaPi pH 7.0 loading buffer (four-fold dilution) and loaded on a protein G column (5 ml; Amersham). Sample was eluted with 0.1 M glycine-HCl pH 2.7 elution buffer and neutralized with 1 M Tris-HCl pH 9.0 neutralizing buffer, followed by dialyzation against PBS (3x 1L). Sample was sterilized (0.2 µm filter) and absorbance at A280 nm was measured (GeneQuant; Amersham) to determine protein concentration and stored at 4oC.

Isolation of human monocytes

PBS (Mg^{2+}/Ca^{2+}-free; Lonza) supplemented with 0.1% BSA (Sigma) and 2.0 U/mL heparin (Leo Pharma) was added to buffy coat blood (blood bank Sanquin, Amsterdam) resulting in a final volume of 120 mL. The diluted blood was layered onto Lymphoprep gradient (1.077 g/mL; Axis Shield) and centrifuged for 15 minutes at 1000g at RT (no braking). Peripheral blood mononuclear cells (PBMC) were harvested and washed in excess of PBS/0.1% BSA by three centrifugation steps, i.e. 10 minutes at 750g, 5 minutes 500g, and 10 minutes 250g at RT (last step, no braking). The pellet

(PBMC) was placed onto a Percoll gradient (stock 1.130 g/mL; GE Healthcare) consisting of 1.076, 1.057, and 1.045 g/mL layers and subsequently centrifuged for 45 minutes at 1,750g at RT (no braking). Enriched monocytes were washed (low density fraction) in excess of PBS/0.1% BSA (5 minutes 500g RT).

Activation of human monocytes

106 monocytes/ml were allowed to adhere for 1.5 hour at 37˚C in serum-free CellGro DC culture medium (CellGenix) supplemented with 50 µg/mL gentamycin (Gibco). After 1.5 hour of incubation non-adhering lymphocytes were removed by washing 4 times with PBS containing 0.5% BSA. Subsequently, 250 µg/mL or 50 µg/mL bovine IgG1 was added from colostrum and purified bovine immunoglobulins with or without 1 ng/ml LPS from Escherichia coli (serotype 055:B5; Sigma) to monocytes. In addition, 50 or 250 µg/ml human IgG (Jackson ImmunoResearch) with or without 1 ng/ml LPS was added. Non-stimulated monocytes and 1000 ng/mL LPS treated monocytes were included as negative and positive controls respectively. Cells were incubated for 2 days in a 5% CO2 incubator at 37˚C. After 2 days supernatants were collected and stored at -20˚C until determination of cytokine levels.

Generation and activation of human immature monocyte-derived DC

0.5x106 monocytes/ml were added to a culture flask and allowed to adhere. After adherence non-bound cells were removed and serum-free CellGro DC culture medium (CellGenix) supplemented with 50 µg/mL gentamycin (Gibco) and 20 ng/mL GM-CSF (PeproTech) and 20 ng/mL IL-4 (PeproTech) was added to monocytes. Cells were incubated for 5 days in a 5% CO_2 incubator at 37˚C. Medium containing IL-4 and GM-CSF was refreshed at day 2.

After 5 days of differentiation immature DCs were harvested and adjust to 1.0x106 DC/mL in CellGro DC medium supplemented with gentamycin and 20 ng/mL GM-CSF and 20 ng/mL IL-4. 250 µg/mL or 50 µg/mL bovine IgG1 from colostrum and purified bovine immunoglobulins with or without 100 ng/ml LPS from Escherichia coli (serotype 055:B5; Sigma) were added to the DCs. Also human IgG (Jackson ImmunoResearch) at 250 µg/mL or 50 µg/mL with or without 100 ng/ml LPS were tested. Non-stimulated DC and 100 µg/mL polyI:C (Sigma) treated DC (PeproTech) were included as negative and positive controls respectively. Stimulated cells were incubated for 2 days (5% CO2 incubator at 37˚C). After 2 days supernatants were collected and stored at -20˚C until determining cytokine levels. DCs were harvested for analysis of surface activation markers.

Determination of surface activation markers on human DC

After 2 days of DC activation DCs were collected, washed in PBS/BSA/NaN3 at 4°C, and adjusted to 105 DC/100 µL in PBS/BSA/NaN3 at 4°C per tube. 5 µL mouse anti-human CD40, CD80, CD83, and CD86 antibodies conjugated to FITC or to PE (Becton & Dickinson) were added to 1 * 10^5 DC/100 µL/tube. Mouse IgG1 isotype controls conjugated to FITC or to PE (Becton & Dickinson) were included to determine non-specific background staining. Cells with antibodies were incubated for 30 minutes at 4°C, washed (3 minutes 500g) in excess (4 mL/tube) of PBS/BSA/NaN3 at 4°C. Cells were resuspended to 10^5 cells/200 µL in PBS/BSA/NaN3/2% formaldehyde at 4°C and incubated for 30 minutes at 4°C. MFI of 10,000 events were recorded to determine DC surface activation markers using a flow cytometer (Becton & Dickinson).

Determination of IL-10 and TNF-α levels

Supernatants from cell cultures were serially diluted in IMDM (Lonza), i.e. for IL-10 determinations at 1:5, 1:10 and 1:50, and for TNF-α determination at 1:10, 1:50 and 1:250. IL-10 and TNF-α levels in diluted supernatants were measured according to manufacturer's instructions (ELIPAIR from Diaclone). IL-10 and TNF-α standards were also diluted in IMDM.

Binding of bovine and human IgG to myeloid cells

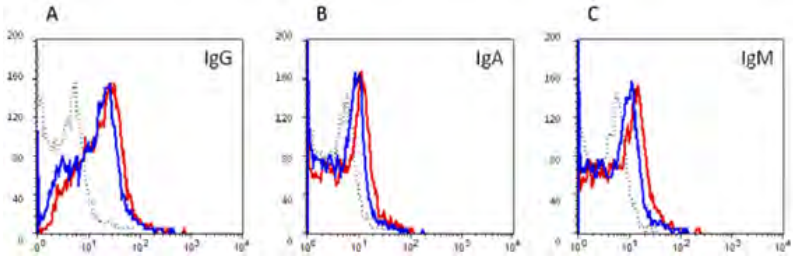

Figure 1. *Binding of bovine IgG (A), of bovine IgA (B), and of bovine IgM (C) from colostrum (red histograms) and purified bovine immunoglobulin (blue histograms) on human monocyte-derived immature DC. Dotted black histograms represent background staining. Data from one representative donor of two is shown.*

250 µg bovine IgG1/mL from colostrum and purified bovine immunoglobulins, and 250 µg native (monomeric) or denatured (20 minutes at 65°C; to mimic immune complexes) human IgG /mL (Jackson ImmunoResearch) was added to enriched monocytes and immature DCs (1 * 105 cells/100 µL) in PBS/BSA/NaN3 at 4°C. Cells with immunoglobulins were incubate for 30 minutes at 4° C, followed by washing (3 times; 3 minutes 500g) in excess (4 mL/tube) of PBS/BSA/NaN3 at 4°C. Cells were resuspended to 1 x 105 cells/100 µL PBS/BSA/NaN3 at 4°C and 10 µl 1:10 sheep anti-bovine IgG (Bethyl), 10 µl 1:10 sheep anti-bovine IgA (Bethyl), and 10 µl 1:10 sheep anti-bovine IgM (Bethyl), or 10 µl 1:10 goat anti-human IgG-FITC (Jackson ImmunoResearch) was added and incubated for 30 minutes at 4°C. Cells were washed (3 minutes 500g) in excess (4 mL/tube) of PBS/BSA/NaN3 at 4°C and resuspended to 1 x 105 cells in 100 µL PBS/BSA/NaN3 at 4°C. 10 µl 1:10 donkey anti-sheep IgG-FITC (Jackson ImmunoResearch) was added and incubated for 30 minutes at 4° C. Cells were washed and resuspended 105 cells/200 µl PBS/BSA/NaN3 containing 2% formaldehyde at 4°C. Cells were incubated for 30 minutes at 4° C and analysed for MFI (10,000 events) MFI to determine Ig binding using a flow cytometer (Becton & Dickinson).

FcR Blocking

Immature moDCs were incubated with blocking antibodies to CD32 (clone AT10, GeneTex), CD64 (clone 10.1, BioLegend, San Diego, CA, USA) (or IgG1 isotype control, clone MOPC-21, BioLegend for 30 minutes on ice. Cells were washed (MEDIUM) and 100 µl 1 µg/ml monomeric (µg/ml) or bovine IgG in complex with 1ng/ml LPS was added and incubated for 30 minutes on ice. Cells were washed again and incubated with 1:100 sheep anti-bovine IgG (Bethyl), washed and incubated with 1:100 FITC conjugated donkey anti-sheep IgG (Jackson). Cells were incubated 30 minutes on ice, washed and resuspended in 200 µl FACS buffer (PBS, 0.5% BSA, 0.5 mM EDTA). MFI was determined of 10 000 events recorded (BD FACS Canto).

RESULTS AND DISCUSSION

Binding of bovine Ig to human monocytes and dendritic cells

Knowing that bovine milk and milk-derived products contain immunoglobulins of various subclasses that can bind to pathogens and allergens that are also encountered by humans, the ability of bovine Ig to bind to human monocytes was studied. Bovine IgG (250 µg/mL), IgA (31 µg/mL), and IgM (125 µg/mL) and purified bovine immunoglobulins did not bind significantly to freshly isolated human monocytes (data not shown).

In contrast, bovine IgG (250 µg/mL) from colostrum as well as purified immunoglobulins did clearly bind to immature monocyte-derived DC (Figure 1). The binding of bovine IgG to moDC was dose dependent (data not shown). Bovine IgA and IgM bound only marginally to moDCs. The

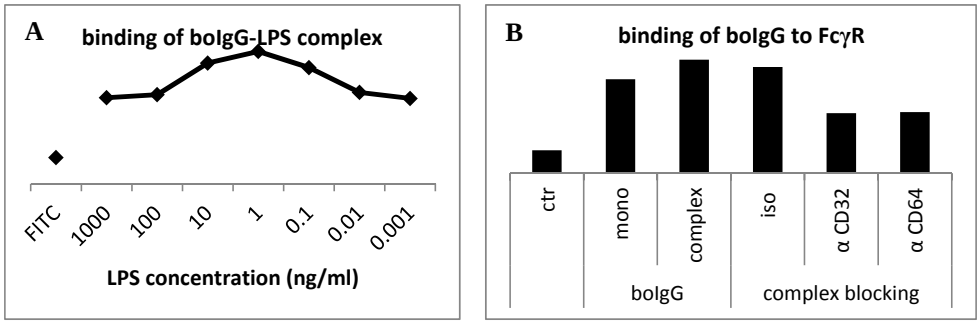

Figure 2. *Binding of bovine IgG-LPS immune complexes to human moDCs. 1 µg bovine IgG was incubated with different doses of LPS and subsequently added to moDCs (A). IgG –LPS immune complexes were added to moDC in the presence or absence of IgG receptor blocking antibodies (B). Ctr shows background staining, boIgG mono is monomeric bovine IgG and boIgG complex shows bovine IgG pre-incubated with LPS prior to addition to the DCs. Blocking of CD32 and CD64 was performed with LPS-IgG complexes. For both panels an example of three experiments is shown.*

lack of CD89/FcαRI expression by these immature DC (data not shown) indicates that the low level of binding of bovine IgA was most likely non-specific. However, involvement of other known human IgA and IgM receptors like asialoglycoprotein receptor, CD71/transferrin receptor, and Fcα/µR cannot be excluded as some of those receptors have been identified on myeloid cells [22, 23].

Bovine IgG binds to human FcγR

Having observed that bovine IgG binds to human moDCs, experiments were designed to identify to which human FcγR expressed on moDC bovine IgG could bind. IgG that was allowed to form complexes with LPS resulted in increased binding of bovine IgG to human moDCs compared to monomeric bovine IgG, suggesting binding of immune complexes (Figure 2A). There is an optimal ratio between antigen and IgG for formation of immune complexes. Titration of LPS with bovine IgG resulted in a bell-shaped curve with optimal binding of IgG-LPS complexes to DCs at a ratio of 1 µg of bovine IgG and 1 ng/ml of LPS. This binding of bovine IgG could partially be inhibited by blocking monoclonal antibodies (mAbs) directed at the low affinity FcγRII (CD32), but also by mAbs to the high affinity FcγRI (CD64) (Figure 2B). These data suggest that monomeric bovine IgG can bind to the high affinity human FcγRI, and that bovine IgG immune complexes could bind to the human low affinity FcγRII.

The monomeric bovine IgG did not bind to monocytes, likely because FcγRI (CD64) on freshly isolated monocytes were already saturated with monomeric human IgG. moDCs however, have CD64 on the membrane without bound IgG, which explains that bovine IgG was bound by moDCs and not monocytes. Binding of complexed bovine IgG to freshly isolated monocytes could be possible as CD32 does not bind to soluble monomeric IgG present in blood. The observation that bovine IgG binds to FcγRI and FcγRII on DCs strongly suggests that bovine IgG may also bind to FcγRI and FcγRII expressed on other cell types. FcγRI and FcγRII are differentially expressed on dendritic cells in the mucosal tissues. Therefore, dietary bovine IgG may mediate uptake of allergens and pathogenic and non-pathogenic bacteria and viruses. Depending on the expression levels of activating and inhibitory FcγR, inflammatory or non-inflammatory downstream immune responses may be initiated leading to the production of protective IgG or IgA antibodies. This may be influenced by which type of DC the FcγRs are expressed.

Effect of bovine immunoglobulins on IL-10 and TNF-α production by human monocytes

Minimal binding of bovine IgG to monocytes was observed. To study if bovine IgG could modulate LPS-induced cytokine production by monocytes,

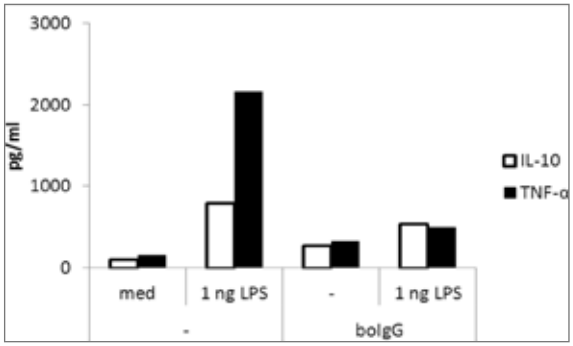

Figure 3. *Effect of purified bovine immunoglobulin, in the absence or presence of a suboptimal concentration of LPS (1 ng/mL), on IL-10 and TNF-α production from human monocytes. Data from one representative donor of three is shown.*

bovine IgG with and without LPS was added to freshly isolated monocytes and incubated for 48 hours. Human monocytes secreted substantial IL-10 and TNF-α upon exposure to LPS (Figure 3). The cytokine secretion levels reached their plateau at a 1000 ng/mL LPS, while 1 ng/mL LPS was suboptimal (n=3; dose response curves not shown).
Interestingly, the cytokine responses evoked by 1 ng/ml LPS were significantly inhibited by co-incubation with purified bovine immunoglobulins. This finding strongly suggests that the anti-LPS specific antibody pool recognized and neutralized a bioactive moiety of LPS.

Bovine immunoglobulins synergistically enhance the activation of human moDC by LPS

As discussed above, bovine IgG-LPS complexes could bind to human moDCs. To check if this results in enhanced responses of the moDC to LPS, bovine IgG alone or in combination with LPS was added to immature moDC. Human immature DC showed no, or only a minimal IL-10 and TNF-α production after stimulation with 100 ng/ml LPS or bovine immunoglobulins alone (Figure 4). However, the combination of 100 ng/mL LPS with purified bovine immunoglobulin resulted in a strong synergistic increase of IL-10 and TNF-α secretion by the moDCs (Figure 4). This observation indicates that immune complexes consisting of bovine LPS-specific immunoglobulin and LPS could activate human moDC via a FcR-signaling pathway. This is consistent with the observation that IgG-LPS complexes result in increased binding to DC compared to monomeric IgG (Figure 2). Consequently, binding of IgG immune complexes seem to result in activation of these DCs.
The induction of increased expression levels of activation- and maturation markers by stimulation of moDC with LSP- bovine IgG immune complexes varied between the donors tested. However, in agreement with the demonstrated synergism on cytokine secretion from human immature DC, bovine immunoglobulins in combination with 100 ng/mL LPS also synergistically increased CD83 and CD86 expression on immature DC (Figure 4B). Apart from CD86 and CD83 bovine IgG also increased expression of CD40 and CD80, but this effect was less prominent (data not shown). CD83 expression is associated with expression of MHC class II, which is required for antigen presentation by DCs to T cells [24]. These data suggest that bovine IgG may mediate FcγR-facilitated antigen presentation to T cells, as was previously demonstrated for human IgG [25].
Two previous reports have shown cross-species binding of bovine IgG to human Fc receptors. Loimaranta et al demonstrated that immunization of cows with Streptococcus mutans and S. sobrinus resulted in increased specific Ab levels in bovine colostrum-derived whey that could mediate phagocytosis by human monocytes, eosinophils and neutrophils [26]. In a more recent paper, colostrum-derived bovine IgG from HIV-1 envelope gp140 vaccinated cows was shown to dose-dependently bind to FcγRs on human neutrophils, monocytes and NK cells, and was able to induce antibody dependent cellular cytotoxicity by monocytes via signalling through the activation-inducing FcγRIIa (CD32a) [1]. The data presented here confirm these previous reports, and extend these

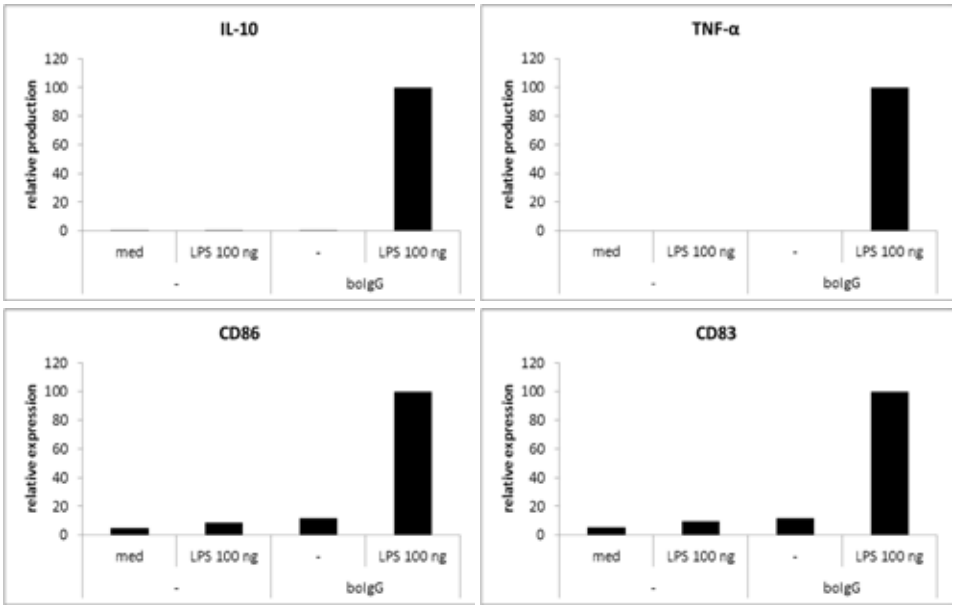

Figure 4. *Effect of colostrum and purified bovine immunoglobulin, and of human monomeric IgG, with or without 100 ng/ml LPS, on IL-10 and TNF-α (A) production and activation marker CD86 and maturation marker CD83 expression (B) by human monocyte-derived immature DC after 2 days. Data from one of three donors is shown.*

findings by showing that the presence of bovine IgG enables DCs to respond more vigorously to LPS (Figure 4).

In addition to classical FcγR, the non-classical IgG-binding neonatal receptor FcRn is expressed in the intestines of neonates, which is involved in induction of oral tolerance to allergens in mice [5]. Binding of bovine IgG to human FcRn does occur, but is relatively low compared to binding of human IgG [27]. We have not been able to show the presence of FcRn on the moDC used in this study, which does not exclude a functional of FcRn expressed in the intestine of humans, in which FcRn is expressed constitutively [28].

Bovine IgG-antigen complexes formed in the intestinal lumen are expected to be bound and subsequently taken up by mucosal FcγR-expressing DCs, that can protrude their extensions through tight junctions between epithelial cells into gut lumen [29]. In addition, goblet cells have been reported to deliver luminal antigen to tolerogenic $CD103^{+}CDd11c^{+}$ DCs [30]. Immune complexed antigens that are internalized via CD32 on immature DC are known to be presented far more efficiently to T lymphocytes than non-complexed antigens [31], suggesting that bovine IgG-antigen immune complexes may also play a functional role in immune defence in the intestine as they can also bind to CD32. In addition to intestinal pathogens or food allergens, airway pathogens and allergens may be encountered by intestinal DCs, as airway content can be swallowed and be bound by immunoglobulin in the intestinal lumen [32].

Induction of immune responses by bovine IgG requires formation of complexes of bovine IgG with a pathogen or allergen. Specific IgG against many different bacterial pathogens has been found in milk of non-immunized cows, including IgG specific for Yersinia enterocolitica, Campylobacter jejuni, Escherichia coli and toxins thereof [33, 34]. Also rotavirus-specific IgG is present in cow's milk [2]. Interestingly, bovine milk also contains specific immunoglobulins against rye-grass pollen, house dust mites, Aspergillus mould and wheat proteins [35]. Levels of specific IgG in bovine milk can be strongly increased by vaccination of the cows. In addition to FcγR-mediated facilitation of immune responses, vaccination results in

strongly increased levels of pathogen-specific IgG that can neutralize pathogens in the intestinal lumen, thereby offering passive protection against infections. For in vivo application of the results reported here, bovine immunoglobulins need to resist industrial processing and the upper digestive tract. Bovine IgG is likely to resist the stomach milieu of infants [36] which has milder conditions (e.g. pH) compared to adults. Indeed several studies have demonstrated that delivering passive protection against human rotavirus by feeding a colostrum formula from vaccinated cows to infants can prevent rotavirus infections [37].

Bovine immunoglobulin preparations from immunized cows have also been applied successfully in treatment of infants that already suffered from rotavirus-induced gastroenteritis [38]. Likewise, an immunoglobulin concentrate from cows hyper immune for Escherichia coli has been shown to protect infants against diarrhoea[39].

CONCLUSION

Bovine colostrum-derived IgG and IgG-LPS complexes bind to FcγRI and FcγRII expressed by human moDC, resulting in cytokine production and upregulation of activation- and maturation-markers.

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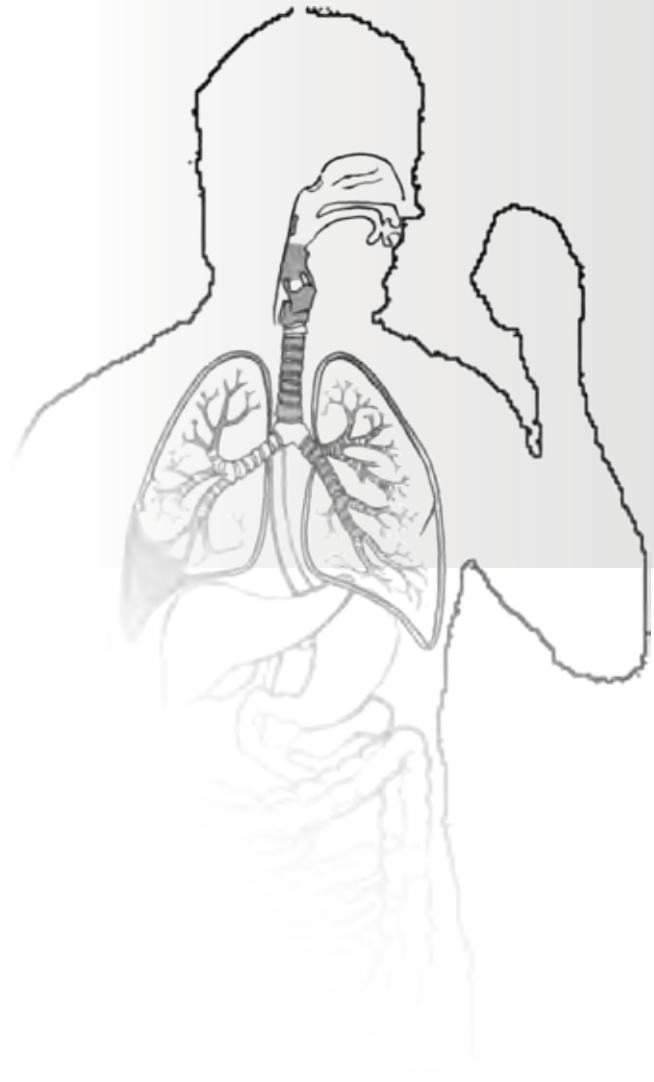

chapter 10

General Discussion

Gerco den Hartog

The mucosal immune system has been studied extensively, but research relating to the role of mucosal B cells in allergy is limited. This chapter discusses the relevance of B cell responses in allergy, with emphasis on mechanisms of IgA induction in mucosal tissues. Reports evaluating if and how IgA may protect against allergy are scarce, especially for mucosal IgA. In this chapter, interaction between the different antibody-isotypes and allergens are discussed. The potential role of dietary ingredients as modulator of the mucosal immune system, as opposed to dietary ingredients causing allergy, is considered. Subsequently, the concept of potential mucosal mechanisms contributing to prevention or protection against allergy are discussed, leading to a new hypothesis about the protective role of mucosal IgA and the difference between IgA1 and IgA2 in protection against allergy.

I SEROLOGICAL DIAGNOSIS OF ALLERGY: BEYOND ALLERGEN-SPECIFIC IGE LEVELS

Epitopes of allergens

Tropomyosin is the major shell fish allergen that is recognized in a cross-reactive manner between different animal species by serum IgE [1-9]. Cross-reactivity can be determined in vitro by inhibition assays. IgE-binding epitopes identified in both crustacean and molluscan tropomyosin cover similar surface areas on the molecule [10]. Techniques to identify IgE-binding epitopes vary. The methods that are used most frequently rely on linear synthetic peptides, and enzymatic digestion of native tropomyosin followed by ELISA analysis. The latter technique was used by Ishikawa et. al [5, 11] to identify epitopes in molluscan species. Using this technique, only one IgE-binding epitope was identified for tropomyosin of see snail (Tur c 1) and oyster (cra g 1). The lack of detection of other IgE-binding epitopes may be caused by the limited number of sera used or indicate limited allergenicity of those molluscan allergens compared to shrimp tropomyosin, where five IgE-epitope regions have been identified.

For tropomyosin, a common sequence motif (LEXXL) has been proposed, as it appeared to be present in all IgE epitopes identified [10]. However, this motif is also present in human tropomyosin and therefore unlikely to be a key determinant in allergic responses to shrimp tropomyosin; no serological analysis was performed on human tropomyosin (Figure 1).

IgE-binding epitopes have been identified in tropomyosin in a variety of species. Those epitopes are distributed over the surface of the protein, and therefore enable crosslinking of IgE immobilized on FcεRI on mast cells and basophils. For IgE crosslinking at least two epitopes are required that should be at least 8-10 nm (80-102 Å) and is estimated to be at maximum 20-24 nm apart from each other [12]. An interesting finding from our tropomyosin 3D model (Chapter 2) was the regional distribution of the five IgE-binding epitopes identified. IgE-binding epitopes on tropomyosin were located at a distance of approximately 70 Å apart from each other, whereas the Fab fragment binding to allergens is about 50 Å and therefore steric hindrance is limited allowing binding of up to five IgE-molecules to shrimp tropomyosin simultaneously. This finding shows that serological analysis at the level of IgE epitopes is relevant to predict aller genicity of allergens and potential cross-reactivity. Currently the usefulness of component resolved diagnosis using recombinant allergen proteins is being discussed in the literature [13]. A complicated analysis may be difficult to apply in clinical settings, whereas selection of a number of allergens in relation to IgE-epitope reactivity may greatly enhance specificity of diagnostic tests.

Systemic versus local allergen-specific Ig

For most allergies, presence of allergen-specific IgE is a prerequisite to develop allergy, whereas sensitized individuals can have varying clinical symptoms and these may even be absent. As a result, detection of allergen-specific IgE in serum

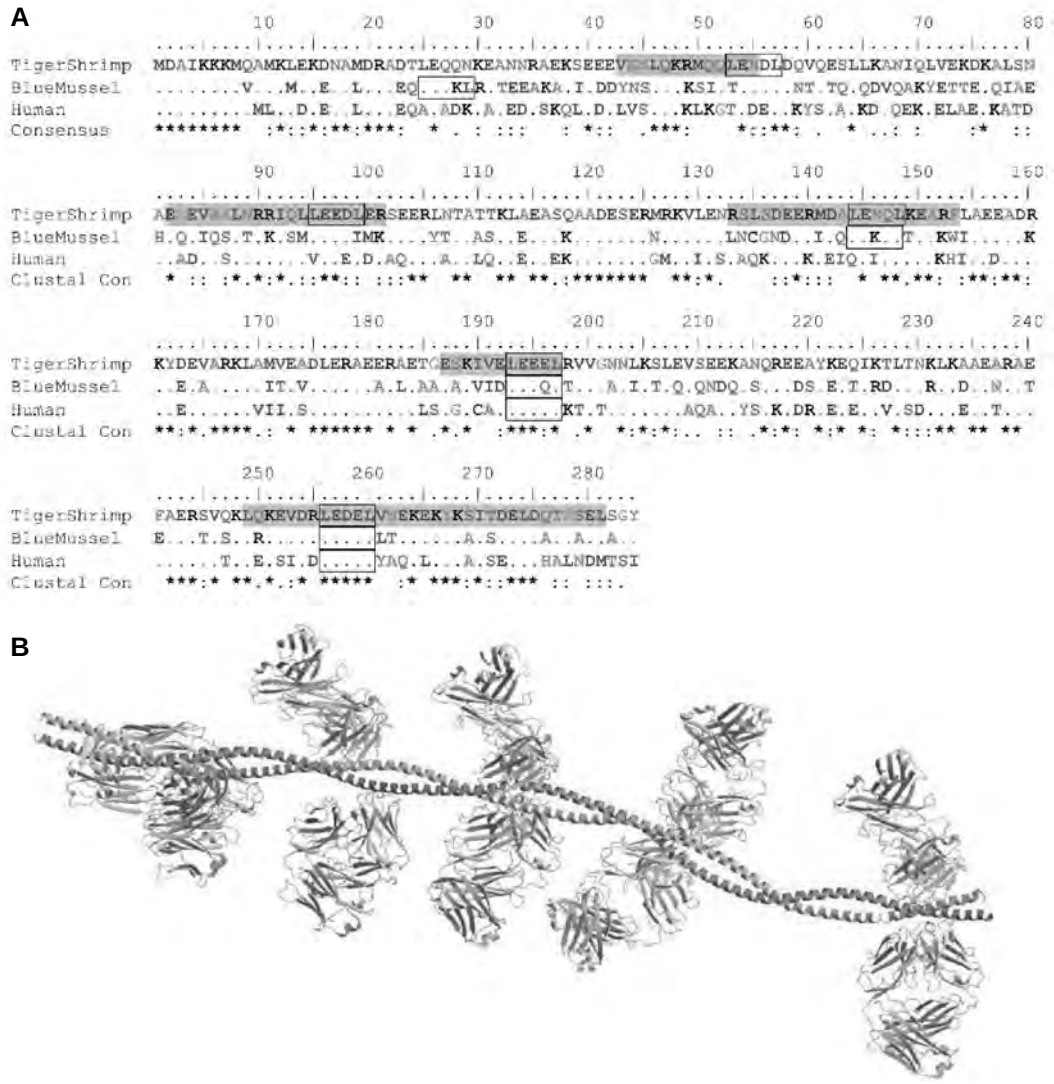

Figure 1. *ClustalW Alignment of tropomyosin sequences of black tiger shrimp, blue mussel and human (A) and a 3D model of IgE Fab fragments binding to tropomyosin (B). The IgE epitope regions identified in shrimp are shaded and the identified common motive LEXXL is boxed, and is shown to be present in human tropomyosin too. The 3D model (B) indicates that multiple IgE molecules can bind simultaneously to tropomyosin, as discussed in more detail in chapter 2.*

has high sensitivity, whilst specificity can be limited. An important explanation for lack of specificity of serological detection of allergen-specific IgE, is the presence of blocking antibodies like IgG4 (Figure 2) [14, 15]. Reduced mast cell and basophil degranulation has been observed after specific immunotherapy prior to visible serological changes in the ratio between IgG4 and IgE [16]. This could be due to the suppressive effect of induced mucosal tolerance mediated by DCs and Tregs. Production of anti-inflammatory cytokines like IL-10 and TGF-ß may inhibit activation of mast cells and basophils or other cellular compartments, like Th2 cells and eosinophils [17].

An alternative, but poorly explored explanation for early clinical protection against allergy during specific immunotherapy, may be the increase in allergen-specific IgA at mucosal surfaces (Figure 2) due

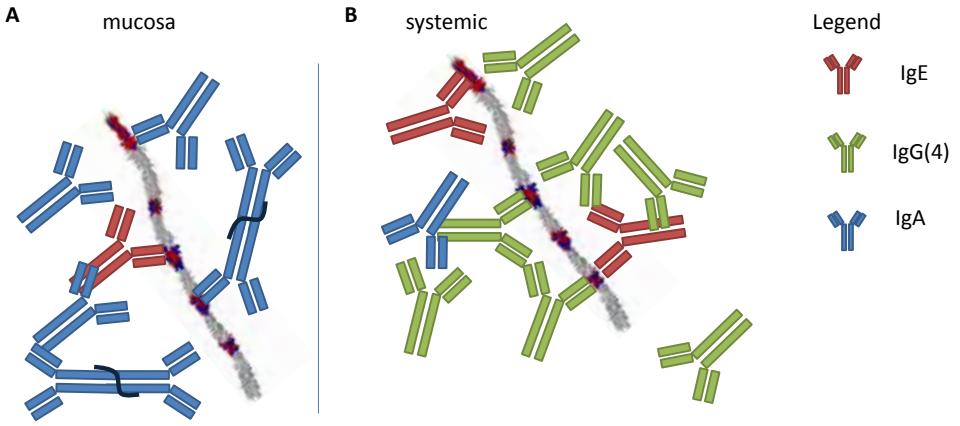

Figure 2 *Isotypes causing or inhibiting allergy. A IgE causes allergy, IgG4 is able to block IgE-allergen interactions, and IgA is proposed in this thesis to be able to protect against allergy. B Presence of immunoglobulin isotypes varies in different parts of the body. In the external mucosal milieu, IgA is the dominantly present isotype, while IgE and IgG4 levels are low. Systemically, IgE and IgG4 levels are higher. Also potential interactions with the allergen tropomyosin are shown, indicating that different isotypes could bind to different epitopes simultaneously, which affect to possibility for IgE crosslinking. Note that picture is not drawn at scale; tropomyosin is 27 kDa and antibodies are ~150 kDA.*

to the tolerogenic cytokines IL-10 and TGF-ß that are crucial for Treg induction and IgA production [17]. Induction of tolerance and production of IgA both occur locally in the mucosa [18, 19]. The fact that the majority of IgA is being produced locally in the mucosa could suggest that the antigenic specificities between systemic and local IgA may vary as these IgA molecules may originate from different lymphoid tissues (Chapter 4). Indeed different antigen specificities have been observed between serum and mucosal fluids like saliva, but no epitope studies that compare allergen-epitope specificity between serum and mucosal fluids have been performed [20-22]. This finding is consistent with the observation that systemically induced allergen-specific B cells are not migrating efficiently to peripheral mucosal sites compared to mucosally induced IgA-producing B cells [23]. Also homing between different mucosal compartments is differentially regulated. IgA plasma cells induced in the gastro-intestinal tract are believed to disseminate to all mucosal sites by expressing integrin α4ß1 and α4ß7, whereas IgA plasma cells induced in the respiratory tract mainly migrate back to the respiratory tract (due to lack of expression of α4ß7) (discussed in chapter 6). That different antigen-specificity repertories exist between serum and saliva could implicate that protection against allergy by IgA would be possible when allergen-specific IgA would be present at the mucosal surface where the allergen is encountered. Indeed, the ratio of systemic IgE over IgA2 in saliva correlated better to clinical symptoms of house dust mite allergic patients, compared to the ratio of IgE over IgA2 in serum (Chapter 4). Next to the differences in antigen specificity between serum-IgA and mucosal secretions, production levels of IgA are much higher in mucosal than systemic tissues. In addition, IgA1 levels are higher in serum, whereas jejunum and especially colon IgA2 levels are higher [22, 24], indicating differential regulation of IgA1 and IgA2 production between systemic and local compartments. In conclusion, IgA1 and IgA2 production is differentially regulated in different tissues and different antigen-repertoires exist between different tissues, which could have functional implications for protection against infections as well as allergies.

Diagnostic *in vitro* assays and immunoglobulin isotypes

In vitro diagnostics ultimately aim at 1) discriminating allergic patients from healthy individuals, 2) predicting the severity of clinical symptoms and 3) identifying to what allergens a person is allergic to. As discussed in Chapter 1, current diagnostic procedures allow discrimination between sensitized and non-sensitized individuals, but poorly correlate with clinical status [25]. Allergen-specific IgE levels seem to have more predictive value for clinical status for inhalant allergens, compared to food-allergens.

To discriminate between the primary sensitizer and potential cross-reactive allergen inhibition assays are used. Serum is incubated with an allergen and subsequently the effect of pre-incubation is determined by measuring the reduction of binding to another allergen. The allergen resulting in most reduction in binding to another allergen is likely the allergen of primary sensitization.

Inhibition methods also can be applied to study if non-IgE immunoglobulin isotypes could reduce binding of IgE to allergens [26]. Specific IgE loaded on cells expressing the IgE receptor will bind to the epitopes of allergens. In case other immunoglobulin isotypes in solution recognize the same epitope, IgE-allergen binding could be inhibited. Inhibition also could occur when other isotypes recognize other parts of the protein, but by steric hindrance reduce the binding of IgE to the allergen [27].

The ImmunoCAP is widely used to determine allergen-specific IgE levels. The ImmunoCAP is also able to determine levels of allergen-specific IgG4 and IgA, but this is not often applied routinely. The CAPS containing the allergens was used to pre-incubate serum, followed by detection of remaining allergen-specific IgE using the default ImmnoCAP procedure (Chapter 3). This method is currently not available at ImmunoCAP, but may be suited to be performed automated after validation.

The basophil activation test (BAT) is increasingly used in experimental settings. The advantage of using basophils over the serological assays is the requirement of FcεR cross-linking to activate the basophils [28]. The test is relatively easy to perform. However, the test should be done relatively shortly after collection of the blood sample (eg preferably the same day) and the sample cannot be frozen for later analysis. IgE immobilized on the membrane of the cell is used, whereas IgE in solution is removed by washing. Like serological tests, the BAT is performed on a systemic sample, thereby omitting the influence of the site where the allergen is encountered.

As different isotypes and isotype subclasses have different effector functions, allergen-specific antibodies other than IgE are relevant in vivo. Allergen specific IgG4 levels are often used as readout in immunotherapy studies [29-31]. Allergen-specific IgG4 is able to prevent cross-linking of IgE immobilized on mast cells and basophils, and the low affinity IgE receptor (CD23) on B cells [15]. Even though allergen-specific IgA is increased locally after specific immunotherapy, there is no evidence that serum-IgA can block IgE-allergen cross-linking. Allergen specific IgA is found in several body fluids, including serum and saliva, but IgA-allergen epitope studies are rather limited. In a study where IgE, IgG4 and IgA epitopes to cow's milk allergy were determined, IgA bound allergens at different regions compared to IgE and IgG4 [32]. It needs to be established whether these results would have been different when mucosal IgA was used instead of serum IgA.

Regardless of the precise Ig epitopes, immunoglobulin isotype (and/or subclass) ratio may enhance the predictive value compared to evaluating allergen specific IgE levels alone. Preliminary data showed that salivary specific IgA2 in relation to serum specific IgE correlated with severity of symptoms upon challenge with intra-nasal house dust mite in HDM allergic patients (Chapter 4). In literature several studies describe that IgG4 can block interaction between allergen and IgE. In immunotherapy, the ratio IgG4 / IgE correlates with effective immunotherapy [33]. No differences in affinity of allergen specific IgE and IgG4 were observed between patients that did receive or did not receive birch pollen immunotherapy [34].

Results from diagnostic assays correlate better with clinical status of patients when the presence of blocking antibodies are taken into account (resulting in enhanced specificity) compared to assays that detect the concentration of allergen-specific IgE present in serum (high sensitivity but less specific). The facilitated antigen presentation (FAP)-assay, detecting binding of allergen-IgE to CD23 on B cells is sensitive for blocking antibodies that interfere with formation of IgE-allergen complexes. FAP by B cells to Th2 cells allows efficient Th2 responses with low doses of allergen, whereas after immunotherapy presence of allergen-specific IgG resulted in inhibition of FAP [15, 35]. In conclusion, several diagnostic assays that correlate better with clinical status of patients are available, but generally are more complicated compared to analyzing allergen-specific IgE levels only.

II MODULATION OF THE IMMUNE SYSTEM

Early life

The immune system of neonates is not fully mature at birth. This delayed immune maturation is influenced by breast feeding. IgG-allergen complexes as well as TGF-ß present in breast milk have been identified as crucial for inducing tolerance to allergy development in neonates [36, 37]. Modulation of the neonatal immune system may have consequences later in life.

After birth or hatch (in avians), IgA-producing B cells are lacking in the intestines. Upon colonization with bacteria, IgA production is initiated in mice [38]. Similar patterns were observed in chickens after hatch. After birth or hatch the availability of maternally derived immunoglobulins disappears; whereas the neonatal immune system isn't mature yet (Chapter 7). In this period we observed a transitionally changed composition of microbiota, which was related to transitionally increased cytokine production, and to the level of IgA. During this period, cytokine production and IgG production was increased. This period could be a time window crucial for maturation of the neonatal immune system with life long lasting consequences.

In humans, the ratio between the IgA subclasses was reported to change with age [39]. Numbers of IgA1 producing B cells remained similar in jejunum, whereas with age, numbers of IgA2 producing B cells increased. As we hypothesize that IgA2 may be mainly induced by innate factors, this increase in IgA2 could either reflect exposure to bacterial antigens in the mucosa, or be derived from initially IgA1 B cells that switch sequentially to IgA2. It remains to be established whether those IgA1 and IgA2 B cells vary in antigen specificity and whether class switch recombination to either subclass are equally likely. The association observed between both the presence of specific bacterial strains in the intestine of infants and increased concentrations of endotoxin in the environment of children, and increased protection against allergic symptoms like eczema, could be partly mediated by the intestinal IgA response [40, 41].

Potential routes for immunomodulation

Modulation of the immune system by dietary components can in theory result in increased or decreased activation of the immune system. The vast majority of antigenic properties of food are eliminated by digestive enzymes that reduce most of the proteins into peptides too small for MHC II presentation. Still, intact or partly intact (thus still functional) ingredients may come into contact with the immune system and modulate its' response, especially in young infants.

Dietary ingredients can have a direct effect on proliferation or induction of apoptosis of immune cells. Another possibility by which dietary ingredients can modulate the innate immune system is by activation of intracellular signaling pathways resulting in for instance up-regulation or down-regulation of T cell co-stimulatory membrane proteins and induction or inhibition of production of T cell activating cytokines.

Food components (e.g. pre-biotics) can affect composition of the intestinal microbiota and therefore

indirectly affect the host's immune system [42]. Next to proteins, oligosaccharides may contribute to induction of allergen specific T regulatory cells that are able to prevent allergic symptoms in whey sensitized mice [43]. ß-glucans from varying sources (oat, barley, mushrooms) have been shown to be bound by dectin-recepors and can induce pro-inflammatory cytokine production by macrophages or macrophage-like cells [44, 45]. Another, indirect effect of food components could be by affecting other physiological conditions like the epithelial barrier. Vitamins D and A for instance, have significant impact on the host immune system as those factors are involved in regulatory immune responses (Chapter 5) [46-49].

The majority of food proteins is (partly) digested by enzymes in the gastro-intestinal tract and therefore presented as peptides to the innate immune system. Short peptides (eg hexa-peptides) are too small to be processed and presented on MHC, but still could exert bioactivity on the human immune system. For instance various short peptide sequences from casein have been shown to have various immunomodulatory effects on the human immune system [50].

Immunomodulation by dietary ingredients

Many pattern recognition receptors involved in recognition of pathogens have been identified [51]. Also some food proteins can be bound by those innate immune receptors, but data available is limited. Glycosylated proteins can be taken up by dendritic cells via the mannose receptor [52]. Involvement of receptors that recognize structures from microbes or food result in efficient phagocytosis. Immunoglobulin (IgG) can serve as soluble receptor that forms immune-complexes which are efficiently phagocytosed via IgG receptors on APC. However, many other interactions with yet unidentified extracellular and intracellular receptors may be involved as well. Also a-specific non-receptor mediated uptake (pinocytosis) could account for the uptake of food-derived fragments by APC [53]. Upon intracellular processing by professional APCs, peptides are presented on the MHC II class and co-stimulatory molecules can be up regulated next to cytokine secretion. These factors together determine if and how the Th cell becomes activated. Th cell activation requires matching specificity of the antigen peptide-MHC complex and the TCR.

Dairy is an interesting part of our diet as it is both widely consumed, can modulate the human immune system, and allergies against proteins present in cow's milk can occur. Recently, promising studies have become available showing that consumption of untreated cows (farm) milk is associated with protection against allergy later in life [54]. This effect could be abrogated when heat processed milk was consumed [55].

Several mechanisms could be involved in the protective effect of farm milk consumption. Immunomodulation may occur via major fraction present in cow's milk; like lactose, phospholipids or major proteins like caseins, α-lactalbumin, ß-lactoglobulin or lactoferrin. Diet directly or indirectly affects the immune system via the microbiota. Dairy products contain a number of immune proteins and molecules like vitamins that can exert bioactivity on the human immune system, which recently has been reviewed by Van Neerven et. al. [56].

Otherwise, bovine milk contains high levels of immunoglobulins that might be involved in inducing tolerance after consumption. In an elegant mouse study, IgG-allergen complexes were involved in inducing tolerance in the offspring [36]. Next to this, cow's milk contains cross-reactive proteins like IL-10 and TGF-ß (Chapter 8) [57]. Bovine IL-10 binds to the human receptor [57], and bovine TGF-ß (1 and 2) in its bio-active form is identical to human TGF-ß [56]. Especially TGF-ß is present in sufficient amounts (several ng/ml) and could pass the acidic conditions in the stomach, especially in infants. Therefore, dietary immunomodulation that depends on bio-active immune proteins may provide opportunities especially in infants.

Bovine IgG does bind to FcγRI and II on human monocytes and dendritic cells (Chapter 9). This is an interesting observation as IgG allergen com-

plexes have been shown to be involved in neonatal tolerance induction in a mouse model [36]. Pathogen- and allergen-specific IgG has been identified in bovine milk, and has immunomodulatory capacity (Chapter 9) [58, 59].

III TOLERANCE

Both central and peripheral tolerance is crucial to prevent auto-immune disease, allergy and massive inflammation of the gastro-intestinal tract. Tolerance in this paragraph refers to peripheral tolerance, not induced in the thymus but mainly by the mucosal tissues. Induction of tolerance prevents inflammation upon encounter of dietary antigens or intestinal bacteria and is a key feature of mucosal tissue. Tolerance induction can occur by administration of low or high doses of antigen. High doses of antigen result in unresponsiveness, anergy or clonal deletion [60], whereas low doses of antigen cause induction of active regulation by T regs [61]. Interestingly, tolerance to food that is induced in the ileum results in systemic tolerance, whereas tolerance to bacteria induced in the colon does not result in systemic tolerance [62]. The term oral tolerance therefore refers to tolerance to soluble dietary proteins rather than tolerance to the intestinal microbiota. Oral tolerance is induced in the intestinal mucosal tissue. Below the mechanism of induction of this peripheral tolerance is discussed.

Supernatants of epithelial cells are able to induce tolerogenic DC's in vitro. One of the phenotypic differences of tolerogenic DC's is expression of the integrin αIEL (CD103) and the absence of CX3CR1. $CD103^{+}CX3CR1^{-}$ and $CD103^{-}Cx3CR1^{+}$ DCs seem to originate from a separate lineage [63, 64]. Amongst the feature of this subset are, expression of TLR2 but lack of TLR4 expression, active pinocytosis of dextran-FITC while they did not express the mannose receptor and production of IL-12 [65]. $CD103^{+}$ DCs like other DC subsets are relatively unresponsive to LPS (Chapter 5) [66]. Those tolerogenic DCs occur in both the intestinal as the respiratory mucosa [63, 65, 67, 68].

Compared to $CX3CR1^{+}$ DCs, mouse bone marrow-derived resting $CD103^{+}$ DCs have higher basal expression levels of costimulatory molecules CD80, CD86 and CD83, but similar levels were observed when CX3CR1 DCs were stimulation with LPS [69]. Compared to CD103 DCs, CX3CR1 DCs were more active in phagocytosis and expression of scavenger receptors. Also expression of almost all TLRs and production of IL-6 and TNF-α, but not IL-10 and IL-12 was elevated in CD103- DC upon stimulation.

A crucial feature of tolerogenic DCs is the ability to induce Tregs. Mucosal induction of Tregs is essential for induction of tolerance to food and other harmless antigens. $CD103^{+}$ DCs and epithelial cells have the capability to convert retinal to retinoic acid (RA) by the NALDH enzyme. This induction of Tregs by $CD103^{+}$ DCs depend on RA and TGF-ß production by mucosal tolerogenic DCs [18].

Several Treg subsets have been described: Th3, Tr1 and $CD4^{+}CD25^{+}$. Tregs can be naturally occurring and induced in response to specific antigens ($CD4^{+}CD25^{+}$). Tolerogenic DCs have the ability to induce antigen-specific FoxP3 expressing regulatory T cells against non-self-antigens.

Allergen specific Tregs are induced upon successful allergen-specific immunotherapy [17, 29]. Those Tregs can produce IL-10 that inhibits IgE production and enhances IgG4 production. IgG4 is able to compete with IgE for allergen binding places, thereby preventing crosslinking of IgE immobilized on mast cells and basophils. Those Tregs also inhibit DC-mediated Th(2) cell activation.

Next to T regulatory cell mediated tolerance, IgA production is involved in maintaining homeostasis by preventing translocation of microbiota over the gut epithelium [70, 71]. Likewise, allergen-specific IgA may prevent allergens to bind to IgE [72, 73].

Recently several regulatory B cell subset (Breg) has been identified, differentiated by expression of combination of markers like CD1d, CD24, CD38 and CD27 [74]. Different subsets of Bregs have the

ability to suppress T cells and APCs and seem to contribute to protection against different immune-mediated diseases like allergy, multiple sclerosis and Systemic lupus erythematosus [75]. It needs to be established if Bregs can produce antibodies, and whether Bregs are a stable subset or derived from a particular differentiation stage of B cells.

Can tolerogenic DCs protect against allergy?

Tolerogenic DCs-mediated tolerance may occur via the induction of Tregs, via non-inflammatory cytokine production or unresponsiveness to antigens. Those DCs may also induce protective IgA production.

TSLP produced by epithelial cells is bound by DCs that in turn enhance Th2 responses. This Th2 immunity can both support tolerogenic Th2 immune responses, and Th2 mediated allergy [76, 77]. TSLP also was found to enhance CSR by increasing BAFF production by DCs [78].

Goblet cells transfer antigen from the lumen to $CD11c^+$ DC's, which are often CD103+ as well [79]. $CD103^+$ DCs have been identified as potent antigen presenters and inducers of Tregs by producing TGF-ß and RA [18, 67]. Under non-inflammatory conditions, the antigens transferred by goblet cells to $CD103^+$ DCs may induce specific regulatory T cells [79]. This mechanism may potently induce Treg mediated tolerance to proteins from food and thereby also prevent allergy. This is consistent with the expression of the high affinity IgG receptor (CD64) that binds to IgG bound to soluble antigen (Chapter 5). $CCR7^+$ $CD103^+$ DCs have been identified in the colon-associated MLN [68]. Those DCs are believed to originate from the intestinal mucosa where those cells could have bound antigens followed by migration to draining lymph nodes. The recent finding that goblet cells transfer antigen to CD103 DCs, combined with the finding that CD103 DCs efficiently induce Tregs could be a key mechanism in mucosal immune regulation.

DCs have been shown to induce mucosal IgA production [80-82]. However the precise subset accountable for DC induced IgA production needs to be identified. Tolergenic $CD103^+$ DCs are a likely candidate, for their ability to produce TGF-ß, RA and IL-10. Also Tregs induced by CD103 DCs may be involved in IgA induction (discussed below).

In vitro we established a monocyte-derived CD103 expressing DC culture. Those DCs were co-cultured with B cells to study the effect of those DCs on IgA production. The plasma cell marker CD38 was highly expressed in the presence of RA-induced CD103 DCs (Chapter 6). In addition, $CD103^+$ DCs seem to enhance IgA2 production, probably due to production of TGF-ß and RA (Chapter 6).

IV MUCOSAL IGA IN RELATION TO ALLERGY

The mucosal immune system harbours a number of unique features to both tolerate harmless antigens and protect against infections. Typically, high levels of IgA are being produced, many tolerogenic APCs are present and Tregs are being induced mucosally. IgA(1) may also leak to systemic fluids, but also can be produced systemic, employing different mechanism compared to mucosally induced IgA in both human and mice (Chapter 6) [80]. In this paragraph the potential role of the mucosal immune system is discussed in relation to IgA-mediated protection against allergy.

Structure and distribution of IgA1 and IgA2

Structurally IgA1 and IgA2 mainly differ in the hinge region connecting the Fab and Fc fragments [83]. Both subclasses bind to the FcαR (CD89) with similar affinity with a region conserved in both IgA1 and IgA2, and IgA bound by FcαR on interstitial DC induces production of the non-inflammatory cytokine IL-10 [84, 85]. The hinge region of IgA2 is shorter compared IgA1, which protects against proteolysis by bacterial enzymes.

IgA2-B cells are particularly abundant in the colon and rectum, which has the highest bacterial load of the gastro-intestinal tract. In serum and ileum,

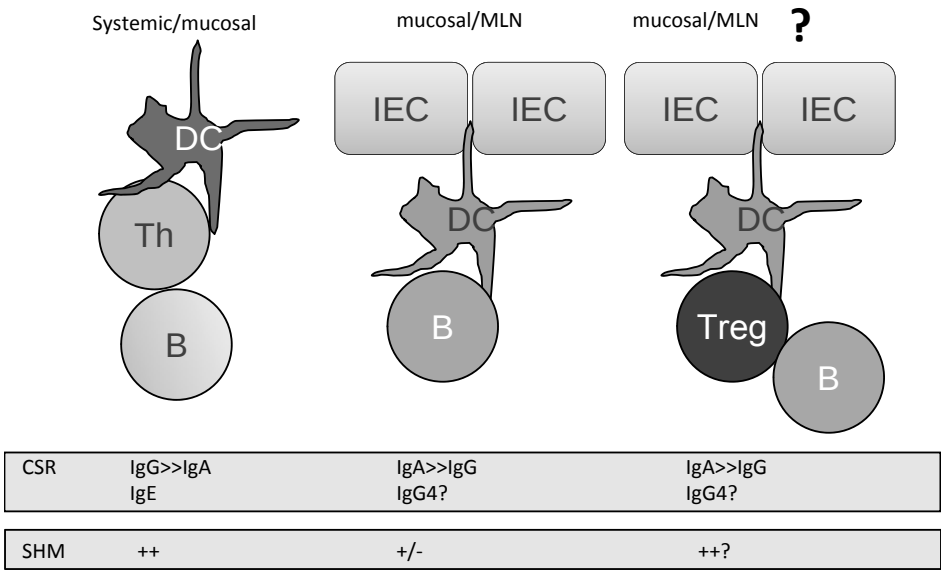

Figure 3. *Ig isotype switching can occur via different routes, both systemically and mucosal and result in different isotypes and degrees of somatic hypermutation (SHM). The most widely studied route is depicted at the left. In the mucosa DCs can induce production of mainly IgA. The role of Tregs in isotype switching is incompletely understood. Tregs may preferentially induce IgA and be is potent as Th cells in inducing SHM.*

IgA1 levels are higher than IgA2 levels, whereas levels in jejunum are relatively similar [24]. In the respiratory tract (tonsil and lungs) IgA1 B cells are more abundant than IgA2 B cells [86]. Also exposure to allergens is different along the gastrointestinal tract as well as other mucosal surfaces. The combination of differential distribution of IgA subclasses and different exposure to allergens on different mucosal regions may have important consequences in relation to a possible role of IgA in protection against allergy.

Differential regulation of IgA1 and IgA2 production

The observation that IgA1 and IgA2 may play different roles in protection against allergy (Chapter 4 and 6) [73] raised the question whether IgA subclasses are induced under the influence of different stimuli. Literature reporting factors that induce IgA CSR is abundant [80, 87-89], whereas literature discriminating IgA1 and IgA2 is limited to a few reports only [19, 90].

CSR occurs both in systemic immune organs and in the mucosa, and in the presence or absence of Th cells (Figure 3). IgA CSR and production can be induced by both T cell dependent and T cell independent stimuli. Activation of CD40 on B cells through ligation with CD40 ligand on T cells leads to TGF-ß1 production which in turn is involved in IgA class switch recombination (CSR) [91]. When B cells were stimulated with irradiated $CD40L^+$ cells only, IgA1 germline and productive IgA1 expression was higher compared to IgA2 [91]. This difference was not visible when exogenous TGF-ß1 was added. Addition of IL-10 enhanced IgA production, but was not crucial under those CD40 dependent conditions. Addition of anti TGF-ß1 antibodies completely inhibited IgA (IgA1 and IgA2 not specified) production and CSR [91].

The role of IL-10 in inducing IgA production and CSR also has been studied under T-cell independent conditions. When instead of CD40L, the cytokines APRIL and IL-10 were added to IgD^+ B cells, IgA2 expression on the membrane and secretion of IgA2 was higher compared to IgA1 [19]. IgA2 secretion and CSR was further enhanced

when flagellin was added. Consistent with our findings (Chapter 6) APRIL and IL-10 resulted in more IgA2 production compared to BAFF and IL-10.

IgA1 production is more easily induced under the influence of CD40 activation compared to IgA2 [19]. IgA2 production and CSR however, seems to be more readily induced under the influence of the T cell independent cytokine APRIL, but not BAFF. BAFF might be more important in regulating plasma cell survival compared to APRIL [92].

Work in mouse models has revealed that both the T cell-dependent and T cell-independent route are involved in IgA induction [93]. The T cell independent route might be especially relevant for inducing natural antibodies (limited SHM, figure 3) that bind to intestinal bacteria, either commensals or pathogens. In our work in chickens, we observed limited changes occurring in the CDR3 region of IgA, together with 70% coated intestinal bacteria (Chapter 7).

Mucosal network favouring IgA induction

The precise location and local environment crucial for induction of IgA production are currently being identified. IgA production depends on a number of factors that are typically present abundantly in the mucosa. Those factors include different cell types and accompanying cytokines and other molecules (summarized in Figure 4).

The list of factors produced by intestinal epithelial cells is growing, of which several can bind to receptors on B cells, like RA, APRIL and BAFF, in addition to cytokines TGF-ß and IL-10. These factors promote IgA1 and IgA2 production either directly or via DCs. IEC produce APRIL, which has been identified as IgA2 switch factor, in combination with IL-10 and TLR5 ligation (e.g. by flagellin) [19]. Also the plasma cell inducing factor RA (Chapter 6) is being synthesized by IEC and DC, which also promotes accessibility of the IgA region on the genome, potentially enhancing IgA CSR [94]. RA also affects homing to the intestine of different cells, including B cells by inducing α4ß7 and CCR9 [95, 96]. Production of BAFF by IEC could promote plasma cell survival that may lead to prolonged production of IgA. IgA CSR is potently induced by the presence of TGF-ß [97, 98] and enhanced by IL-10 (Chapter 6). Both cytokines are produced by IECs [99]. Those factors produced by IEC are also being produced by mucosal DCs but could also be diet-derived (e.g. breast milk or cow's milk) [18]. However, it has not been identified if those factors are differentially produced by DC subsets.

Currently, it is debated in the literature to what extent organized lymphoid structures are required for intestinal T cell-independent IgA CSR [93]. Probably, this debate is partly caused by differences between human and mice. In general, B cells differ between human and mice, especially with regard to immunoglobulin subclasses. Mice produce a single IgA subclass, whereas humans produced IgA1 and IgA2. We have evidence (Chapter 6) that those IgA subclasses are differentially regulated in humans (discussed above). Consequently, the conclusion that T cell-independent IgA2 CSR occurs in the lamina propria may be possible in humans because it occurs independently of T cell help [19]. This is in line with mouse studies showing a crucial role for CD28 in systemic IgA responses, but not in mucosal IgA responses [100]. In mice, IgA production is largely maintained in the absence of germinal centres, but in mice lacking any organized lymphoid structures IgA production was abrogated [93, 101].

Recently, neutrophils have been related to induction of IgA production in the marginal zone of spleen [102]. Marginal zone neutrophils were activated (non-inflammatory) upon intestinal colonization. In vitro co-culturing of marginal zone neutrophils and B cells resulted in production of BAFF, APRIL and IL-21 by neutrophils, which was involved in IgA production. In needs to be established to what extent this mechanism contributes to IgA-production in vivo [103].

Mucosal factors associated with eczema

A number of factors produced in the mucosa have been associated with presence or absence of aller-

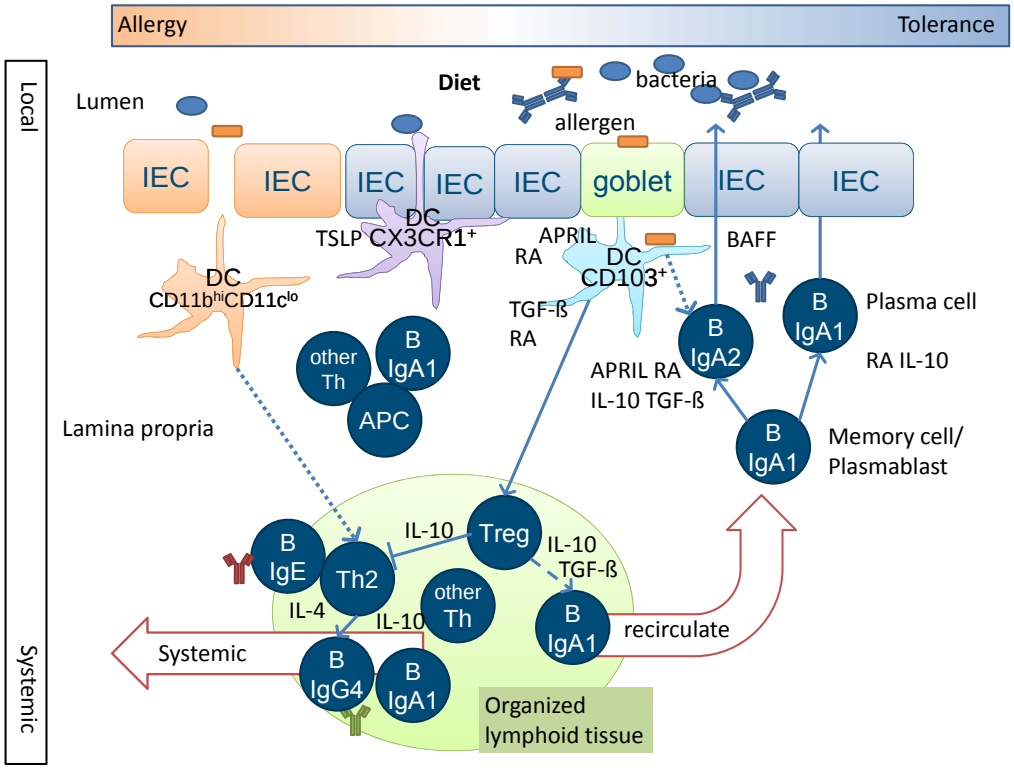

Figure 4. *Local and systemic parts of mucosal B cell responses in allergy and tolerance to allergens. Different DC subsets induce different Th cell responses and downstream B cell Ig production. Activated cells do (solid line) or may (dashed line) migrate to lymph nodes. Depending on the local signals, cells may become disseminated systemically or recirculate to mucosal tissue. In the mucosa, B cells can terminally differentiate into plasma cells or undergo (sequential) switching to IgA(2). Mucosal immunotherapy targets DCs that locally may enhance Treg and B cell IgA induction.*

gic symptoms like eczema. Children having toxic Staphylococcus aureus in the intestine produce increased levels of APRIL and IgA and developed less often eczema later in life [104]. Also an association was found between the composition of intestinal microbiota and IgA production during ontogeny (Chapter 7). In addition, levels of APRIL are reduced in skin biopsies of individuals with allergic eczema, whereas levels of BAFF and TSLP are increased. Currently the exact mechanism behind development of atopic eczema has not been elucidated, but the factors depicted in figure 5, provide a possible model that may explain the association of eczema with decreased levels of APRIL and IgA and increased levels of TSLP. Reduced levels of APRIL and increased levels of TSLP and BAFF may be the result of aberrant immune responses in the tissue, or may be causative for the development of eczema in allergic patients [104-106]. The notion that the presence of specific bacteria early in life (4 months of age) was associated with a reduced frequency of eczema later in life (18 months of age) suggests that those factors may be causally related to eczema development. In our adult house dust mite allergic patients, presence of eczema was associated with reduced levels of allergen specific IgA2 (Chapter 4). This association was even stronger for patients suffering from both eczema and asthma, though this needs to be confirmed in a larger study. IgA2 production is induced by factors like APRIL and inhibited by TSLP (Chapter 6). The reduced levels of serum IgA2 in patients

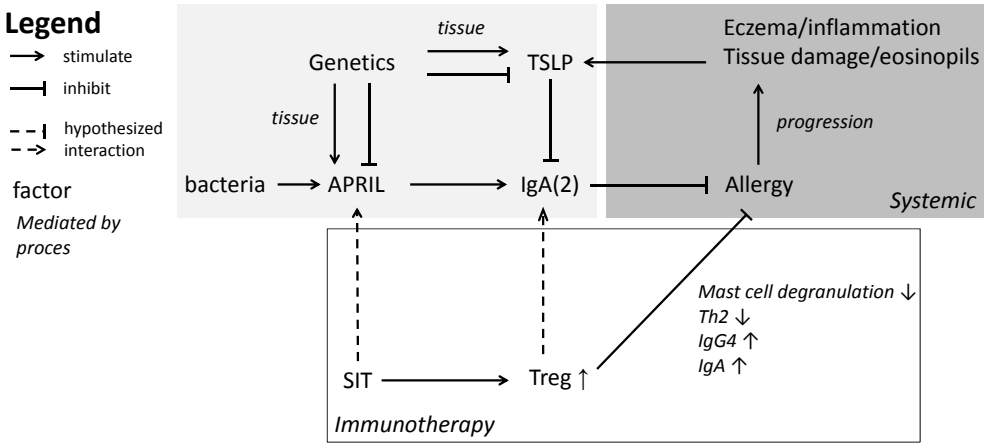

Figure 5. *Hypothetical model linking IgA levels with allergic eczema and its associated decreased levels of APRIL and increased levels of TSLP. Bacterial exposure in early life may prevent allergic sensitization and development of eczema and/or asthma later in life. Immunotherapy increases production of IgA, and inhibits development of allergic symptoms. Those interactions may me mediated by the factors APRIL and TSLP.*

with eczema might be caused by the reduced levels of APRIL and increased levels of TSLP (Figure 5). Alternatively, allergic patients have reduced levels of mucosal (salivary) IgA. Reduced IgA-mediated mucosal protection upon allergen encounter may enhance Th2 mediated eosinophilia and IgE class switching [107] and thereby contribute to development of eczema.

The role of Tregs in IgA induction

Tregs are essential to control inflammatory responses, including production of cytophilic immunoglobulins. Interestingly, regulatory cytokines IL-10 and TGF-ß do not inhibit but rather enhance IgA production. IgA is a non-inflammatory isotype as it does not activate complement and binding of IgA to myeloid cells induces IL-10 production. As Tregs are imported sources of those regulatory cytokines [17], IgA production might be enhanced by Tregs. In addition to producing cytokines, Tregs express co-stimulatory molecules as found on Th cells [108, 109]. Taken together, Tregs are likely potent inducers of IgA production. Surprisingly little reports are available studying the role of Tregs in IgA induction [26, 110]. In vitro culturing of B cells and Tregs showed that Treg blocked IgE production, enhanced IgG4 production, but no evidence was found for changes in IgA production in the presence of Tregs (identified as $CD4^+CD25^+$, while FoxP3 expression was not determined) [26]. However, a profound role for Tregs was found in a mouse study, where IgA production was limited when mice were treated with an anti CD25 antibody [110]. IgA production was restored upon adoptive transfer of Tregs.

Mechanism of IgA2 mediated protection against allergy: a hypothesis

In a mouse model T cells were able to induce significantly more mutations in the complementarity determining region (CDR) of IgA, which determines antigen specificity, compared to T cell-independent induced B cells [93]. It also was observed that T cell-independent signals mainly induce IgA2 CSR. As a result, IgA2 could be of lower affinity when its induction would solely depend on TI activation. However, sequential switching from IgG1 and IgA1 to IgA2 has been reported [19]. IgA1 and IgG1 B cells are most likely to switch to those respective isotypes under the influence of T cells. B cells sequentially switching from affinity matured IgG1 and IgA1 to IgA2 under the influence of APRIL could therefore still contain high numbers of substitutions in the CDR [19]. There

is no evidence that IgE B cells could undergo CSR to IgA2, and this would be hard to prove in vivo due to the very low frequency of IgE-producing B cells. However, the observation that IgG1 B cells T cell-independently switch to IgA2 and that IgA2 correlated with protection in our house dust mite patient data suggests that allergen-specific IgE-producing B cells that reside locally in the mucosa may switch further to IgA2 under T cell-independent conditions. The potential protective role of mucosal IgA has been suggested in the seventies of the 20th century, and was found to be associated with eczema in infants [111, 112]. However, the protective role of IgA against the development of allergy and clinical symptoms upon allergen exposure has been controversial. This might be largely due to the fact that IgA has often been determined in serum, and that IgA combined levels were determined rather than IgA1 and IgA2 allergen-specific levels. Indeed, increased numbers of allergen-specific IgA2, but not IgA1, B cells were present in the nasal mucosa after birch pollen immunotherapy [72]. In addition, there is evidence that pointing to CSR locally in the nasal mucosa and bronchia [113-115]. It needs to be noted that the T cell-independent IgE CSR cytokine APRIL inhibits Th2 responses and production of Th2-dependent immunoglobulins [116]. Therefore increased levels of IgA2 induced by APRIL and IL-10 may correlate with protection against allergy because it is confounded by a simultaneous inhibition of the Th2 compartment. The finding that allergen-specific IgA2-producing B cells have been identified after immunotherapy indicates that IgA2 may indeed exert a functional role in the mucosa by protecting against allergy. In addition, IgA2 levels that seem to be involved in protection against allergy also were associated with progression of allergic disease in HDM-allergic patients (Chapter 4).

IV CONCLUSIONS AND FUTURE DIRECTIONS: MUCOSAL IGA IN RELATION TO ALLERGY

The mucosa harbours a complex immune system that is able to both induce tolerance to dietary ingredients and commensal bacteria, and to mount protective immune responses to pathogens. Dietary ingredients are not only tolerated by the immune system, but also may modulate the immune system. In case of lack of tolerance, diseases like allergy could develop. Mechanisms of immunomodulation by dietary ingredients are only started to be understood. Further understanding of the relation between dietary ingredients and mucosal immune responses may provide opportunities to prevent development of allergy and help maintaining intestinal homeostasis. Intestinal homeostasis requires both tolerance and the ability to recognise potential harmful bacteria, viruses and parasites. Our current understanding of homing of lymphocytes through the body, indicate possibilities to also modulate the respiratory immune system via the intestinal mucosa. This is facilitated by induction of homing receptors on B cells and T cells. Likely, dietary immunomodulation largely depends on DC responses. DCs determine the fate of Th cells by modulation of expression of cell surface molecules and secretion of cytokines. How these cell surface molecules and cytokines are modulated depends among others on the interacting DC subset. The development and responsiveness of DC subsets is in turn affected by the local environment. This local environment consists of several cell types, of which the importance of epithelial cells is increasingly recognised. In addition, the diet influences the local environment and might directly affect cell-responsiveness when dietary ingredients bind directly to receptors on the cell membrane. Dairy products are anticipated to be potent modulators of the human immune system as bovine milk contains cytokines and immunoglobulins that can bind to the human receptors. In addition the vitamins A and D support induction of regulatory tolerance. Bioactivity of immune-reactive ingredients from bovine milk, depend on

Key messages

- Levels of mucosal IgA2, but not IgA1, may be a relevant parameter in the diagnosis of allergy.
- Specific serum-IgA2 levels, but not IgA1 levels, are decreased in patients with eczema, and IgA2 production is affected by the eczema-related factors APRIL and TSLP.
- Detailed IgE-epitope comparison explains limited sensitization of shellfish allergic patients to molluscan allergens.
- Dietary factors may influence mucosal IgA production.

Key questions

- Does allergen-specific mucosal IgA2 protect against sensitization to allergens and is induction of allergen-specific IgA2 a useful target for immunotherapy?
- Does induction of protective IgA in allergy depend on T cells or not, and what is the role of intestinal microbiota on IgA specificity?
- Can IgE-producing allergen-specific B cells in the mucosa switch to IgA2 and could this occur upon local encounter of allergens?
- Are reduced levels of APRIL and increased levels of TSLP causally related to the development of eczema and asthma?

proper processing to prevent degradation resulting in loss of immune reactivity. This could also be the reason that consumption of raw but not heated farm milk early in life is associated with reduced allergic symptoms later in life.

Reviewing the literature about B cells and B cell responses in relation to allergy, a number of issues arise that need further research. The importance of the location of allergen encounter in relation to the locally present immunoglobulin isotypes and subclasses is still underestimated. Serological levels of allergen-specific IgA are most likely inaccurate to estimate the protective role IgA could have against allergy. In addition, IgA subclass levels differ in relation to the clinical status of the patients. Therefore, allergen-specific detection of mucosally present IgA subclasses could provide opportunities for finding a better association between Ig levels and clinical status of patients.

Mechanisms of T cell-independent induction of IgA are currently being identified [19, 80, 102]. These mechanisms seem to be relatively similar between intestinal and the nasal respiratory mucosa. In both cases, epithelial cells produced APRIL and/or BAFF, and TGF-ß1 and RA, and have DCs in place producing cytokines favouring IgA induction. Also DC responses in relation to allergy are increasingly becoming elucidated [76, 117-119]. However, data linking DC-dependent induction of IgA in relation to allergy is lacking. Studying DC- dependent mucosal IgA induction in relation to allergy is important, as the specificity of B cells undergoing DC-mediated but Th cell independent IgA CSR might well be different compared to Th or Treg-dependent IgA CSR. The specificity of de novo induced IgA is relevant as mucosal protection against allergy is likely more effective when the IgA produced is allergen-specific. In addition, understanding of mucosal IgA CSR in relation to allergy is important to be able to more efficiently develop strategies that enhance induction of IgA protective against allergy. Until now it is not clear to what extent TI IgA-CSR contributes to mucosal IgA production in humans, but innate cells like ep-

ithelial cells and DCs are likely major contributors to in vivo IgA production in both the intestinal and respiratory mucosa [120]. An interesting question is to what extent T cell-independent IgA production is able to prevent development of allergy and clinical symptoms upon allergen exposure, and to what extent allergen-specific T cell help is required. The relation between allergen-specific IgA and the clinical status of allergic patients may well be different between the different allergies (e.g. food and inhalation). Though the immune system and the luminal content greatly vary between the respiratory and intestinal tract, the general principle of protective recognition of IgA by allergens may be consistent.

Mechanistic parameters to assess the success of immunotherapy include IgE and IgG4 formation, and the presence of allergen specific Tregs. Peripheral induction of Tregs upon mucosal immunotherapy depends on DC responses. Those DCs also affect Th cell reactivity and DCs can induce IgA production. Also Tregs likely enhance production of IgA. In addition Tregs might be able to also induce somatic hypermutation (in contrast to DC-mediated IgA induction) and thereby allow induction of high affinity allergen specific IgA in the mucosa. Until now, these mechanisms are rarely considered as part of the success of immunotherapy. Clinical improvement as the result of immunotherapy is associated with IgE-IgG4 ratios. In addition, induction of mucosal IgA production upon mucosal (e.g. sublingual immunotherapy) needs further investigation (Figure 5). Allergen-specific IgA at mucosal surfaces can bind the allergen and thereby prevent formation of IgE-allergen complexes (Chapter 4, figure 5). Allergen-specific IgG4 induced after immunotherapy can block formation of IgE-allergen complexes systemically. To our current understanding, mucosal protection against allergy depends on IgA2 rather than IgA1. In addition, allergen-specific mucosal IgA may prevent progression of allergic status into eczema and asthma. Though allergen-specific IgA2-producing B cells could be induced by immunotherapy, it is unclear from what type of B cells those cells originate and through what mechanism these IgA2 B cells are induced.

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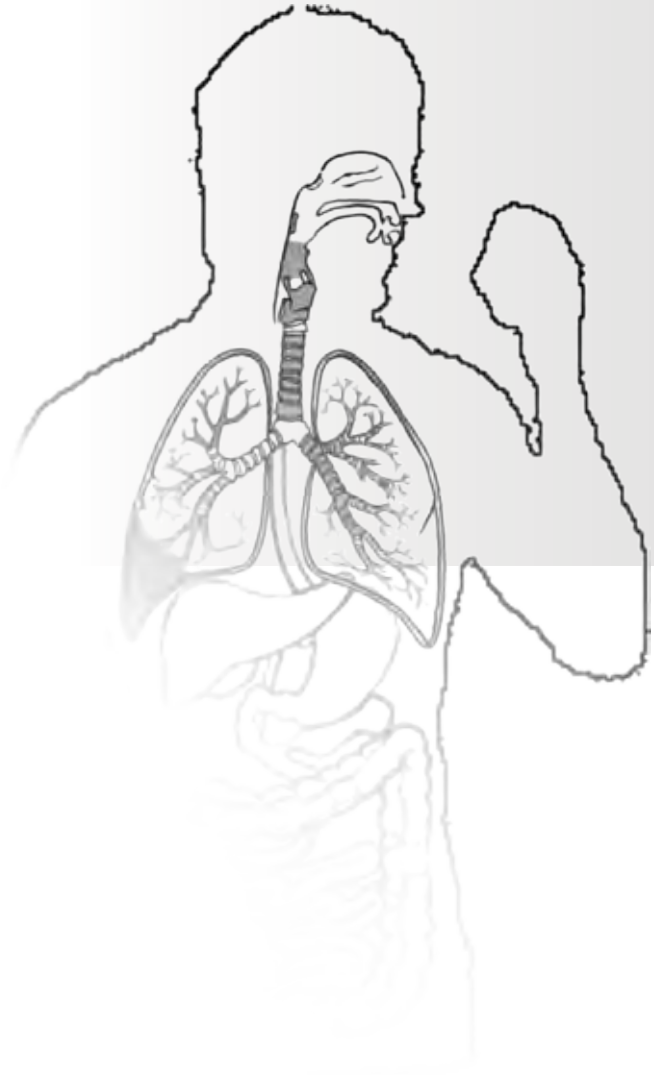

chapter 11

Miscelleneous

English summary
Nederlandse samenvatting (Dutch summary)
Acknowledgments
Curriculum Vitae
Publications
Education certificate

Summary

Type I allergy is mediated by allergen-specific IgE antibodies that are produced by B cells upon stimulation by T- helper type 2 cells. Immunoglobulins have varying antigen- and allergen specificities (introduced in **chapter 1**). IgE needs to be specific for an allergen and bind simultaneously to distinct epitopes on the allergen to induce degranulation by cross-linking of IgE immobilized on high affinity IgE receptors expressed on mast cells and basophils (**Chapter 2**). IgE molecules can be specific for a protein of a single species or bind to similar regions on allergens from different species, thereby causing cross-reactivity. This is discussed for shrimp and mussel tropomyosin in chapter 2, and fig and ficus in **chapter 3**. In most cases of cross-reactivity the primary sensitizing allergen can be identified by inhibition assays (**Chapter 3**).

The presence of allergen-specific IgE in serum of sensitized patients does not necessarily cause clinically relevant symptoms of allergy. For example, when blocking immunoglobulins of non-IgE isotypes are present, like IgG4 which is induced by specific immunotherapy, IgE-mediated effector functions can be inhibited. Until now, serum-derived IgA has not been shown to be able to block IgE-allergen interactions, and a protective role of allergen-specific IgA is still debated. The aim of this thesis was to study how the mucosal immune system and especially the induction of allergen-specific IgA could protect against allergy.

Compared to non-house dust mite allergic controls, patients with house dust mite allergy had decreased levels of allergen-specific IgA2, but not IgA1 (**Chapter 4**). The mechanisms underlying the differential regulation of the two IgA subclasses IgA1 and IgA2 in humans are largely unknown. Factors in the local mucosal tissue are involved in differentiating between IgA1 and IgA2 production. Evidence for a role of dendritic cells (DC) in IgA production is increasing. We generated and characterized two DC types not previously described in literature by using the mucosal factors retinoic acid (RA) and transforming growth factor (TGF)-ß (**Chapter 5**). Those RA- and TGF-ß-derived dendritic cells have tolerogenic characteristics, as shown by reduced inflammatory cytokine production (IL-12 and TNF-α), but non-significant reduction in IL-10 production, and reduced expression of activation- and maturation markers after stimulation with bacterial ligands.

To study the role of epithelial cell- and DC-derived molecules in the regulation of production of IgA by B cells and plasma cells, the mucosa-related cytokines APRIL, BAFF, IL-10, TGF-ß, and vitamin A-derived RA were added to B cells. Addition of RA resulted in differentiation of B cells into plasma cells ($CD38^+$) and enhanced secretion of IgA1, and enhanced secretion of IgA2 when also IL-10 and APRIL or BAFF was present (**Chapter 6**). Our data indicate that production of IgA1 is increased in the presence of BAFF (probably by plasma cells), whereas IgA2 production is increased in the presence of APRIL which is produced by either DCs or epithelial cells in vivo (probably by *de-novo* induction). When RA and IL-10 were added, B cells upregulated homing integrin ß7 on the membrane, which is believed to preferably direct homing to the small intestine. In literature, increased levels of APRIL have been associated with decreased prevalence of eczema, whereas increased levels of TSLP have been associated with the presence of eczema. In **chapter 6**, it is shown that APRIL is involved

in induction of IgA2, and that TSLP potently inhibits IgA1, and to a lesser extent also IgA2 production, which is consistent with reports showing that increased levels of IgA inversely correlate with eczema. In **chapter 4**, we described that indeed an association between reduced levels of allergen-specific IgA2 in serum and presence of eczema and asthma can be demonstrated. Also the tissue (systemic, e.g. serum versus mucosal, e.g. saliva) where IgA is measured is differentially linked to the clinical status in house dust mite-allergic patients. Interestingly, whereas allergen-specific levels of IgA2 appeared to be associated with absence of allergy, IgA1 levels were not. In house dust mite-allergic patients the ratio between serum-IgE and saliva-IgA2 was associated with the severity of local responses to a nasal allergen challenge.

IgA production is decreased in the absence of intestinal bacteria, and colonization of the gut induces production of IgA. The ontogeny of the bacteria-specific repertoire of intestinal IgA in relation to the composition of the intestinal microbiota has not been studied before. In chickens we analysed the CDR3-repertoire development in the first ten weeks post hatch. No association between bacterial composition and IgA CDR3 repertoire was found, indicating that bacteria may induce IgA production but not cause extensive modification in the specificity of IgA (**Chapter 7**). Transitional changes in the composition of the microbiota were restored once IgA production was initiated, suggesting that IgA is directly involved in regulation of the intestinal microbiota composition.

Diet-derived RA is a key regulator of tolerance and IgA production in the mucosa. In addition, dietary ingredients can directly interact with cells of the intestinal immune system. In **chapter 8** we show that bovine IL-10 binds to the human IL-10 receptor and thereby inhibits bacterial ligand-induced activation of monocytes and DCs. Bovine milk also contains immunoglobulins that are specific for bacterial ligands and inhalation allergens that are also encountered by the human mucosal immune system. Bovine IgG efficiently binds to human IgG receptors, and can modulate myeloid cell activation by LPS (**Chapter 9**). Immunoglobulins from bovine milk may therefore provide potent opportunities to either induce tolerance or antigen-specific immune responses in the intestinal mucosa resulting in the production of protective IgA, thereby contributing to protection against infections. This protection may be mediated by bovine IgG alone, or in cooperation with IL-10, TGF-ß and vitamin A-derived RA; factors that are involved in induction of IgA.

The findings from chapters 2-9 are discussed (**Chapter 10**)and applied to the field of allergy, both for the clinic and experimental research. The finding that human IgA1 and IgA2 are differentially regulated by innate factors and differentially correlated with the presence or absence of allergy and with the severity of clinical symptoms is a promising finding. This finding may provide new opportunities for diagnosis of allergy as well as allergen-specific immunotherapy. Finally, the efficacy of mucosal vaccination strategies could be affected by the innate mucosal mechanisms of regulation of IgA production and the effects of dietary components as described in this thesis.

Samenvatting (Dutch Summary)

IgE is een antistof van het type E, verder zijn er nog IgG, IgM, IgA en IgD.

In het immuunsysteem regaeren cellen op **stimulatie**. Stimulatie gebeurt door eiwitten op de celwand en door eiwitten (cytokinen) die uitgescheiden worden door de cel.

Th2 zijn T helper cellen die specifiek zijn voor een stukje van een eiwit, in dit geval een eiwit wat allergie kan veroorzaken. De aanduiding 2 in Th2 geeft aan wat voor soort afweerresponse de Th cel kan maken.

Een **inhibitie assay** test of hetzelfde IgE molecuul aan eiwitten van verschillende plant of diersoorten kan binden.

In tegenstelling tot IgE, kan **IgG4** niet binden aan (IgE)receptoren op mestcellen.

DCs zijn immuuncellen die belangrijk zijn aan het begin van de immuunresponse. DCs zijn niet specifiek (zoals Th en B cellen), maar wel essentieel om Th cellen te stimuleren, en blijken nu ook B cellen te kunnen stimuleren.

TGF-ß1 is een eiwit dat een belangrijke rol speelt in het immuun systeem. TGF-ß1 valt onder de zogenaamde cytokinen, die reguleren of er ontsteking (inflammatie) of juist tolerantie geinduceerd wordt door het immuun systeem.

De meest bekende en meest voorkomende allergie is Type I allergie. Type I allergie wordt veroorzaakt door antistof type E (**IgE**). IgE wordt, net als andere antistoffen, geproduceerd door zogeheten B cellen, meestal na **stimulatie** van T helper type 2 (**Th2**) cellen. Deze Th2 en B cellen reageren heel specifiek op een stukje (epitoop) van een bepaald eiwit (een eiwat dat allergie veroorzaakt wordt allergeen genoemd), wat verklaart waarom iemand voor een bepaald eiwit allergisch kan zijn, zonder last te hebben van andere eiwitten. Om allergie te veroorzaken, moet IgE niet alleen een allergeen herkennen, maar het allergeen moet tegelijkertijd door meerdere IgE moleculen gebonden worden. Als een allergeen tegelijkertijd door meerdere IgE moleculen herkend wordt, kan het allergie veroorzaken (***Hoofdstuk 2***). Het allergeen tropomyosine (speelt een rol bij functionering van spieren) is een belangrijk allergeen voor schaal- en schelpdier allergie, dat grote gelijkenis vertoont tussen dieren. Daardoor kan een specifiek IgE molecuul zowel tropomyosin van bijvoorbeeld een garnaal als van een mossel herkennen. In de praktijk lijkt dat echter weinig voor te komen in Nederland, doordat de IgE-epitopen voldoende verschillend zijn (***Hoofdstuk 2***). Het principe van kruisreactiviteit komt voor tussen schaal- en schelpdieren maar ook tussen vijg (vrucht) en ficus (plant). Kruisreactiviteit kan met specifieke **inhibitie assays** onderzocht worden (***Hoofdstuk 3***). De inhibitie assay beschreven voor twee patiënten in hoofdstuk 3, toont aan dat waarschijnlijk de ficus plant de primaire oorzaak is voor de allergie, waarna het IgE van de patiënt ook kruisreageert met vijg.

Voor de diagnose van allergie wordt vaak gekeken of een patiënt allergeen-specifiek IgE in het bloed heeft. De aanwezigheid van dit specifieke IgE wordt sensibilisering genoemd, maar bewijst nog niet dat iemand daadwerkelijk allergisch is. Dit komt onder meer doordat er ook andere (met name **IgG4**), antistoffen aanwezig kunnen zijn die het allergeen binden, en voorkomen dat IgE eraan kan binden. Er is nog geen overtuigend bewijs dat IgA antistoffen in bloed ook allergie kan voorkomen bij gesensibiliseerde mensen.

In dit proefschrift is onder andere gekeken of er een relatie is tussen de aanwezigheid van allergeen specifiek IgA en allergie voor huisstofmijt. Daarbij werd een verband gevonden tussen IgA in speeksel en bescherming tegen allergie, en dat allergische mensen die ook eczeem hebben, in hun bloed lagere levels hebben van allergeen specifiek IgA (***Hoofdstuk 4***). Mensen maken twee soorten IgA: IgA1 en IgA2. De genoemde relaties tussen allergie en IgA, waren gevonden voor IgA2 en niet voor IgA1.

Een interessante vraag is dan, of er factoren zijn die specifiek de productie van IgA1 of IgA2 beïnvloeden. Het meeste IgA wordt geproduceerd in de zogenaamde mucosale weefsels van de longen en vooral de darmen. Productie van antistoffen wordt deels gestimuleerd door Th cellen, maar kan ook gestimuleerd worden door **dendritische cellen** (DC), vooral in de mucosa. In ***Hoofdstuk 5*** laten we zien dat de mucosale factoren **TGF-ß1** en retinoic acid (RA), wat gevormd wordt uit vitamine A, beïnvloeden hoe een DC reageert op stimulatie met bacteriële producten. Als DCs gedifferentieerd worden in de aanwezigheid van TGF-ß1 en RA, produceren ze minder ontsteking stimulerende cytokinen. Ook de expressie van **co-stimmulatoire moleculen** op het membraan van de DCs was verminderd onder invloed van TGF-ß1 en RA.

Zoals beschreven is in ***hoofdstuk 6***, spelen TGF-ß1 en RA ook een rol in de productie van IgA1 en IgA2. Ook andere mucosale cytokinen als APRIL, BAFF, TSLP en IL-10 zijn in deze experimenten gebruikt. Deze mucosale factoren worden geproduceerd door epitheliale cellen, die het lichaam scheiden van de buitenomgeving, en DCs. De aanwezigheid van RA zorgde ervoor dat de B cellen verder differentieerden naar plasma cel en daarmee samenhangend, meer IgA uitscheidden. De productie van IgA1 was het hoogste in de aanwezigheid van BAFF, terwijl IgA2 levels het hoogst waren in de aanwezigheid van APRIL. Voor de maximale productie van IgA1 en IgA2 waren niet alleen BAFF of APRIL nodig, maar ook RA en IL-10. De combinatie van RA en IL-10 zorgde ook voor verhoogde expressie van integrine ß7, wat de B cellen helpt om naar de (dunne) darm te migreren. IgA producerende B cellen zijn van belang voor de bescherming van de grote oppervlakte van de darm tegen pathogenen, en naar we vermoeden ook allergenen.

Co-stimmulatoire moleculen zijn nodig om Th cellen te kunnen activeren. De Th cellen kunnen na activatie vervolgens antistof productie stimuleren.

TSLP remde de productie van IgA, wat een interessant bevinding is omdat TSLP vaak verhoogd is in mensen met eczeem. Huisstofmijt allergische patiënten met eczeem, hadden lagere levels van allergeen-specifiek IgA2 in hun bloed. Aanwezigheid van eczeem correleert ook met afwijkende levels van APRIL en BAFF, die ook invloed hebben op IgA productie. Omdat dezelfde factoren die IgA productie beïnvloeden afwijken in patienten met eczeem, en omdat patienten met eczeem verlaagde niveaus van IgA hebben, toont dit mogelijk een verband aan tussen factoren die invloed hebben op IgA productie en eczeem.

De productie van IgA wordt gestimuleerd door de aanwezigheid van bacteriën in de darm, en andersom helpt IgA de darm te beschermen tegen pathogene bacteriën. In ***hoofdstuk 7*** worden de resultaten beschreven van het volgen van IgA productie, IgA specificiteits-**repertoire** en bacteriesamenstelling in de darm van kuikens vanaf dat ze uit het ei komen, tot de leeftijd van 10 weken. Het repertoire van het IgA in de darm van deze kuikens konden we niet koppelen aan de samenstelling van de bacteriepopulatie in de darm. Wel was er een verband tussen de productie niveaus van IgA en de bacteriesamenstelling. De samenstelling van de bacteriën veranderende met de leeftijd, en werd stabiel wanneer productie van IgA op een hoog niveau was gekomen. Het dieet heeft ook invloed op het immuun systeem. Een speciale situatie doet zich voor als eiwitten uit koemelk ook binden aan receptoren op bijvoorbeeld DCs bij de mens, oftewel, als cytokinen of antistoffen kruis-reageren. Het ontstekingsremmende cytokine IL-10 van koeien bindt ook aan de receptor voor IL-10 van mensen (***Hoofdstuk 8***). Bovine IL-10 is ongeveer evengoed in staat als humaan IL-10 om de ontstekingsreactie te remmen van humane immuuncellen. Ook IgG van koeien bindt aan receptoren voor IgG van de mens (***Hoofdstuk 9***). Daarnaast is RA en TGF-ß1 beschikbaar via koemelk. Deze bevindingen bieden mogelijkheden om het menselijke immuun-systeem te reguleren met behulp van koemelk.

Repertoire: Een antistof-producerende B cel, maakt één type antistof tegelijkertijd, met 1 specificiteit. Om het brede scala aan antigenen te kunnen herkennen zijn er veel verschillende B cellen nodig die elk een andere specifiteit voor een bacterie of eiwit hebben, wat het repertoire genoemd wordt.

De bevindingen uit de hoofdstukken 2 – 9 worden geïntroduceerd (***Hoofdstuk 1***) en bediscussieerd (***Hoofdstuk 10***) in de context van onderzoek dat gaande is binnen het vakgebied van de allergie. De algemene conclusie is dat dieet en regulatie van het mucosale immuunsysteem mogelijkheden bieden om de ontwikkeling van allergie te beïnvloeden.

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My enthusiasm for immunologic research started with lectures of Aart Lammers and Henk Parmentier as part of the course Adaptation Physiology, and from the lectures of Huub Savelkoul as part of the course Comparative Immunology (now called Human and Veterinary Immunology). Aart, we still have a nice collaboration.
My first MSc thesis I did with Aart, where I also met Henk Parmentier and Richard Crooijmans. During this thesis I developed my interest in B cells and mucosal immunology. Thank you all for the nice time and for all I learned. Another experience I still regularly think of is my time in Tanzania with Hans Verhoef and Jacobien Veenemans (and Erasto, Laura, Inge and Gertie!). Thank you for the nice time, the opportunity to get a little glimpse of all details involved in conducting a clinical trial.
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Currently I work at the Immunotherapy group of the UMC Utrecht, in a nice collaboration with Laurien Ulfman and Joost van Neerven from FrieslandCampina. Jeanette and colleagues, thanks for the nice working environment and help. I learn a lot and enjoy working at your lab.
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Curriculum Vitae

Gerco den Hartog was born on July 28th 1983 in Gorinchem, The Netherlands. He received his high school diploma (VWO) in 2001 at the Gomarus ScholenGemeenschap in Gorinchem, and started his course Biology at Wageningen University in the same year.
In his first MSc thesis he studied the development of IgA repertoires in the ileum of chickens in the first ten weeks post hatch. His internship and second thesis was about the levels of IgG subclasses against the malaria parasite *Plasmodium facliparum* in children aged 5-50 months. Those IgG levels were related to micronutrient status and health status. For this project he stayed half a year in Tanzania, where the field work was conducted.
In 2008, he started with his PhD project at the Cell Biology and Immunology Group of Wageningen University, under the supervision of Prof. Huub F. J. Savelkoul and Dr. R. J. Joost van Neerven. In his PhD project he collaborated with FrieslandCampina, the allergist Ad P. H. Jansen MD of the Allergologie Praktijk Arnhem and with Dr. Janneke Ruinemans of the Clinical Chemistry and Haematology Department of Rijnstate Hospital, Arnhem. The results of the PhD research are described in this thesis.
Currently Gerco den Hartog works at the Immunotherapy group of Dr. Jeanette Leusen of University Medical Center Utrecht, on a project about antibody responses to respiratory viruses.

Publications

- Gerco den Hartog, Huub F. J. Savelkoul, Vitamine D en allergie [Vitamine D and allergy], Nederlands Tijdschrift voor Allergologie [Dutch journal for allergology], 2012
- Gerco den Hartog, Huub F. J. Savelkoul, Ruud Schoemaker, Edwin Tijhaar, Adrie H. Westphal, Talitha de Ruiter, Elise van de Weg-Schrijver, R. J. Joost van Neerven; Modulation of human immune responses by bovine interleukin-10, PLoS ONE 6(3): e18188, 2011
- Aart Lammers, Willemien H. Wieland, Leo Kruijt, Arne Jansma, Trudy Straetemans, Arjen Schots, Gerco den Hartog, Henk K. Parmentier, Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken, Developmental and Comparative Immunology 34 (2010) 1254–1262
- Gerco den Hartog en Huub Savelkoul, Book chapter, Aanleg en allergie: de allergische mars [Predisposition and allergy: the allergic march], Het Allergie Boek [book about allergy for high school education], ISBN 978 90 313 7752 7, Houten 2010, The Netherlands

Publications and chapters part of PhD thesis

- Gerco den Hartog, Richard P. M. A. Crooijmans, Ger de Vries-Reilingh, Ariane M. Wehrmaker, Nicolaas A. Bos, Huub F. J. Savelkoul, Henk K. Parmentier, Aart Lammers, The level of IgA but not the Ig-repertoire is related to intestinal microbial composition during ontogeny, submitted
- Gerco den Hartog, Lara Cornel, Janneke Ruinemans-Koerts, Ad P. H. Jansen MD, Huub F. J. Savelkoul, Limited cross-reactivity of shellfish tropomyosin-specific IgE to molluscan tropomyosin, submitted
- Gerco den Hartog MSc, R. J. Joost van Neerven PhD, Diderik Boot PhD, Ad P. H. Jansen MD, Huub F. J. Savelkoul PhD, Allergen-specific IgA2, but not IgA1, is associated with protection against eczema in house dust mite allergic patients, submitted
- Gerco den Hartog, R. J. Joost van Neerven, Huub F. J. Savelkoul, The mucosal factors retinoic acid and TGF-ß1 induce phenotypically and functionally distinct tolerogenic DC types, submitted
- Gerco den Hartog, R. J. Joost van Neerven, Huub F. J. Savelkoul, Differential regulation of IgA2 production by factors associated with eczema, submitted
- Gerco den Hartog, Huub F. J. Savelkoul, Ad P. H. Jansen MD, Janneke Ruinemans-Koerts, An alternative inhibition method for diagnosis of allergy: Two patients with a ficus (*Ficus benjamina*) and fig (*Ficus carica*) sensitization, manuscript in preparation
- Gerco den Hartog, Christine van Altena, Huub F. J. Savelkoul, R. J. Joost van Neerven, tentative title: Bovine IgG modulates human myeloid cell activation by binding to human Fc receptors, manuscript in preparation

Education Certificate
Issued by WIAS

Discipline related courses		
Immunology Course	ES	2009
Nutrient Density of Milk	VLAG	2009
Epigenesis - Epigenetics: Physiological consequences of perinatal programming	WIAS/VLAG	2008
(Shell)fish immunology Workshop	WIAS	2008

General courses		
WIAS Introduction Course	WIAS	2008
Course on philosophy of science and/or ethics	WIAS	2009
Statutory course: Use of Laboratory Animals (art 9)	WU	2008

Scientific meetings		
European Academy of Allergy and Clinical Immunology (poster)	EAACI	2010
World Immune Regulation	SIAF	2011
EAACI Winterschool (poster)	EAACI	2012
Beneficial Microbes (oral and poster)	TNO	2012
Dutch Research School of Allergy (poster)	NVvA	2008
Annual Meeting, Dutch Society for Immunology (poster)	NVVI	2008
WIAS Science day (oral)	WIAS	2011
MACS User day	Myltenyi Biotec	2008
WIAS symposium, Corticosteroid recepters in carp	WIAS	2008
WIAS symposium Immunomodulation and Allergy	WIAS	2008
IBH Symposium genetics and immunology	WIAS	2009
WIAS symposium, Of fish and men: curiosities of the immune system	WIAS	2009
Immuno Valley, Intestinal immunity	VUMC	2009
Scientific meeting NVvA	NVvA	2010, 2011
The 4th Symposium & Master classes on Mucosal Immunology (oral)	Erasmus	2011
IMARES PhD Science Day: Bottom-up (oral)	IMARES	2011

Research Skills Training		
Preparing own PhD research proposal	WIAS	2008
BD FACS diva training (flowcytometry)	BD	2008
Phadia, Immunocap training (clinical IgE anti allergen measurements)	Phadia	2008
Scientific Writing	WGS	2011
NWO Talentdays	NWO	2010
Mobelising your - scientific - network	WGS	2011
WIAS Advanced Statistics Course: Design of Animal Experiments	WIAS	2009
Introduction to R for statistical analysis	WIAS	2008
Statistics of the life sciences	WIAS	2009
Statistical analysis of ~omics data	VLAG/EPS	2010

Didactic Skills		
Supervising practicals and excursions (real time)	CBI	2008-2011
Course Supervising MSc thesis work	WGS	2009
Supervising theses (5 BSc, 8 MSc theses)	CBI	2008-2012
Tutorship (research master training)	CBI	2011
Preparing course material	CBI	2011

total educational certificate		***72 ects***

Abbreviations

BD	Becton Dickinson, company
CBI	Cell Biology and Immunology group
EAACI	European Academy Allergy and Clinical Immunology
EPS	Experimental Plants Sciences
ES	Eijkmanschool (UMC Utrecht)
MolMed	Molecular Medicine (Erasmus MC)
NVvA	Dutch Association of Allergy
NVVI	Dutch association of Immunology
NWO	Dutch Academy of Sciences
Phadia	now part of Thermofisher
SIAF	Swiss Institute of Allergy Research
TNO	Organisation for Applied Scientific Research
VLAG	Food Technology, Agrobiotechnology, Nutrition and Health Sciences
WIAS	Wageningen Institute of Animal Sciences
WGS	Wageningen Graduate School
WU	Wageningen University

Design and layout by Gerco den Hartog

The back shows the allergen tropomyosin (this thesis, long streched molecule), bound by monomeric IgE (top) en dimeric IgA (bottom). The Ig molecules were modified from PDB (http://www.rcsb.org).

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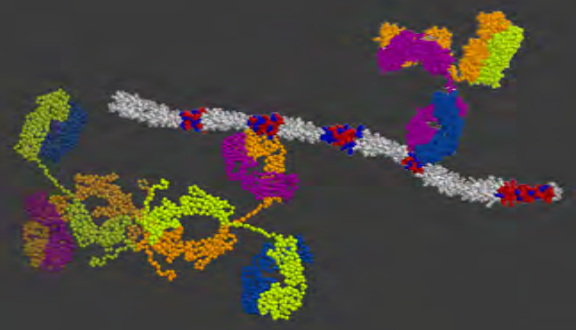
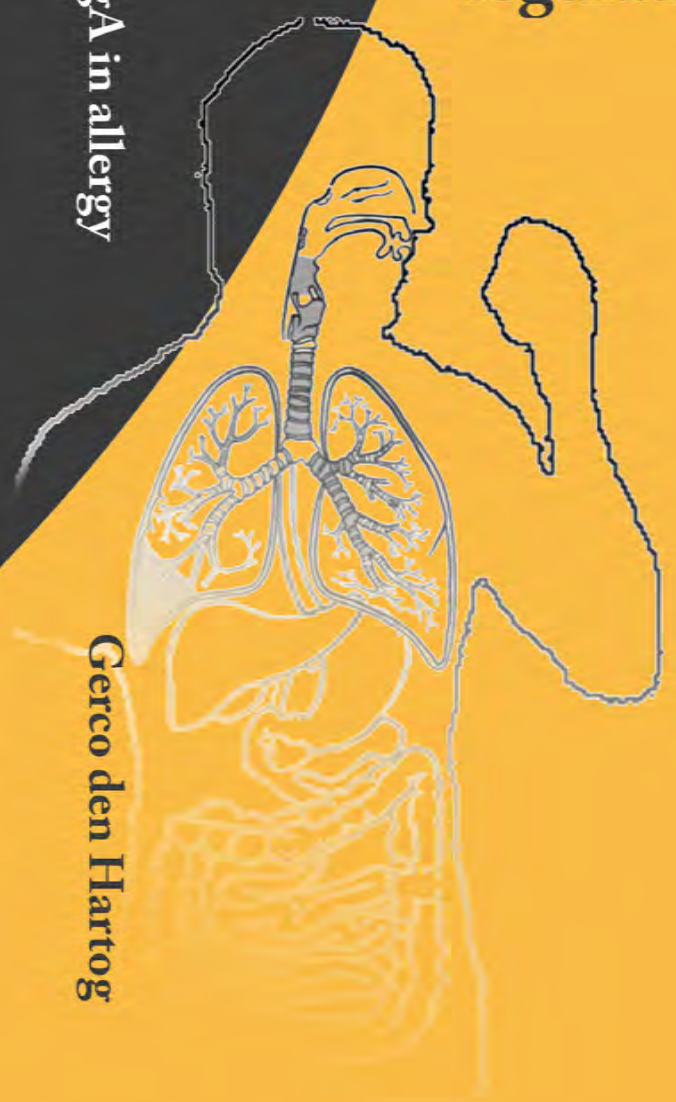


The role of IgA in allergy



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