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INDUCTION OF IMMUNOGLOBULIN A AS A THERAPEUTIC INTERVENTION IN ALLERGIC ASTHMA

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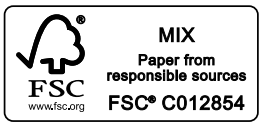

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Induction of Immunoglobulin A as a Therapeutic Intervention in Allergic Asthma

Stimuleren van immunoglobuline A als therapeutische interventie tegen allergisch astma

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GENERAL INTRODUCTION

Allergic asthma is a chronic inflammatory disorder of the airways in response to inhaled allergens and is characterized by airway inflammation, bronchial hyperresponsiveness and a variable degree of airflow obstruction, leading to episodes of wheezing, coughing and breathlessness. In addition, structural changes ('airway remodeling') in the airway including subepithelial and airway wall fibrosis, goblet cell hyperplasia/metaplasia, smooth muscle thickening and increased vascularity are observed (1). In susceptible individuals, repeated exposure to harmless environmental allergens like house-dust mites (HDMs), molds, plant pollen and animal dander can lead to sensitization and development of a chronic immune response consisting of an effector cascade leading to immediate and late-phase reactions to allergens and subsequent characteristic clinical symptoms (2).

Currently approximately 300 million people worldwide suffer from asthma, with estimates suggesting that asthma prevalence increases globally by 50% every decade. This increase in prevalence of asthma has been associated with an increase in atopic sensitization, and is paralleled by similar increases in other allergic disorders such as rhinitis (3). Prevalence has reached a peak in developed countries but rates are rising in developing regions (Africa, Latin America and parts of Asia) as they become more westernized. This has reduced the global differences in prevalence, however the global burden of asthma and allergies continues to rise and new therapies are warranted (4,5). Genetics cannot account for the rapid increase in prevalence over time, but epidemiological evidence supports the idea of a combination of environmental and genetic risk factors (6). Epidemiological studies have shown negative associations between the prevalence of allergic asthma and growing up on traditional European farms or rural tropical areas, traditionally exposed to higher ambient concentrations of microbial pollutants and higher rates of parasitic infections (7,8). Therefore, hyperinflammatory diseases are thought to result from insufficient maturation of the regulatory arm of the immune system due to reduced infectious pressure during childhood. This may cause disbalance of the immune system, allowing for uncontrolled expression of inflammatory responses against innocuous antigens later in life ('Hygiene hypotheses') (9).

New therapies should focus on reducing inflammatory responses against allergens at an early age preventing the onset of structural damage and changes to the lungs, by targeting natural tolerizing mechanisms as found in healthy individuals.

Inflammatory immune responses in infection and allergy

The role of effector T helper cells and humoral immune responses

The human body is continuously exposed to a variety of environmental particles, which can be potentially harmful (pathogenic viruses, and bacteria) or relatively harmless (certain allergens, commensal bacteria). Our immune system has developed to discriminate between noxious and innocent antigens and to prevent the establishment of a potentially life-threatening infection

while avoiding deleterious inflammation. The induction of specific protective immune responses requires the activation and maintenance of specific T helper (Th) cell subsets that control the different types of infections. Th1 cells produce high amounts of interferon (IFN)-γ and play an important role in immunity against intracellular pathogens such a viruses and intracellular bacteria or parasites. Th2 cells secrete interleukin (IL)-4, IL-5, IL-9 and IL-13, and are instrumental in providing protection against certain helminth infections, through activation of eosinophils and induction of mucus hyperproduction at the mucosal surfaces. Th17 cells are characterized by the secretion of IL-17 and are associated with the clearance of extracellular bacteria and fungi. In contrast, regulatory T (Treg) cells are more involved in the downregulation of potentially harmful inflammatory responses mediated by other effector Th cells, through cell-cell contact or by the cytokines IL-10 and transforming growth factor (TGF)-β (10). When the induction of tolerance fails and immune responses are directed against self antigens or to harmless environmental allergens, this can result in autoimmune diseases (Th1 and Th17) or allergic diseases like allergic asthma (Th2 and Th17), respectively. The different effector Th cells also provide help to B cells for survival and immunoglobulin isotype class switching resulting in different types of immunoglobulins each with a distinct function to support immune responses to and elimination of pathogens. Those include neutralization of bacterial products and toxins, activation of effector cells, and marking microorganisms for recognition by phagocytes (opsonization). As such, in humans Th1 cytokines are associated with IgG1, IgG2 and IgG3 production, Th2 cytokines IL-4 and IL-13 drive IgE class switching, while IL-10 from Treg cells induce B cells to switch to IgG4. In mice, Th2 cytokines drive production of IgE and IgG1.

Dendritic cells bridging innate and adaptive immunity

Induction of adaptive Th responses does not occur via direct recognition of the TCR with antigen. Antigen need to be processed into small peptides and presented on the surface of major histocompatibility complex (MHC) I (for CD8 T cells) and MHCII (for CD4 T cells, the Th cells) molecules. The processing and antigen presentation of antigen is the task of professional antigen presenting cells (APCs), like macrophages, B cells and dendritic cells (DCs). DCs are a heterogeneous population of hematopoietic cells that belong to the innate immune system, but have the unique capacity to also translate their function to the adaptive immune system. Immature DCs reside in peripheral and mucosal tissues at sites where there is possible exchange with the outside world and danger is constantly at bay. Here, DCs continuously sample the environment for foreign soluble antigens and small particles (11,12). Like other cells of the innate immune system, DCs express different types of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors and C-type lectin receptors, that allow the recognition of molecules that are broadly shared by pathogens but distinguishable from host molecules (pathogen-associated molecular patterns (PAMPs)) (13,14). Upon encounter of non-self material and danger signals, DCs become activated and upregulate the chemokine receptor CCR7, which directs their migration to the T cell area of draining lymph nodes (15). During their

journey a maturation process is started that strongly down regulates the antigen uptake capacity of DCs but qualifies them uniquely to activate antigen-specific naïve Th cells and drive their development in effector T helper cells (16,17). DCs provide three signals that are crucial to drive the polarization of naïve Th cells, including the presentation of processed peptides in the context of MHC molecules (signal 1), the expression of costimulatory molecules necessary for avoidance of anergy and full T cell activation (signal 2, like CD40 or molecules from the B7 family (e.g. CD80, CD86)), and different sets of T cell polarizing molecules (both cytokines and membrane bound molecules) (signal 3) (18,19). The T cell polarizing capacity of mature DCs will be strongly influenced by signals encountered during their stay in the peripheral tissues. This can include microbial signals induced by the ligation of PRRs on the DCs or alarming signals from structural cells like local epithelial cells of the airways (20-22).

Inflammatory immune responses in Asthma

Immediate and late phase reactions against inhaled allergens

Chronic inflammation in allergic asthma is controlled by effector Th2 cells and characterized by eosinophilic airway inflammation and high levels of allergen-specific IgE antibodies, hallmarks of a persistent Th2 response (2) (**Figure 1**). In addition, in severe forms of asthma also Th17 cells are found (23,24). Upon encounter with the allergen, effector responses can be divided into immediate and late phase reactions. The immediate allergic inflammatory reaction is initiated by crosslinking of IgE molecules which are bound to IgE receptors on basophils and mast cells. As a result, these cells will degranulate and release preformed mediators from vesicles or secrete cytokines (IL-6, TNFα, MIP1α) (25). Histamine, leukotrienes, prostaglandins and platelet-activating factor cause immediate vascular permeability, blood vessel dilation, bronchoconstriction, and smooth muscle contraction (26). This immediate reaction may be followed by the late phase response, initiated by inflammatory cytokines and type 2 cytokines which recruit and activate eosinophils and basophils, and induce goblet cell metaplasia and overproduction of mucus (27,28). Th17 cells can enhance the effects of the Th2 cytokines and recruit neutrophils and other inflammatory leukocytes (23,24,29).

Not only distortion of immunological pathways during allergen sensitization and challenge are important for development of asthma, but also aberrant structural airway remodeling is involved. Some groups have even suggested that the airway structural changes occur before the deranged immune response is present. Indeed, basement membrane thickening is detectable in children younger than three years old with persistent wheezing before the diagnosis of asthma (30,31). Airway remodeling includes marked changes in the airway wall, like epithelial injury, extracellular matrix deposition under the epithelial basal membrane, goblet cell hyperplasia and increased smooth muscle mass. These changes lead to a defective physical and functional barrier of the airway epithelium in severe asthma. Various studies point at dysfunctional injury/repair

mechanisms in response to damaging stimuli and/or respiratory viruses in asthmatics, which may only be in part explained by allergic airways inflammation (32,33) (**Figure 1**).

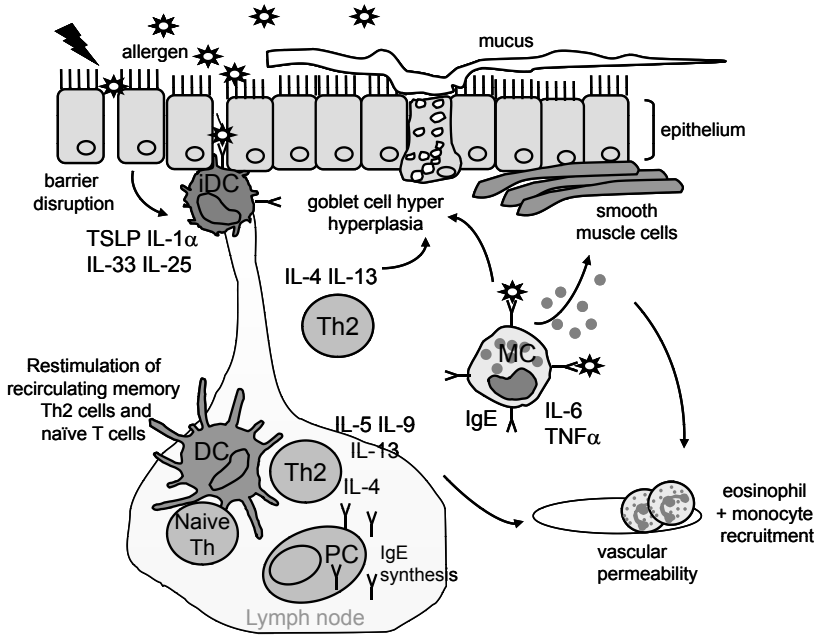

Figure 1. Upon allergen exposure, crosslinking of IgE molecules which are bound to IgE receptors on basophils and mast cells (MC) results in degranulation and the release of inflammatory mediators and cytokines, responsible for vascular permeability and smooth muscle contraction. Meanwhile, after allergen uptake dendritic cells (DCs) migrate to the draining lymph nodes where they activate and differentiate specific naïve T helper cells into T helper 2 (Th2) cells, which enhance the inflammation by recruiting and activating other inflammatory cells, stimulating IgE synthesis by plasma cells (PC) and overproduction of mucus. These immunological changes are accompanied by aberrant structural airway remodeling.

The role of crosstalk between epithelial cells and dendritic cells

Epithelial cells (ECs) line the cavities and surfaces of tissues throughout the body, and form many glands. In healthy individuals they provide a tight physical barrier, preventing the entry of harmful particles and airway epithelial cells play an integral role in airway defense mechanisms via the mucociliary action. Interestingly, ECs express different sets of PRRs and after exposure to PAMPs, they produce a wide range of molecules, including chemokines, cytokines, antimicrobial peptides, prostaglandins, MHC class II and costimulatory molecules and thereby influence the functions of local immune cells. Closely attached to the lung epithelium different DC subsets have been identified (34). Crosstalk between airway ECs and DCs may form a critical link for the induction and continuation of allergic inflammation in the lungs as several EC-derived molecules can influence DC migration, differentiation and function (12,35). For example, airway ECs can prime for Th2-polarizing DCs by the enhanced production of innate cytokines like IL-1α, GM-CSF,

TSLP, IL-25 and IL-33 (33,36). Indeed, a dysregulated production of these cytokines has been found in several inflammatory disease states including inflammatory bowel disease, atopic dermatitis and asthma. TSLP is the one most studied, and was enhanced in lung biopsies from asthmatics and correlated with the severity of airflow obstruction, as assessed by FEV_1 (37). The same link with asthma severity has also been observed in the mouse (20). IL-1 has also been identified as a potent upstream inducer of TSLP production, and IL-1α was shown to cause cytokine release by ECs and activate DCs in response to inhaled house dust mite allergen (20). Strong stimuli for EC-derived innate cytokine production are damage, TLR3 ligation by viral infections or TLR4 ligation by inhaled endotoxin in combination with allergens, processes that are further potentiated by Th2 cytokines, sustaining allergic inflammation (**Figure 1**).

Tolerogenic immune responses to environmental allergens

Immunological responses to allergens in healthy individuals: role of Treg cells and IgA

In healthy individuals, T cell responses to allergens are commonly observed, yet are usually dominated by Treg cells, that can suppress different effector Th cell subsets. Allergen-specific Treg cells can suppress Th2 cells by cell-cell contact or release of the anti-inflammatory and immunoregulatory cytokines IL-10 and TGF-ß. In almost all patients with asthma, one can find the counterregulatory Treg cells, but these fail to or insufficiently suppress allergic inflammation (38). It has therefore been suggested that asthma may result from aberrant of defective Treg mechanisms.

Humoral responses of healthy individuals consist of mainly low IgG1, IgG4 and secretory IgA (sIgA) antibodies to allergens in the presence or absence of low amounts of IgE (39-41). The presence of allergen-specific IgA has drawn relatively little attention, and it is still controversial how IgA actually contributes in the protection or exacerbation of allergic disease. Although most individuals with immunoglobulin A (IgA) deficiency are asymptomatic, allergic disorders appear to be more common among patients with IgA deficiency (42). Balzar et al found lower IgA levels in brochoalveolar lavage of severe asthmatics than in healthy subjects, which correlated with lung function and asthma symptoms (43). Conversely, high salivary secretory IgA levels were associated with less development of allergic symptoms in sensitized Swedish children (44). Furthermore, high levels of specific IgA antibodies in salivary of sensitized infants were associated with significantly less late-onset wheezing (45). In addition, allergic patients who naturally develop tolerance responses towards cow's milk concomitantly undergo a shift towards IgA dominance in serum (46).

Taken together, these data show an inverse relationship between IgA and allergy development, suggesting a protective role for IgA in allergic diseases such as asthma.

Therapeutic manipulation of immune tolerance

Treatment provided by allergen-specific immunotherapy (IT), which involves incremental delivery of the allergen to which the individual is sensitive, effectively suppresses the clinical symptoms of allergy (38). After successful IT components of the regulatory network such as Treg cells and the cytokine IL-10 are elevated, while allergen-specific IgE levels are reduced. It is hypothesized that the enhanced immunoregulatory network is instrumental in suppressing allergen-specific effector T cells which are responsible for many of the characteristics of allergic diseases. IL-10 does not only contribute to T cell tolerance, but potently suppresses total and allergen-specific IgE and it simultaneously increases IgG4 and IgA production in cultures (39,47). Interestingly, successful immunotherapy is also associated with increases in IgA responses *in vivo*. In a 2-year double blind trial, grass-pollen immunotherapy induced a shift in allergen-specific antibody response towards IgA2, which correlated with increased local TGF-β expression and induced monocyte IL-10 expression (48). Another study using sublingual grass pollen immunotherapy (SLIT), reported increases in allergen-specific IgG4 and IgA (49). Moreover, in an experimental setting, Schwarze was able to protect mice against the development of eosinophilic airway inflammation and hyperresponsiveness by treating with antigen-specific IgA during challenge (50).

Thus, stimulating the development of regulatory immune responses, including IgA responses, against allergens at relevant mucosa can prevent the development of allergic diseases.

Immunoglobulin A

Different forms of IgA

IgA is by far the most abundantly produced immunoglobulin isotype at mucosal surfaces, but can also be found in the circulation. IgA can occur as a monomer (**Figure 2A and B**), but also, through interactions of the Fc region with the joining chain (J-chain) in dimeric or even polymeric forms (**Figure 2C**). In human serum, IgA occurs mainly in a monomeric form, while in mice polymeric IgA is the main isotype in serum. Furthermore, human IgA, but not mouse IgA, is divided into closely related subclasses, IgA1 and IgA2. The main difference between the 2 subclasses is an extra 13-amino acid sequence in the hinge region of the IgA2 molecule which makes this form less susceptible for proteolytic degradation (**Figure 2A and B**). In serum, the subclass IgA1 is dominant, while in secretions the main isoform found is IgA2, although both IgA1 and IgA2 can be detected as secretory IgA (SIgA). Dimeric or polymeric IgA will bind via the J-chain to the polymeric immunoglobulin receptor (pIgR) at the basolateral side of the epithelium and is transported to the luminal side (**Figure 2D and E**). Here, IgA is released at the mucosal surface (lumen) by cleavage from the pIgR. In this process part of the pIgR, called the secretory component (SC), remains attached to the IgA molecule and together they form the molecule secretory IgA (SIgA). Some IgA can enter the mucosal lumen by passive diffusion, and 'free' SC can also be present in mucosal lumen when a pIgR releases its SC part (51).

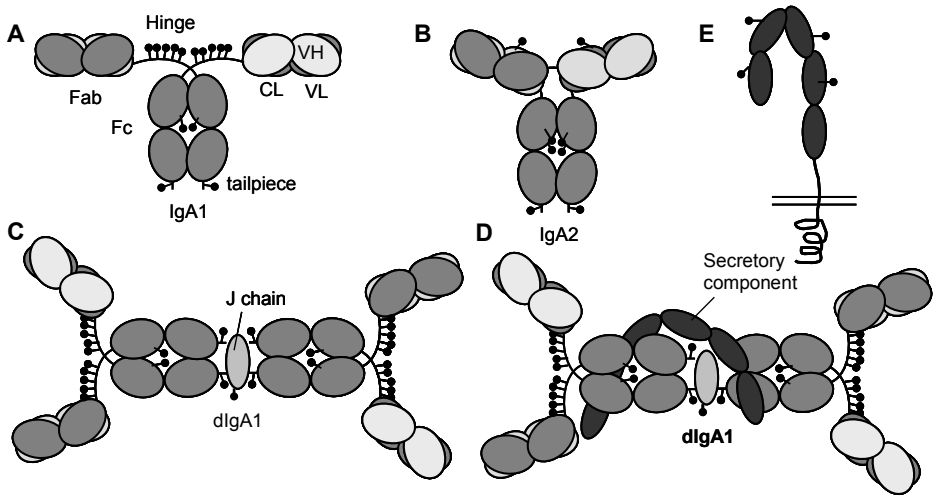

Figure 2. Human IgA structure. Schematic diagrams of A) IgA subclass 1 (IgA1), B) IgA2, C) dimeric IgA1 (dIgA1), D) Secretory IgA1, and E) Polymeric immunoglobulin receptor (pIgR) [Adapted from *Woof Nature Immunology 2011*].

FcalphaRI: Receptor for IgA

The varied interaction of the Fc region of IgA with receptors confers this antibody class with many of its unique properties. IgA has been described to interact with various host receptors, i.e. pIgR, FcαRI (CD89), transferrin receptor (CD71), asialoglycoprotein receptor (ASGPR) and receptor specific for secretory component (SCR), Fcα/μR. The consequences after ligation are not very clear for most of these receptors. However, the FcαRI allows both inhibitory and activating signals and is therefore important for the role of IgA in preserving homeostasis and tolerance at mucosal sites (52,53).

FcαRI (CD89) is the only IgA Fc receptor expressed on (blood) myeloid cells, including dendritic cells, monocytes/macrophages, neutrophils and eosinophils. In mucosal areas under homeostatic conditions, only few cells are positive for FcαRI. Intriguingly, this receptor has not been identified in mice. Although this receptor is associated with an immunoreceptor tyrosine-based activation motifs (ITAM) (**Figure 3A**), its signaling can be activating as well as inhibitory. This depends on the ligand and subsequent configuration (involving Syk or SHP-1 phosphatase) of the ITAM, resulting in an activating or an inhibtory ITAM motif (**Figure 3**). The inhibitory ITAM (ITAMi) pathway takes place in the absence of receptor coaggregation and of an immunoreceptor tyrosine-based inhibitory motif (ITIM) which is known for inhibiting immune responses (**Figure 3B**). All forms of IgA can ligate to FcαRI, but they differ in their binding capacities. Monomeric IgA only binds with low affinity to the FcαRI and activates the ITAMi which does not lead to cell activation or degranulation/oxidative burst (in the case of granulocytes) (54). In contrast, IgA complexes show a stronger binding and subsequent activating signal, resulting in cell activation (55,56) (**Figure 3**). In addition to the subform of IgA, environmental factors may also contribute to the effect

of IgA ligation to FcαRI. Proteases, LPS, chemoattractants, inflammatory cytokines or adaptor protein binding to the intracellular domain of FcαRI modulate the expression of FcαRI and the outcome of IgA- FcαRI interaction. This can have a beneficial contribution to the preservation of homeostasis as for example, inflammatory sites may activate the anti-inflammatory FcαR1-IgA interaction and thereby help to downregulate local inflammatory reactions (55,57).

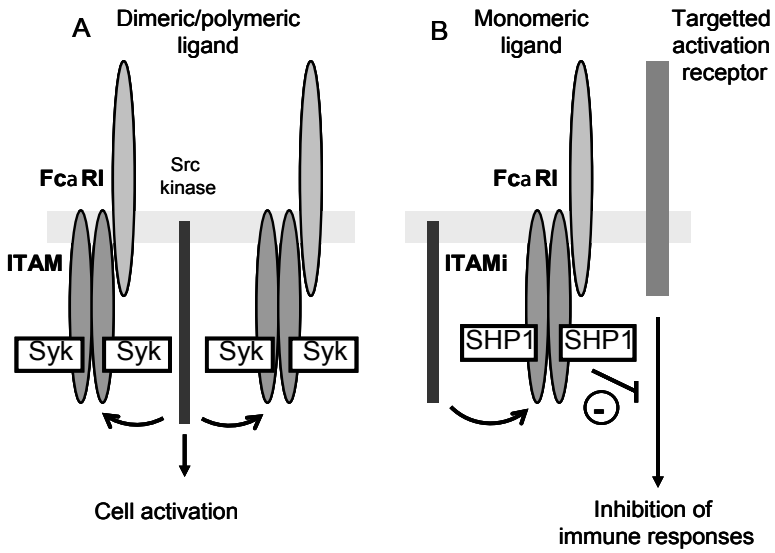

Figure 3. Dual role of FcαRI in the immune system. A) Cell activation by ITAM signaling. This involves receptor aggregation and crosslinking by IgA immune complexes, resulting in full (src-kinase-mediated) phosphorylation and recruitment of Syk. B) Cell inhibition by ITAMi signaling. Monomeric or low-affinity ligand binding to the receptor induces an inhibitory ITAM (ITAMi) configuration, resulting in recruitment of the inhibitory signaling effector SHP1 phosphatase [adapted from *Blank Immunological Reviews 2009*, and *Monteiro JCI 2010*].

Function of IgA

IgA is classically known for neutralizing toxins and bacteria (viruses) at mucosal surfaces (58,59), by interfering with their motility, by competing for epithelial adhesion sites, and by improving the viscoelastic properties of the airway secretions (60). The SC protects SIgA from proteolytic degradation and is involved in establishing local interactions with bronchial mucus, thereby contributing to the 'trapping' and removal of the antigen ('Immune exclusion'). When an antigen has passed the first barrier and gets into the tissue, it can be bound by IgA and transcytosed into the mucosal lumen via the pIgR as described, leading to removal of antigen from the infected tissue (61). Interestingly, it has been suggested that IgA can also directly reduce inflammatory responses by inhibiting effector functions of inflammatory cells. For example, anti-FcαRI Fab treatment, by initiating ITAMi signaling, suppressed manifestations of allergic asthma in FcαRI

transgenic mice immunized with anti-IgE immune complexes (62). Triggering ITAMi signaling also prevented marked inflammation and leukocyte infiltration in kidney inflammation models such as glomerulonephritis (63). Furthermore, *in vitro* cross-linking of FcαRI on human monocyte-derived DCs leads to internalization of IgA complexes and antigen presentation, resulting in DC maturation and IL-10 production (64,65). (Serum) IgA ligation on monocytes also induces IL-10 expression (66), and inhibits inflammatory cytokine (IL-6 and TNFα) release (67,68). Importantly, IgA has only limited capacity to activate the complement system, in contrast to IgG and IgM. Furthermore, it can competitively block the IgG-mediated activation of complement (53,69). Of note, a few specific diseases are associated with an increase in serum IgA levels, often paralleled by IgA tissue deposition (70). In IgA nephropathy, the formation of aggregated IgA immune complexes in the kidney causes severe inflammatory responses (71,72). However, there are indications that in these patients glycosylation of the circulating IgA antibodies is abnormal, which may explain the pathogenic potential (73).

Collectively, these data suggest that under homeostatic conditions secretory IgA contributes to the maintainance of mucosal tolerance by dampening immune responses. Therefore, IgA can have a role in preventing the development of hyperinflammatory responses towards environmental allergens, that otherwise could cause allergic inflammation as observed in allergic rhinitis or asthma.

Regulation of Immunoglobulin A responses

T cell dependent and T cell independent IgA class switching

Mature B cells can acquire IgA expression by undergoing class switch recombination (CSR). This process allows isotype switch of antibodies from one class to another; e.g. from IgM antibodies to IgA. It involves the exchange of the upstream donor Cμ and Cδ genes with a downstream acceptor Cα gene through a DNA recombination process guided by switch regions (74). In response to repeated exposure to Ag, somatic hypermutation and clonal selection optimize the binding specificity and affinity of the antibody (affinity maturation).

In contrast to other isotypes, IgA class switching can occur via a conventional T cell dependent (TD) pathway and an alternative T cell-independent (TI) pathway. TD IgA class switching occurs primarily in special inductive sites (mucosal follicles, such as intestinal Peyer's Patches and mesenteric lymph nodes) from which the resulting IgA^+-B cells will migrate to the draining effector sites, where they become plasma cells and start to secrete IgA. TD class switch is induced by CD40-CD40L ligation and specific cytokines secreted by T cells as a result of activation by DCs or other APCs. The major cytokine signal for α-CSR is TGF-β with contributions from IL-2, IL-4, IL-5, IL-6, IL-10, and IL-21 (**Figure 4** TD pathway).

The alternative TI pathway is a much faster mechanism to generate IgA and can occur locally at effector sites, like the lamina propria. TI class switching is induced independently of CD40-

CD40L engagement and needs alternative costimulatory signals, such as B cell activating factor of the TNF family (BAFF, also known as BLyS), a proliferation-inducing ligand (APRIL), Retinoic Acid (RA), TGF-β, Nitric Oxide (NO), and/or IL-6. These IgA costimulatory factors can be produced in part by both resident epithelial cells of mucosal organs and by local DCs. In fact, mucosal DCs, from Peyer's Patches (PP), gut lamina propria (75) or lungs (76), are the primary APCs able to drive TI IgA class switching (**Figure 4** TI pathway). Once CSR has taken place, most of these factors, including BAFF and APRIL, further enhance both TD and TI IgA responses by providing survival signals, and/or inducing plasma cell differentiation and IgA secretion, pointing at an additional and crucial role of structural cells and DCs at a later stage of IgA development (77-79).

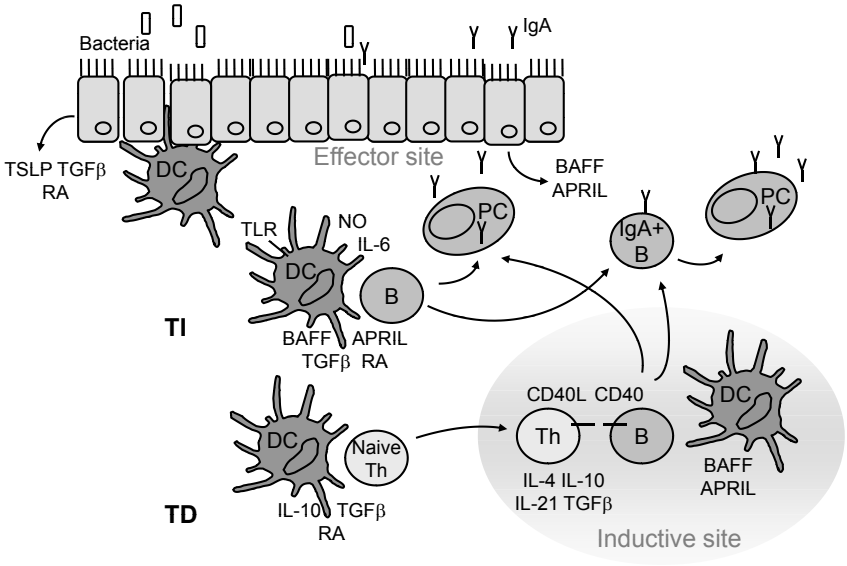

Figure 4. After conditioning by tissue-derived factors and TLR ligands, DCs activate T cells which provide for co-stimulatory signals (CD40-CD40L) and specific cytokines (TD), or DCs provide for alternative co-stimulatory signals, like BAFF and APRIL (TI) to initiate class switch recombination and expression of IgA by the mature B cells (IgA+ B). From the inductive site, the IgA^+-B cells will migrate to the draining effector sites, where, in response to additional signals, they become plasma cells (PC) and start to secrete IgA.

It might be possible to induce in situ CSR of existing allergen specific IgE^+ B cells into IgA_2^+ cells. Because $C_H\alpha_2$ is the last exon located downstream from $C_H\varepsilon$ in the human heavy chain locus, it is the only alternative for CSR in IgE^+ B cells. Shifting the allergen-specific antibody response from IgE to IgA2 would result in neutralization of allergen in the mucosal lumen, before it could interact with IgE, and could constitute a therapeutic target. Potentially, IL-21 in combination with TGF-β could be involved in inducing IgA2 production in already class-switched B cells (80).

Dendritic cells and TLRs

Mucosal conditioning of DCs occurs via resident tissue-derived factors, such as Thymic Stromal Lymphopoietin (TSLP), IFN-β, RA and TGF-β, but also by ligation of Toll-like receptor (TLR) ligands expressed by (commensal) bacteria (81-83). Epithelial cells release these DC conditioning factors in addition to other IgA stimulatory factors in response to TLR ligands (**Figure 4**) (84). In the gut lamina propria, several specialized DC subsets are described with an enhanced intrinsic capacity to drive IgA CSR. For example, Tip DCs express inducible Nitric Oxide Synthase (iNOS) in response to TLR signaling and initiate TI IgA production by releasing BAFF and APRIL (85). $CD11c^{hi}CD11b^{hi}$ DCs induce TI IgA production upon sensing bacteria through TLR5, a process that elicits release of RA and IL-6 (86). $CD103^{+}$ DCs are known for driving the differentiation of FoxP3+ Treg cells. As the main RA producing subset, they are also responsible for imprinting gut-homing molecules on B cells, and support IgA synthesis (75). How these DCs acquire their tolerogenic properties is not yet fully understood, but a role for microbial activation was suggested (87,88). Recently, it was published that *in vitro* mouse $CD11b^{hi}$ lung DCs induce IgA more efficiently, than $CD103^{+}$ lung DCs (89). The two main DC subsets, myeloid DCs (mDCs) and plasmacytoids DC (pDCs), respond differently to TLR ligands. In contrast to mDCs, activated pDCs mediate B cell differentiation and IgA production (90) via APRIL and BAFF expression (91).

Altogether DCs form a crucial cell type in the differentiation of IgA responses and their IgA inducing capacity can be enhanced by both local factors produced by mucosal tissues and by (local) microbial products such as TLR ligands.

Early priming and the microbiota

The establishment of commensal flora in the intestine, and most likely also the respiratory tract (92), starts at birth and is considered to be crucial for stimulating and directing the development of the host immune system. Animals raised under germ-free conditions have an undeveloped immune system with fewer germinal centers and decreased number of IgA-producing plasma cells (93). Interestingly, gut microbiota are necessary for a protective immune system, including mucosal IgA responses, in the airways. In response to OVA, germ-free (GF) mice developed more severe features of airway inflammation compared to control specific pathogen free (SPF) mice, which could be reversed by recolonization of GF mice with complex commensal flora. Furthermore, the absence of commensal bacteria was associated with less pDCs and attenuated production of IgA in the airways in the (94). Human studies have also suggested the link between commensals and allergy. Indeed, children who developed allergy had significantly less diverse gut microbiota and lower levels of salivary SIgA (95), while, intestinal colonization by *Staphylococcus aureaus* was associated with high circulating IgA levels and with a lower frequency of eczema (96). The exact number and diversity of an individual's community of commensals seem to be determined by factors occurring in early childhood (97). Several mouse and human studies have shown that early life (prenatal, preconception) exposure to environments characterized by a diverse and concentrated microbial milieu such as traditional farming sites may protect against

the development of allergic diseases (98-101). Breast milk contains many Igs, and may have a collective tolerogenic effect acting via sIgA, cytokines and/or immune complexes (102).

A rich microbial environment contributes to mucosal tolerance and protective IgA responses, which are associated with protection against allergic asthma. The ideal candidate for an adjuvant stimulating protective IgA responses and thereby preventing development of allergic asthma could therefore be a microbial derived molecule.

Cholera Toxin B

Cholera toxin (CT) is the most widely experimentally used mucosal adjuvant, potentiating serum and local immune responses to co-administered antigens. It has been reported that oral and/or nasal immunizations of antigens with CT induces strong mucosal IgA and serum IgG and IgE responses (103). Although some investigators have observed Th1 responses using CT as an adjuvant (104), most reports have shown a bias towards Th2 responses. A more recent study, Lavelle demonstrated that CT also promotes the induction of Treg cells specific for co-administrated antigen KLH by modulating DCs (105). The enterotoxin Cholera Toxin is produced by the bacterium *Vibrio Cholera,* and consists of an A and B subunit each with distinct effects on cells of the immune system. The A subunit is known for its toxic (side) effects: after entering the cell cytosol, the A subunit triggers electrolyte efflux via activation of adenylate cyclase and increased cyclic AMP (cAMP) production, resulting in severe watery diarrhea. The B subunit of CT (CTB) is more considered as a non-toxic subunit, as it is not linked to the activation of cAMP. It binds to the GM1- ganglioside receptor, which is found ubiquitously on the cell surface of all mammalian cells, including T cells, B cells and DCs. Initially, it was thought that the B subunit only mediated receptor interactions that result in internalization and uptake of the toxic A subunit, explaining the adjuvant activity of the holotoxin CT. However, to avoid toxicity, isolated B subunits of CT have been explored for their ability to augment immune responses against co-administered antigens. Interestingly, the adjuvant activity of CTB seems to be mainly associated with immunoregulatory events (106,107). For example, feeding of CTB conjugated to myelin basic protein before or after disease induction protected rats from experimental autoimmune encephalomyelitis (108), and nasal administration of CTB-insulin significantly delayed incidence of spontaneaous diabetes in NOD mice (109). In these models, protection against autoimmunity by CTB/Ag conjugates was associated with the formation of Treg cells expressing IL-10 and/or TGF-β (108). The tolerizing effect of CTB has also been shown to extend to other immune-mediated diseases. In a delayed type hypersensitivity model, (prolonged) oral treatment with low doses of OVA conjugated to CTB prevented sensitization and suppressed IgE antibody responses in sensitized mice (110). Furthermore, intranasal pretreatment of CTB linked to the BetV1, a major allergen of birch pollen, prevented sensitization to the antigen and subsequent IgE antibody development by shifting the Th2 response towards Th1. In addition, they found

that CTB conjugates markedly enhanced allergen-specific IgA responses (111). CTB can modulate specific B cell and T cell activation by directly acting on these cells, but its primary action as an adjuvant is probably mediated through a direct effect on APCs such as DCs. Administration of CTB leads to recruitment of DCs to the exposed areas (112,113) which would greatly facilitate Ag uptake and MHC class II restricted Ag presentation by DCs. In contrast to CT, CTB can induce TGF-β and IL-10 production by DCs, while suppressing IL-6 (106). Of note, the effect of CT/CTB (on DCs) seems to be very much dependent on route of administration, type of antigen, nature of coupling to the antigen and the disease model used (114).

The non-toxic microbial compound CTB could form an interesting candidate to study for its potential to increase mucosal IgA responses in the lung, and thereby suppressing the development of allergic diseases like allergic asthma.

Aim of this thesis

In this introduction it was pointed out that there is an inverse relationship between IgA and allergy development, suggesting a protective effect of IgA against allergies including asthma. Indeed, IgA contributes to maintaining immune tolerance at mucosal surfaces by dampening immune responses. DCs are a crucial cell type in the regulation of IgA synthesis and signals from the microbiota contribute to proper protective IgA responses. Based on this, we hypothesized that actively stimulating mucosal IgA responses by microbial adjuvants, such as Cholera Toxin B (CTB), will contribute to the development of tolerance to inhaled allergens and that this may provide a novel approach to prevent or treat allergic asthma. The studies described in this thesis aimed to investigate this hypothesis.

First, we addressed the preventive and therapeutic potential of the mucosal adjuvant CTB in an ovalbumin driven mouse model of allergic airway inflammation (AAI). In particular, it was studied whether the effect of CTB was associated with the induction of regulatory immune responses such as IgA and/or Treg cells (**Chapter 2**). Using *in vitro* and *in vivo* experiments, we aimed to gain insight into the mechanism by which CTB induces IgA responses and to identify the cell types and the critical signals involved (**Chapter 3**). It is suggested that first line defense and protection against infection by commensal bacteria and certain intestinal pathogens is mediated by IgA antibodies with a polyreactive and/or irrelevant specificity profile. In **chapter 4** it was explored whether this non-specific IgA response similarly protects against allergens and the development of allergic asthma, or that allergen-specific responses are required. Therefore, we studied the effect of CTB on Birch Pollen (BP) induced allergic asthma in WT and transgenic (Tg) mice with a restricted B cell receptor repertoire recognizing an in this context irrelevant antigen (**Chapter 4**). In Chapter 5, we aimed to investigate the IgA-priming capacity of human blood DC subsets of healthy and house dust mite allergic asthmatic individuals and how this capacity is modulated by the TLR7/8 ligand CL97 (**Chapter 5**). Finally, the main findings presented in this thesis are evaluated in a summarizing discussion (**Chapter 6**).

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2

CHOLERA TOXIN B SUPPRESSES ALLERGIC INFLAMMATION THROUGH INDUCTION OF SECRETORY IGA

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Abstract

In healthy individuals, humoral immune responses to allergens consist of serum IgA and IgG4 whereas cellular immune responses are controlled by Treg cells. In search of new compounds that might prevent the onset of allergies by stimulating this type of immune response, we have focused on the mucosal adjuvant cholera toxin B (CTB), as it induces the formation of Treg cells and production of IgA. Here, we have found that CTB suppresses the potential of dendritic cells to prime for Th2 responses to inhaled allergen. When we administered CTB to the airways of naïve and allergic mice, it strongly suppressed the salient features of asthma such as airway eosinophilia, Th2 cytokine synthesis and bronchial hyperreactivity. This beneficial effect was only transferable to other mice by transfer of B but not T lymphocytes. CTB caused a transforming growth factor-β-dependent rise in antigen-specific IgA in the airway luminal secretions which was necessary for its preventive and curative effect, as all effects of CTB were abrogated in mice lacking the luminal IgA transporting polymeric Ig receptor. Not only do these findings show a novel therapeutic avenue for allergy, they also help to explain the complex relationship between IgA levels and risk of developing allergy in humans.

Introduction

Atopy is commonly defined as a tendency of individuals to mount serum IgE responses and/or positive skin prick tests to inhaled or ingested antigens, such as house dust mite, pollen, animal dander or peanut. The type of immunoglobulin produced by allergen-specific B cells is heavily influenced by help from T-helper 2 lymphocytes providing cognate costimulatory molecules and cytokines for immunoglobulin class switching. In allergic individuals, T cells proliferate in response to allergen and are biased for interleukin (IL)-4 production, thus stimulating IgE synthesis. Several studies have showed that non-allergic individuals mount a different type of immune response to allergens, as sometimes reflected by high serum levels of allergen-specific IgA and IgG4 in the absence of specific IgE (1;2). In healthy individuals, T cells fail to vigorously proliferate *in vitro* in response to allergens (3), possibly explained by the low frequency of allergen-reactive T cells in these individuals, or by the existence of counterbalancing regulatory T (Treg) cells actively suppressing responses against allergens through IL-10 and/or transforming growth factor-β (TGF-β) (4;5). The type of immune response to inhaled allergens is influenced by several factors, including genetic makeup, timing and level of allergen exposure in early life, and exposure to exacerbating or protective environmental triggers (6). Central to the process of allergic sensitization are dendritic cells (DCs) that pick up inhaled or ingested allergens. DCs integrate many of the above factors into a signal that is sensed by T cells, and promote a stable Th2 response and long-lasting IgE response or promote a balanced immune response free of pro-inflammatory effector cells (7).

The incidence of allergic diseases, such as asthma, has risen dramatically over the last 50 years, particularly in westernalized countries (6). This rise is thought to result from a disbalance in the immune system, caused by a diminished infectious pressure during childhood. New preventive strategies that might interfere with DC-driven Th2 sensitization are urgently needed to halt this epidemic. As DC function is heavily influenced by microbial danger signals (8-10), we searched for microbial compounds that could prevent DC-driven Th2 responses and allergic asthmatic reactions to inhaled allergens, and at the same time could directly stimulate the immune response to a type observed in healthy individuals, that is, dominated by Treg cells and IgA/IgG4 responses.

In our search, we narrowed down on the non-toxic mucosal adjuvant cholera toxin B (CTB), produced by *Vibrio Cholera*. The enterotoxin cholera toxin is a holotoxin that consists of an A and B subunit, each with distinct effects on cells of the immune system (11). The B subunit has immunomodulatory capacities, through its binding to the asialo-GM-1 receptor on B cells, T cells and DCs (11;12). When CTB conjugated to allergen is administered through the oral or nasal route, it stimulates the formation of $Foxp3^+$ IL-10-producing regulatory Treg cells, in a process requiring TGF-β and/or IL-10 (13-18). In addition, CTB is a classical mucosal adjuvant (12;19) that stimulates the formation of IgA responses to ingested antigens, a process known to require TGF-β for class switching. Here, we have addressed the preventive and therapeutic potential of

CTB given to the airways in a mouse model of asthma. We focused on the relative contribution of altered cellular or humoral immunity and the importance of IL-10 and TGF-β when studying how CTB suppresses asthma. Together, our data uncovered a prominent role for mucosal IgA responses in protection from allergic disease by CTB.

Materials & Methods

Animals

Female BALB/c mice (6-week-old) were purchased at Harlan (Horst, The Netherlands) and kept under specific pathogen-free conditions according to the institutional guidelines of the animal ethics and welfare committee. $PIgR^{-/-}$ mice were provided by Dr Johanson (Oslo, Norway).

A mouse model of asthma induced by adoptive transfer of bone marrow-derived DC

The DCs were generated by culturing bone marrow cells in a medium containing recombinant granulocyte-macrophage colony stimulating factor, as described (20). After 7 days, cells were pulsed overnight with PBS, OVA (100 μg/ml, Worthington Biochemical Corp., Lakewood, NJ), or OVA + CTB (10 μg/ml, Sigma-Aldrich, St. Louis, MO). DCs ($1*10^6$) were instilled through the opening vocal cords, as described (20). On day 10-12, the mice were exposed to OVA aerosols and killed 24 hour after the last aerosol. BAL was carried out and lungs and MLNs were removed and digested using collagenase/DNAse, as described (35). In some experiments, TGF-β-Ab (50 μg, clone 1D11, obtained from ATCC, Manassas, VA) or mouse IgG (isotype control) was daily administered intraperitoneally (IP) at day -1 till day 5 (before and during priming) or from day 10 till day 13 (before and during challenge), or IL-10R-Ab (250 μg; clone 1B1.3a, obtained from Dr. K. Moore (DNAX, Palo Alto, CA)) or rat IgG1 (isotype control) was administered IP at day -1 (before priming) or day 10 (before challenge).

A mouse model of asthma-induced systemic IP sensitization

Mice were sensitized by two IP injections of OVA (10 μg) emulsified in $AL(OH)_3$ (1 mg; Imject Alum, Pierce, Rockford, IL) on day 0 and 7. At day 17-19, mice were challenged with either PBS or OVA aerosols (grade III, 10 mg/ml in PBS; 30 min, Sigma). In some experiments, mice were isofluorane-anesthetized 30 min before each aerosol and received an intratracheal injection of PBS or purified CTB (Sigma). At 24 h after the last exposure, BAL was carried out and lungs and MLNs were removed, as described (35).

Airway hyperresponsiveness

Airway hyperreactivity was assessed by invasive measurements of dynamic airway resistance in response to increasing concentrations of intravenously administered metacholine (Sigma) during mechanical ventilation with a Flexivent apparatus (SCIREQ, Montreal, QC, Canada), as described earlier (36).

Adoptive transfer of MLN cells

At day 17, OVA-sensitized mice (two IP injections of OVA/alum; day 0 and day 7) received intravenously $5x10^6$ MLN $CD19^+$ B cells (positive isolation by $CD19^+$ MACS microbeads; Miltenyi, Bergisch Gladbach, Germany) or $5x10^6$ MLN $CD4^+$ T cells (positive MACS isolation kit for $CD4^+$ T cells; Miltenyi) from donor mice treated as indicated in the legends and challenged with OVA aerosols. After 2 days, recipient mice were challenged with daily OVA aerosols for 3 consecutive days.

Flowcytometry and sorting

BAL cells were stained with antibodies directed against MHCII, CD11c, CD3, CD19 (all from eBioScience, SanDiego, CA), and CCR3 (R&D systems, Abingdon, UK), as described before (37). For isolation of B cells from lung suspensions, B cells were pre-purified using $CD19^+$ MACS microbeads (Miltenyi) and subsequently sorted on B220 and CD19 positivity using a FacsARIA flowcytometer (BD Biosciences, Franklin Lakes, NJ).

Culture of B cells and IgA measurements

The $CD19^+$ lung B cells ($2*10^5$ cells) were stimulated with 10 μg/ml LPS (Sigma). After 7 days, supernatant was harvested and total IgA or OVA-specific IgA was measured by ELISA (BD Biosciences, Franklin Lakes, NJ).

Differentially pulsed or unpulsed DCs were co-cultured with MACS-sorted splenic $CD19^+$ B cells ($1*10^6$ cells per cell type in a ratio 1:1) and anti-mouse IgM $F(ab')_2$ (10 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA). In some conditions, SB431542, a SMAD3 inhibitor (5 μM; Sigma-Aldrich) or a DMSO control was added. After 7 days, supernatant was harvested and total IgA, IgG1, IgG2a or IgE were measured by ELISA (BD). The detection limit was 2 ng/ml.

Cytokine measurements

Cells were plated at a density of $2x10^5$ cells per well and restimulated with 10 μg/ml OVA. After 4 days, IL-4, IL-5, IL-10, interferon-γ (OptEIA, Pharmingen, Becton Dickinson, San Diego, CA) and IL-13 (R&D systems) were measured in supernatants by ELISA. The detection limit of the ELISA-kits is generally 5-10 pg/ml.

Statistical analysis

For statistical analysis, Kruskal-Wallis one-way ANOVA (analysis of variance) was used to calculate the overall P value between the groups. In case of a significant difference in the Kruskal-Wallis analysis, the Mann-Whitney U test was used for unpaired, non-parametric data to compare individual groups separately. P values less than 0.05 were considered significantly. P-values less than 0.05, 0.01 or 0.001 are indicated by one, two or three asterisks.

Results

CTB modulates the potential of DCs to induce allergic sensitization

To test whether CTB has the potential to interfere with DC-driven Th2 responses in the airways, we used an reported model in which inflammatory type DCs (generated *in vitro* by granulocyte-macrophage colony stimulating factor treatment of bone marrow precursors) were pulsed with ovalbumin (OVA) allergen and transferred into the lungs of mice, thus generating Th2 immunity and asthma features (20). In mice receiving OVA-pulsed DCs, strong lymphocytosis, eosinophilia, perivascular and peribronchial inflammation, combined with goblet cell hyperplasia, were induced upon allergen challenge (**Fig 1A and C**). Cultures of mediastinal lymph node (MLN) cells showed the presence of Th2 cytokines IL-4, IL-5, IL-13 and IL-10 (**Fig 1B**). None of these were seen when PBS-pulsed DCs were injected, followed by OVA allergen challenge. The pretreatment of OVA-pulsed DCs with CTB before transfer dramatically altered their pro-inflammatory potential such that it prevented eosinophilic airway inflammation upon rechallenge (**Fig 1A**) and decreased OVA-specific MLN Th2 cytokines (**Fig 1B**). *In vitro* CTB treatment had no toxic effects on DCs and did not alter the expression level of co-stimulatory molecules (MHCII, CD40, CD80 and CD86) known to be important for induction of T-cell responses by DCs (Supplementary Fig 1). Again, in the absence of antigen pulsing, CTB-pulsed DCs failed to induce any changes typical of allergic asthma.

Despite the increase in interferon-γ production in MLN cultures, there were no signs of perivascular or peribronchial inflammation in the lung sections of mice receiving OVA+CTB-pulsed DCs (**Fig 1C**). Moreover, lung mRNA analysis on several inflammatory cytokines and chemokines that are known to regulate allergic inflammation revealed lower levels of IL-6, MCP1, MCP3, eotaxin and its receptor CCR3 and TARC (thymus and activation regulated chemokine), indicatives of a Th2 signature, in mice injected with OVA+CTB-primed DCs compared with those injected with OVA-primed DCs. Importantly, IP-10 and MIG, signature of a type-1 response, were reduced after adoptive transfer of OVA+CTB pulsed DC compared to OVA pulsed DCs (Supplementary Fig 2), arguing against a deviation of Th2 responses towards more Th1 type of inflammation.

CTB modulates the potential of DCs to induce Treg cells, yet Treg cells fail to transfer protection

Having found that CTB treatment of DCs abolished their potential to prime for Th2 responses in the lung, we next addressed the mechanism of action. Earlier findings indicated that CTB-allergen complexes given orally induced the formation of $Foxp3^+$ Treg cells (13;14), which had strong inhibitory effects *in vitro*, partly dependent on TGF-β and IL-10. In accordance, when carrying out *in vitro* co-cultures of OVA+CTB-pulsed DCs together with OVA-specific T cells taken from T cell receptor Tg mice, we observed an increased percentage of IL-10-producing T cells (Supplementary Fig 3A) and an elevated percentage of $FoxP3^+$ cells (Supplementary Fig 3B), when compared with OVA-pulsed DCs. However, when we transferred OVA+CTB pulsed DCs into the airways of naïve mice *in vivo*, followed by OVA challenge, we found no differences in the

number of $CD4^+CD25^{high}FoxP3^+$ T cells in the broncho-alveolar lavage (BAL) compartment (**Fig 1D**) in parallel to reduced $CD4^+CD25^+$ T cells and increased B cells – but instead small differences were found in the percentage of $CD4^+CD25^{high}FoxP3^+$ T cells in the draining MLN from OVA+CTB-DCs-immunized mice (**Fig 1E**). When MLN $CD4^+$ T cells were taken from OVA+CTB DC-immunized mice after OVA challenge, they showed a small, yet significant capacity to reduce OVA-specific T cell proliferation by approximately 30% in *ex vivo* co-cultures of naïve T cells and antigen presenting cells (Supplementary Fig 4A), similar to earlier reports (13,14). This inhibitory capacity was not observed for splenic $CD4^+$ T cells from the same mice (Supplementary Fig 4A).

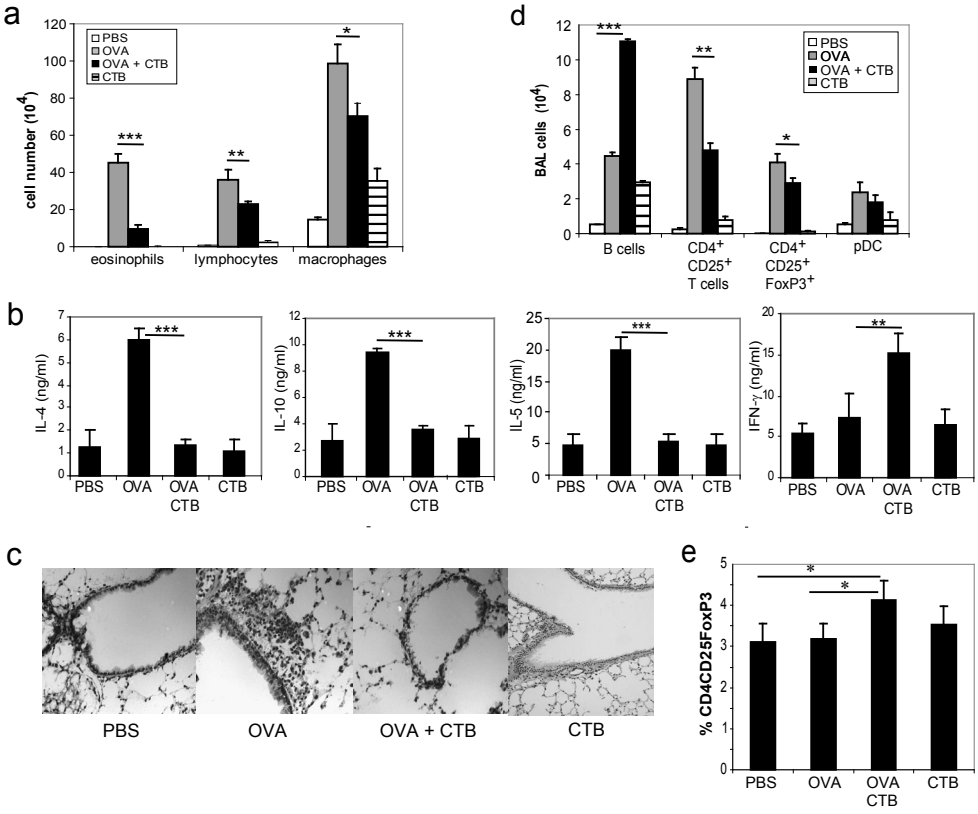

Figure 1: Cholera Toxin B (CTB) inhibits the potential of dendritic cells (DCs) to induce allergic sensitization. DCs were pulsed overnight by phosphate-buffered saline (PBS), ovalbumin (OVA) (100 µg/ml), OVA + CTB (10 µg/ml) or CTB (10 µg/ml). One million DCs were instilled in the airways of naïve mice. After 10 days, the mice were exposed to three OVA aerosols. At day 1 after the last aerosol, the mice were killed and lung lavages were taken. (A) The cellular composition of the BAL was determined by flowcytometry. (B) Mediastinal lymph node (MLN) cells were cultured with OVA (10 µg/ml) for 4 days. Cytokine production was determined by ELISA (enzyme-linked immunosorbent assay). (C) Lungs were fixed with OCT and snap-frozen. Sections of 3-µm thickness were cut and stained with hematoxilin and eosin. The number of $CD4^+CD25^+FoxP3^+$, pDC and B cells in BAL (stained for CD4, CD25, FoxP3, CD19, B220 and 120G8) (D) and the number of $CD4^+CD25^+FoxP3^+$ T cells in MLN (E) was determined by flowcytometry. Data are mean +/- s.e.m, n=4 to 6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of three are shown.

Regulatory T cells exert their regulatory capacity through many mechanisms, including the secretion of immunoregulatory cytokines. Sorted lung $CD4^+$ T cells taken from OVA+CTB DC immunized mice expressed more IL-10 and TGF-β RNA copies compared to mice that received OVA-pulsed or unpulsed DCs (Supplementary Fig 4B). Next we addressed the contribution of Treg cell-derived immunoregulatory cytokines, IL-10 and TGF-β, during either priming dose or challenge. Administration of a neutralizing anti-IL10R antibody did not abolish the inhibitory effect of CTB treatment on allergic responses when given during priming or challenge (Supplementary Fig 5A and B). Remarkably, TGF-β blockade at the time of administration of OVA+CTB pulsed DCs restored eosinophilic airway inflammation, lymphocytosis, and OVA-specific Th2 cytokines in the MLN cells (**Fig 2A and B**). In contrast, blocking TGF-β antibodies were again without effect when given during the challenge phase (Supplementary Fig 5C), arguing against the induction of TGF-β secreting Treg cells as an explanation for allergy suppression. A classical hallmark of Treg cells is their capacity to transfer suppression when adoptively transferred to immunized recipient mice. Adoptive transfer of purified MLN T cells of OVA+CTB-DCs immunized mice into OVA-sensitized recipient mice failed, however, to influence eosinophilic airway inflammation (Supplementary Fig 5D), again arguing against a prominent role for Treg cells in mediating the protective effects of CTB *in vivo*.

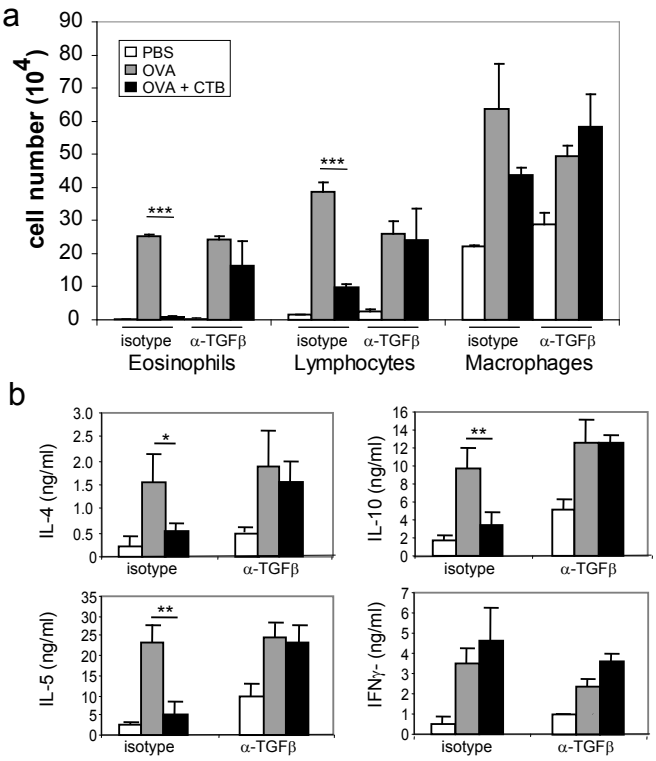

Figure 2: Involvement of TGF-ß in mediating the protective effect of Cholera Toxin B (CTB) on sensitization. Mice were injected with anti-TGF-ß antibodies (11B1; 20 µg) or mouse IgG (isotype control) at day -1 till day 5. At day 0, mice were injected with OVA-pulsed or PBS-pulsed DC, treated or not with CTB and challenged as described in figure 1 legend. (A) BAL cell differential counts were determined by flowcytometry. (B) MLN cells were cultured with OVA (10 mg/ml) for 4 days. Cytokine production was determined by ELISA. Data are mean +/- s.e.m, n=4 to 6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of three are shown.

CTB stimulation of DCs suppresses allergy through induction of IgA-secreting B cells

The only consistent finding in mice receiving OVA+CTB-pulsed DCs was a 3-fold increase in B cells in the BAL fluid, compared with mice receiving OVA-pulsed DCs (**Fig 1D**). This finding together with the crucial early dependence of the protective effect of CTB on TGF-β, made us hypothesize that CTB suppressed allergy development mainly through effects on mucosal humoral immunity. TGF-β is an important switch factor for B cells to produce IgA molecules (21) and CTB is used as a gastro-mucosal adjuvant to induce production of IgA antibodies (12;19). To examine the possible involvement of IgA, we set out to investigate whether CTB treatment of DCs could induce local IgA production in lung mucosal tissues (**Fig 3A**). When OVA-pulsed DCs were exposed to CTB before transfer to the lungs, there was a rise in BAL levels of total IgA and OVA-specific IgA (**Fig 3A**), as well as an increase in total IgA production by *ex-vivo* LPS-stimulated $CD19^+$ lung B cells (**Fig 3B**), compared with mice receiving untreated OVA-pulsed or PBS-pulsed DCs.

To directly address the potential of OVA+CTB pulsed DCs to stimulate immunoglobulin class switching of B cells, we carried out *in vitro* co-culture of DCs with purified $CD19^+$ spleen B cells, in the presence of anti-IgM Fab fragments (22). In these cultures, OVA+CTB treatment of DCs induced an increase in IgA production, an effect not seen when cells were unpulsed or pulsed with CTB alone or OVA alone (**Fig 3C**). Importantly, under conditions of OVA+CTB stimulation of DCs, the production of IgG1, IgG2a or IgE was hardly induced (Supplementary Fig 6) and could not be explained by differences in B cell proliferation as equal proliferation was induced by either OVA or OVA+CTB pulsed DCs. Control cultures of splenic or lung B cells stimulated with CTB directly in the absence of DCs did only induce background levels of total IgA (data not shown). Although we could not detect differences in DC-TGF-β mRNA levels between the groups, the IgA induction by OVA+CTB pulsed DCs appeared to be dependent on TGF-β receptor signaling, as the addition of a SMAD3 inhibitor (a TGF-β -specific signaling molecule) substantially abolished the IgA production induced by OVA + CTB pulsed DCs (**Fig 3C**).

To investigate whether B cells were responsible for the reduction in eosinophilic airway inflammation upon allergen challenge in OVA + CTB-DCs-treated mice, MLN B cells taken from mice instilled with differently treated DCs were adoptively transferred to OVA-sensitized mice. Upon OVA challenge, eosinophilia and lymphocytosis were significantly reduced in mice injected with B cells from OVA+CTB-DCs immunized mice, in contrast to mice injected with B cells from OVA-DCs immunized mice or mice receiving no B cells (**Fig 3D**). The injection of naïve B cells or B cells from KLH+CTB-DCs immunized mice was also without effect (data not shown). Similarly, adoptive transfer of *in vitro* modulated B cells from co-cultures of OVA+CTB pulsed DC and $CD19^+$ spleen B cells reduced eosinophilic airway inflammation (**Fig 3E**) and Th2 cytokine production (**Fig 3F**) to the same extent, as *ex vivo*-isolated MLN B cells (**Fig 3D**).

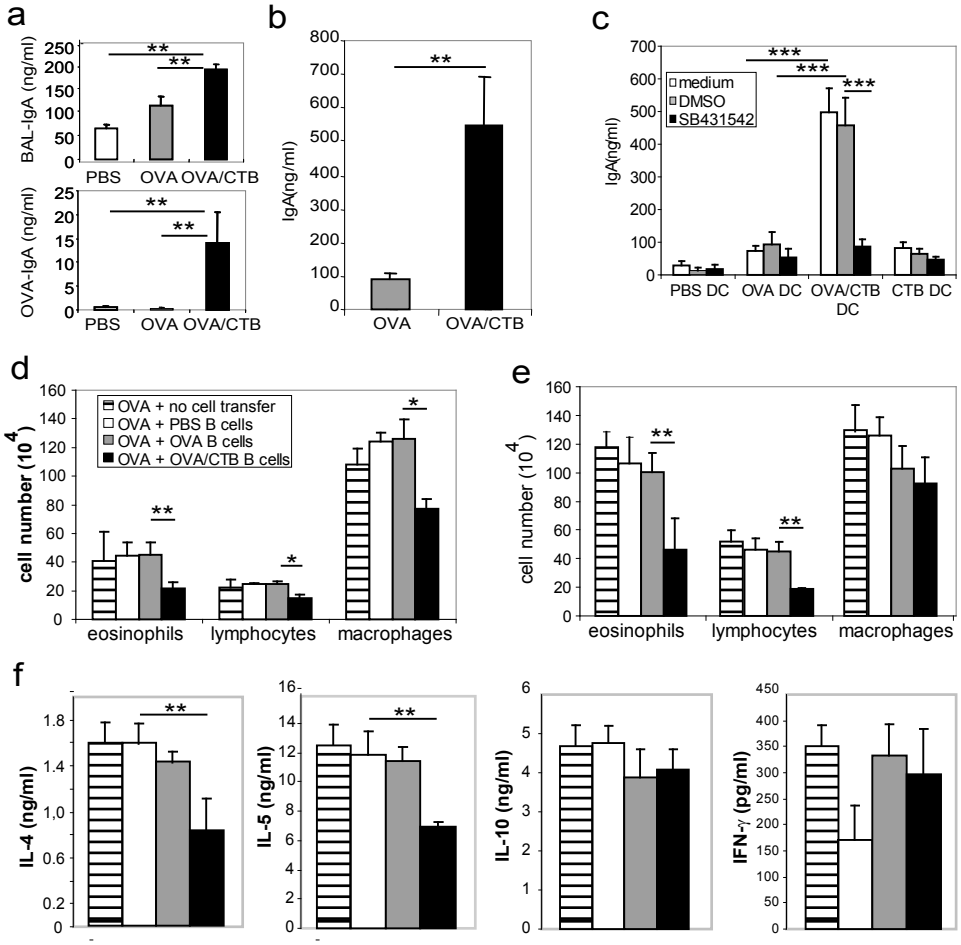

Figure 3: Contribution of IgA and B cells to the protective effect of Cholera Toxin B (CTB). (A) Mice were treated as described in the legend of figure 1. Total IgA and OVA-specific IgA in BAL fluid were determined by ELISA. (B) $CD19^+$ lung B cells were sorted. B cells were stimulated by LPS (10 mg/ml) for 7 days. Total IgA was determined by ELISA. (C) Bone marrow-derived DC were cultured and pulsed as described in the legend of figure 1. One million DC were co-cultured with $1*10^6$ $CD19^+$ spleen B cells, anti-IgM Fab-fragments (10 µg/ml), and in some conditions SB431542 (5 µM) or a DMSO control was added. After 7 days, supernatants were harvested and total IgA was measured by ELISA. (D) MLN B cells were isolated by CD19-microbeads and injected intravenously ($5*10^6$) in previously OVA-sensitized mice at day 10. After two days the mice were challenged and BAL cell differential counts were determined by flowcytometry. (E) Cells were treated as described in C. After 7 days, the B cells were harvested and injected intravenously ($5*10^6$) as in D. Mice were treated as in D. BAL cell numbers were determined by flowcytometry. (F) MLN from experiment described under E were taken and cells were stimulated by OVA (10 mg/ml). After 4 days, supernatants were taken and cytokines determined by ELISA. Data are mean +/- s.e.m, n=4 to 6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of two or three are shown.

The protective effect of CTB induced B cells on allergy depends on IgA secretion in the luminal compartments

As it was shown earlier that IgA administered directly into the lung can capture and neutralize inhaled allergen (23), it was important to know whether the protective effect of IgA-producing B cells on allergic inflammation was because of secretory IgA being present in the airway lumen. For this, we used mice deficient for the polymeric Ig receptor (pIgR), which is necessary for transport of dimeric IgA across the epithelium into the lumen of mucosal areas (24). The B cells taken from OVA- or OVA+CTB-pulsed DCs immunized mice were adoptively transferred into OVA-sensitized pIgR-deficient mice. In contrast to transfer into wild-type (WT) mice (**Fig 4A**), we found no reduction in eosinophilic airway inflammation, airway lymphocytosis and MLN Th2 cytokines by adoptive transfer of B cells into pIgR-deficient mice (**Fig 4B**). It is noteworthy that OVA sensitization by OVA-alum injections in pIgR-deficient mice resulted in much weaker eosinophilic airway inflammation and MLN Th2 cytokines (in particular IL-5) than those in WT mice.

The inhibitory effects of CTB on DC-driven Th2 development were also observed when we used CTB *in vivo*. In a model of sensitization to inhaled OVA through the respiratory route, we could show that intratracheal administration of CTB prevented the onset of Th2 sensitization, as a dose-dependent decrease in BAL eosinophilia, lymphocytosis and LN Th2 cytokines was observed (Supplementary Fig 7A and 7B), whereas LN interferon-γ secretion remained unchanged. In addition, IgA production by *ex vivo* LPS-stimulated B cells was increased by increasing dosages of intratracheally applied CTB (Supplementary Fig 7C).

Intrapulmonary administration of CTB reduces allergic airway inflammation in OVA-sensitized mice

In view of the powerful preventive effect of CTB on development of DC-driven allergic inflammation by induction of IgA, we also wanted to obtain information whether CTB could be used in a secondary prevention setting, that is, in mice that are already sensitized to OVA. To this end, OVA-alum sensitized mice were injected intratracheally with PBS or CTB before each OVA aerosol challenge. CTB induced a significant and dose-dependent decrease in BAL eosinophilia and lymphocytosis (**Fig 5A**), LN Th2 cytokines (**Fig 5B**), airway resistance in response to metacholine (**Fig 5C**), and a reduction in the BAL levels of Th2-selective chemokines (MDC and TARC; **Fig 5D**), than in PBS-treated mice, thus showing that CTB can also reduce Th2-mediated allergic features in sensitized mice. To address a possible role for secreted IgA in the therapeutic efficiency of CTB in already primed mice, we also applied CTB into the lungs of OVA-sensitized WT or $pIgR^{-/-}$ mice before each OVA challenge, revealing a significant reduction in airway eosinophilia (**Fig 5E**) and LN Th2 cytokines (data not shown) in WT mice, but unchanged parameters in $pIgR^{-/-}$ mice.

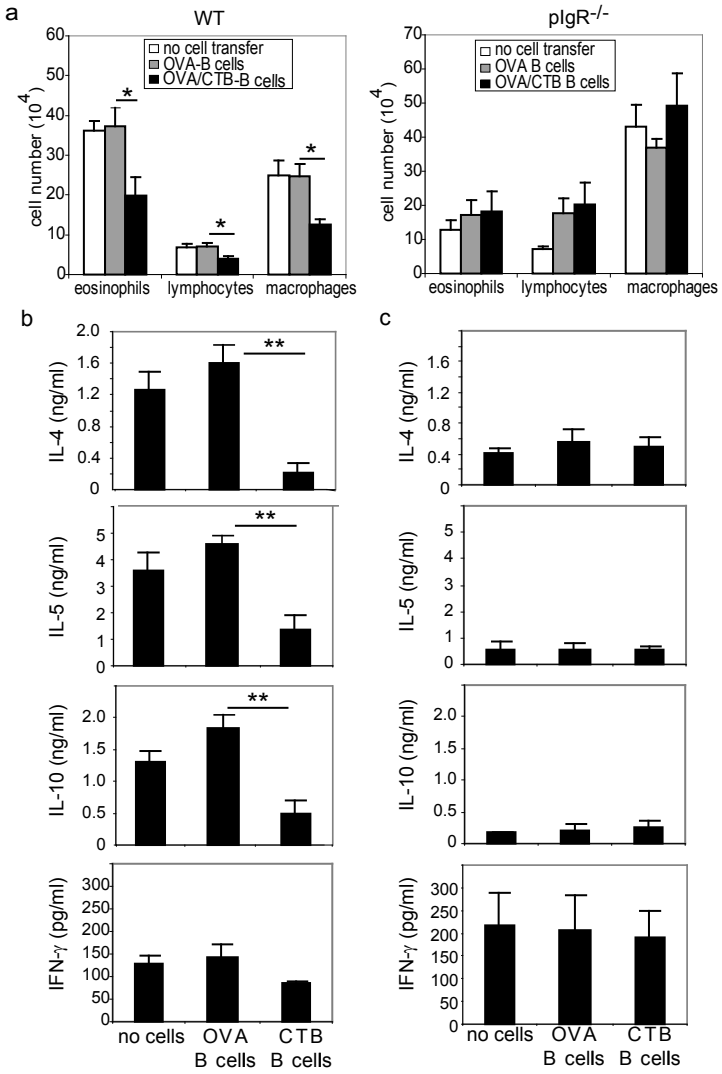

Figure 4: Cholera Toxin B (CTB)-induced B cells transfer suppression of allergy via secretory IgA. Mice were injected with pulsed BM-DC and challenged as described in figure 1 legend. MLN B cells were isolated by CD19-microbeads and intravenously injected ($5*10^6$) in previously OVA-sensitized wild type (WT) or $pIgR^{-/-}$ mice at day 10. After 2 days, the mice were challenged and BAL cell differential counts were determined by flowcytometry (A). MLN cells were cultured with OVA (10 mg/ml) for 4 days. Cytokine production was determined by ELISA (B: WT; C: $pIgR^{-/-}$). Data are mean +/- s.e.m, n=4 to 6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of two or three are shown.

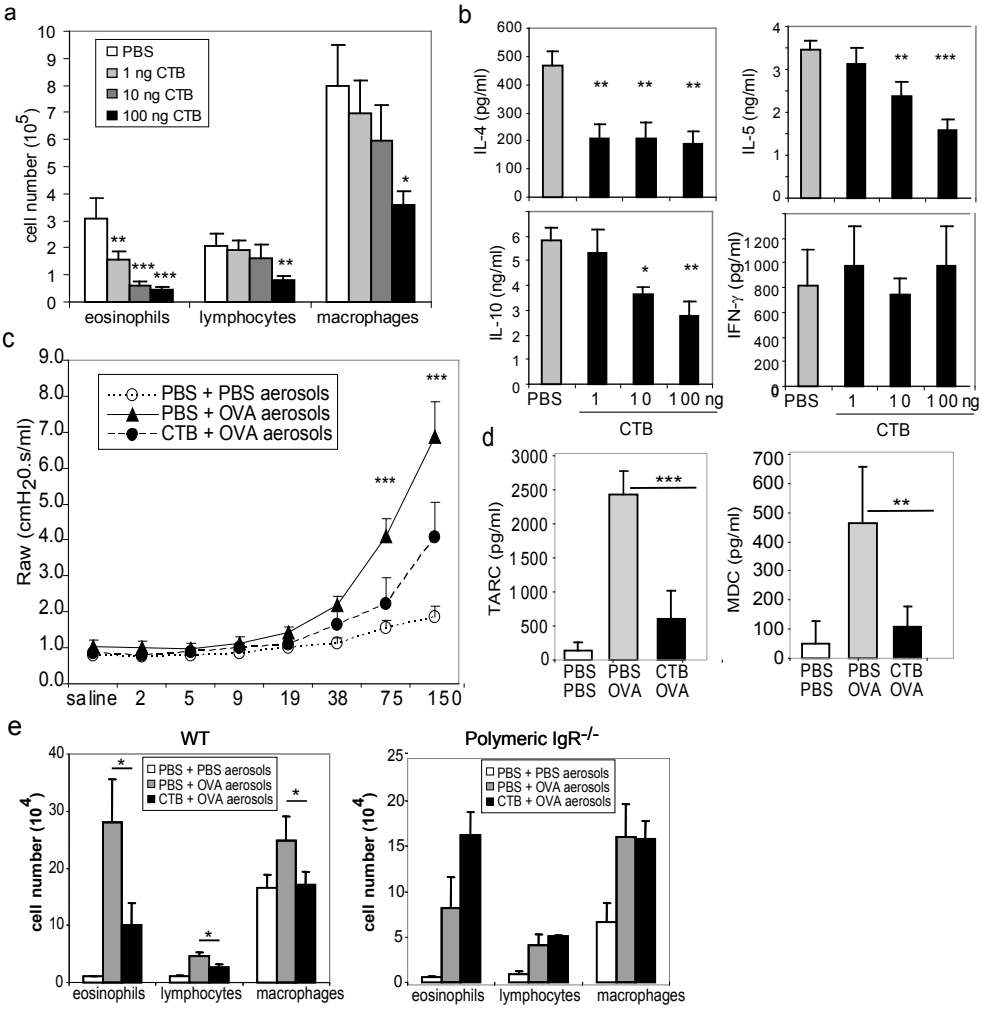

Figure 5: Intrapulmonary administration of Cholera Toxin B (CTB) reduces allergic airway inflammation and bronchial hyperreactivity in already sensitized mice. Mice were OVA-sensitized by two injections of OVA/Alum 1 week apart. At day 17, the mice were challenged by three OVA aerosols. Before each aerosol mice were intratracheally instilled with PBS or CTB. (A) BAL cell differential counts were determined by flowcytometry. (B) MLN cells were cultured with OVA (10 mg/ml) for 4 days. Cytokine production was determined by ELISA (C) At 1 day after challenge, bronchial hyperreactivity was measured by invasive measurements of dynamic airway resistance (Flexivent system) in response to increasing concentrations of intravenously administered metacholine (Sigma) during mechanical ventilation. (D) Chemokine production (TARC and MDC) in BAL supernatant was determined by ELISA. (E) WT and pIgR-/- mice were OVA-sensitized. At day 17, the mice were PBS or OVA challenged. Before each aerosol mice were instilled with PBS or CTB. BAL cell differential counts were determined by flowcytometry. Data are mean +/- s.e.m, n=6 to 8 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of two or three are shown.

Discussion

We show here that CTB strongly suppresses the cardinal features of allergic airway inflammation when applied locally to the lung through a mechanism involving IgA-secreting protective B cells. Although we cannot exclude the indirect stimulatory effects of CTB by interaction with other cell types, such as bronchial epithelial cells, we believe that CTB has this effect by altering DC function, thus programming DCs to induce IgA-producing B cells. This conclusion is based not only on the fact that *in vivo* adoptive transfer of CTB-pulsed DCs leads to an induction of IgA-producing B cells but also on the fact that *in vitro* co-culture of bone marrow-derived DCs that were exposed to CTB with purified $CD19^+$ B cells lead to a similar induction of IgA-producing B cells. IgA-producing B cells that were induced under both conditions protected from allergic inflammation upon transfer in OVA-sensitized recipient mice (**Fig 3**). Furthermore, we observed inhibitory effects of pulmonary administration of CTB on DC-driven Th2 development (Supplementory Fig 7) in a recently described model of respiratory sensitization to inhaled allergen that depends totally on the function of conventional lung DCs, as plasmacytoid DCs are depleted using depleting antibodies (25). Supporting the idea that CTB can alter the function of DCs, other investigators showed that CTB induces the accumulation DCs in the exposed epidermis and dermis (9), whereas oral feeding of CTB leads to the recruitment of $CD11c^+$ DC in the intestinal epithelium (10) and migration of subepithelial DCs into mucosal lymphoid tissues of Peyer's patches (26).

Earlier reports have suggested that sublingual or oral application of CTB resulted in enhanced Treg cell development, as increased FoxP3-expression was demonstrated in spleen, Peyer's patches and mesenteric LN (13;14). In those studies, the Treg cells had strong inhibitory effects *in vitro*, that was, in part, dependent on TGF-β or IL-10. In our study, CTB-modulated DC also primed for the development of IL-10 producing $FoxP3^+$ Treg cells both *in vitro* and *in vivo*. However, the *in vivo*-generated Treg cells only displayed a mild inhibitory function in *ex vivo* cultures with OVA-specific T cell receptor transgenic T cells and antigen presenting cells (Supplementary Fig 4). The differences in Treg cell development observed between the various studies that have employed CTB as an adjuvant for immunotherapy might be explained by differences in route of application, the nature of the allergen, or its covalent binding to CTB, as already suggested by Wiedermann et al (16).

Secretory IgA is a crucial component of first-line immune mechanisms at mucosal surfaces and has many anti-inflammatory functions that might suppress allergy (27-29). Supporting evidence for a regulatory role of IgA against allergic diseases comes from patients with selective IgA deficiency, who are at increased risk for auto-immune and allergic disorders (30). Furthermore, antigen-specific IgA is detected in higher levels in the BAL from healthy individuals than from allergic subjects (31) and in serum from tolerized allergic patients following successful allergen immunotherapy (32;33). Our data suggest that intrapulmonary administration of CTB can prevent the onset of allergic inflammation predominantly by inducing this secretory IgA response. CTB or CTB-induced protective B cells clearly do not affect eosinophilic airway inflammation in

pIgR$^{-/-}$ mice, despite the fact that allergic airway inflammation in OVA-sensitized pIgR$^{-/-}$ mice was much lower than that for WT mice. Future studies will have to address in greater detail the reason for this reduced allergic lung inflammation in pIgR$^{-/-}$ mice subjected to a classical asthma protocol, although the extremely high polymeric (but non secretory) IgA levels might be involved in altering the immune responsiveness of these mice to the asthma protocol (24).

One of the mechanisms by which secretory IgA could reduce allergic airway inflammation includes the capturing and neutralization of the allergen, in this way avoiding IgE mediated effector responses. Nevertheless, intranasal treatment of mice with antigen-specific monoclonal IgA antibody also resulted in increased IgG2a serum concentrations, suggesting the influence of IgA on systemic immune responses, beyond the point of molecular allergen avoidance (23). Indeed, recent studies have shown that FcαRI (CD89) can act as an inhibitory receptor in humans. Serum IgA or anti-FcαRI Fab inhibits activating responses of heterologous FcγR or the high-affinity IgE receptor, FcεRI (34). Most strikingly, anti-FcαRI Fab treatment suppressed allergic asthma manifestations in humanized FcαRI-transgenic mice (34). Unfortunately, no mouse counterpart or alternative IgA receptors for the human CD89 have been identified yet.

One aspect of our study that clearly stands out is the fact that CTB programs bone marrow DCs to instruct B cells for IgA class switching. Earlier, it was reported that B cells class switch to IgA production only when exposed to mucosal DC such as Peyer's patch DCs or lung DC (22), switching being explained through the production of mucosal-specific specializations of DCs, such as production of retinoic acid and/or TGF-β. As our data show that CTB exposure of bone marrow DCs endows them with the capacity to promote IgA class switching, it will be very interesting to study whether CTB also induces instructive signals normally associated with mucosal Peyer's patch DCs. At least the signaling through the TGF-β receptor on B cells was necessary to induce IgA production *in vitro*, as revealed by the use of a SMAD3 inhibitor. Future studies will have to address the functional importance of retinoic acid metabolites in this process.

In conclusion, we show that CTB is endowed with the potential to inhibit Th2 sensitization while at the same time stimulating features of the response of non-allergic individuals to allergens, one being the induction of Treg cells (13;14) and the other being the induction of an immunoglobulin response dominated by IgA. Owing to the absence of a correlate for human IgG4 in the mouse, we could not address any effects that CTB might have on IgG4. In search for alternative therapies for allergic diseases, CTB forms a highly promising lead molecule as it uniquely induces protection against allergic airway inflammation through a novel mechanism targeting DC function and resulting in protective IgA.

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Ref Type: Generic
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Supplementary Online Material

Methods:

DCs culture and flowcytometric analysis.

DCs were generated by culturing BM cells in medium containing recombinant GM-CSF, as described(20). After 7 days, cells were pulsed overnight with PBS, OVA (100 µg/ml, Worthington Biochemical Corp., Lakewood, NJ), or OVA + CTB (10 µg/ml, Sigma-Aldrich, St. Louis, MO) or CTB alone. The next day, the cells were incubated with anti-CD11c-APC, anti-MHCII-Fitc and anti-CD40-PE, anti-CD80-PE or anti-CD86-PE (all purchased by BD).

In vitro culture of DCs and OVA-specific T cells.

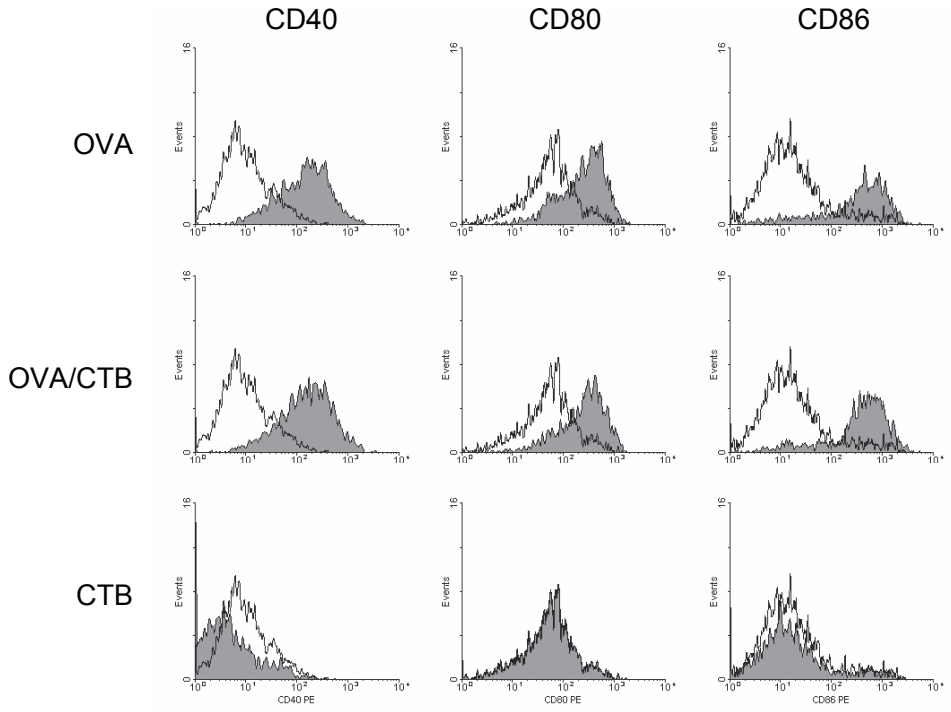
Unpulsed DCs, OVA pulsed, OVA+CTB pulsed or CTB alone pulsed DCs ($1*10^5$ cells/well) were co-cultured with MACS-sorted OVA-specific $CD4^+$ T cells ($1*10^6$ cells/well) from OVA TCR transgenic mice (DO.11.10 strain). After 7 days, T cells were harvested and either fixed by a fixation buffer from the FoxP3-staining kit from eBioscience or restimulated with PMA (100 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) for 6 hours. Brefeldin A (10 µg/ml) was added during the last 4 hours, followed by fixation in 4% paraformaldehyde. Cells were either stained for KJ1-26 (marker for OVA specific TCR transgenic T cells), CD4, CD25 and FoxP3 (all antibodies purchased from BD) or by KJ1-26, IL-4, IFN- γ and IL-10 (all antibodies purchased from BD). Cells were analyzed on a FACS Calibur.

mRNA analysis of lung (T) cells.

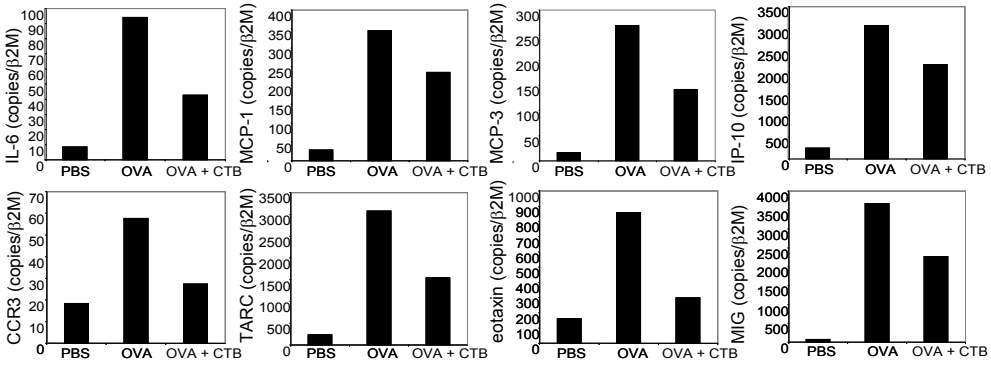
Mice were treated as described in the legend of figure 1. After challenge, lungs were flushed and digested with collagenase/DNAse, as described (36). Subsequently, RNA was isolated from lung cell suspensions or sorted $CD3^+CD4^+$ T cells with RNeasy midi-prep columns (Qiagen, Hilden, Germany), and treated on-column with DNase I, according to the manufacturer's protocol. RNA (1 µg) was reverse-transcribed using superscriptII (Invitrogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 120 min at 42°C. PCR conditions and primer sequences for IL-6, MCP-1, MCP-3, IP-10, CCR3, TARC, eotaxin and MIG were used as described by Kuipers *et al.* (J Leukoc Biol 2004; 76:1028-38).

A mouse model of asthma induced by endogenous airway myeloid DC (mDC).

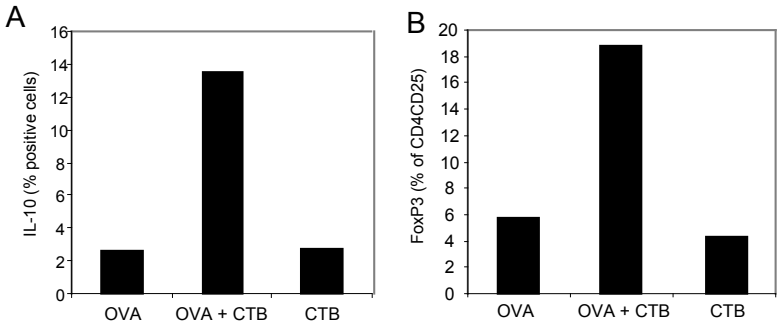
Naïve mice were injected 4 times with a GR1-depleting Ab (RB6-8C5) on days -2, -1, 0, and 1, as described (25). On day 0, 800 µg of OVA (Worthington Biochemical Corp.) was injected i.t., mixed with different concentrations of CTB (Sigma) or PBS. After 10 days the mice were challenged with OVA aerosols on three consecutive days. One day later, BAL was performed and lungs and mediastinal lymph nodes (MLN) were removed and digested using collagenase/DNAse, as described (35).



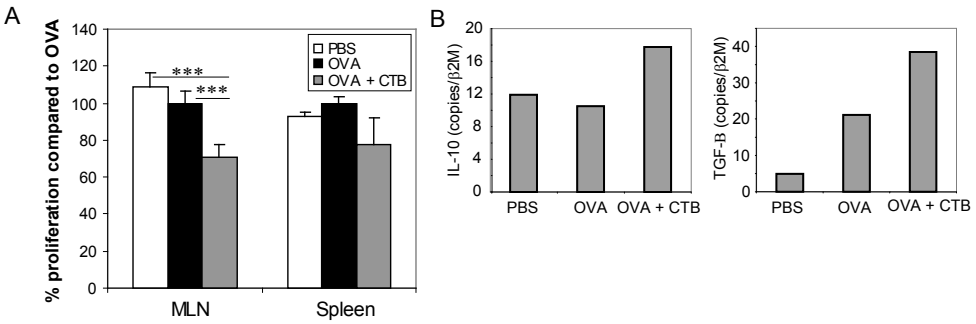
Supplementary Figure 1: CTB does not inhibit OVA-induced DC maturation and activation. DCs were pulsed overnight by medium, OVA (100 µg/ml), OVA + CTB (10 µg/ml) or CTB (10 µg/ml). The next day, CD11c, MHCII, CD40, CD80, CD86 levels were assessed by flowcytometry. The open histogram represents medium or unpulsed DCs. Data from one representative experiment out of ten are shown.



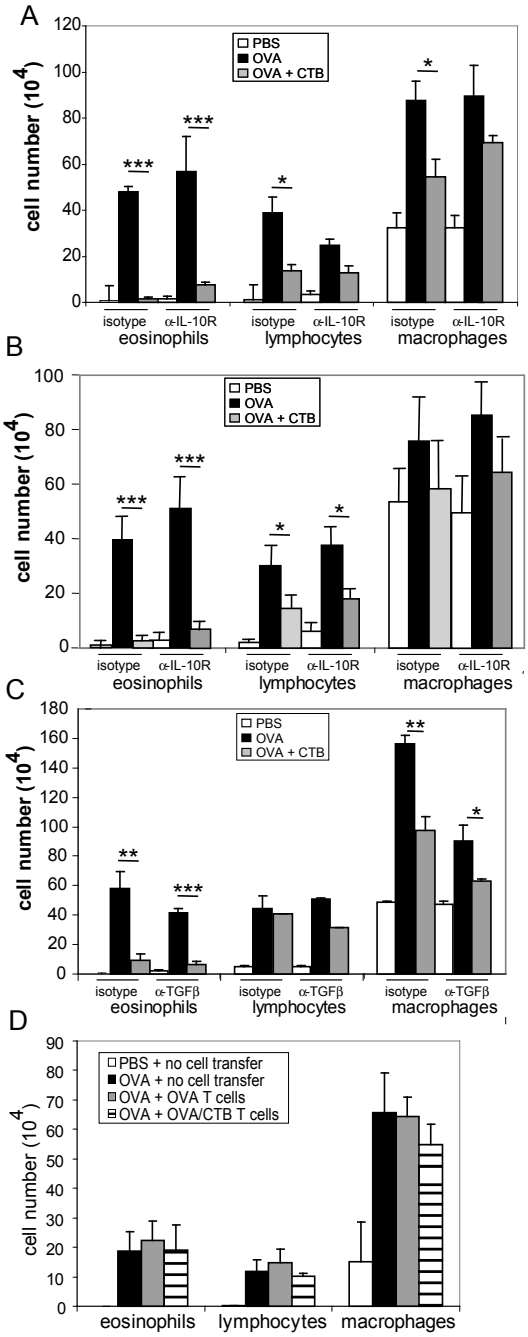
Supplementary Figure 2: OVA plus CTB pulsed DCs do not induce inflammation in the lungs. Mice were treated as described in the legend to figure 1. Lungs were flushed and digested using collagenase/DNAse. Cell homogenates were used to isolate mRNA followed by RT-PCR for IL-6, MCP-1, MCP-3, IP-10, CCR3, TARC, eotaxin and MIG. Data of one representative experiment out of two is shown



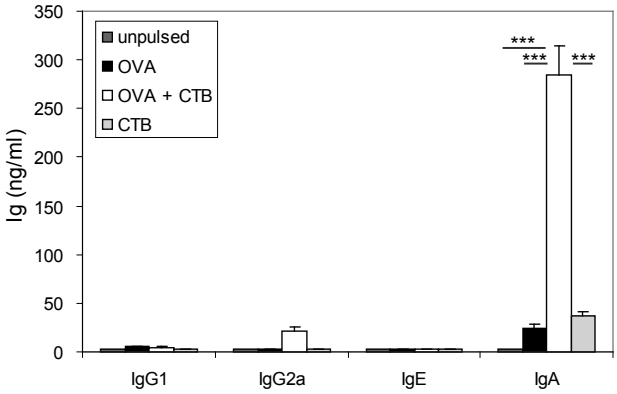
Supplementary Figure 3: OVA+CTB pulsed DCs drive IL-10 producing regulatory T cells *in vitro.* DCs were treated as described in the legend to supplementary figure 1. OVA or OVA + CTB pulsed DC ($1*10^5$ cells/well) were co-cultured with OVA-specific TCR transgenic T cells ($1*10^6$ cells/well). At day 7, the cells were either fixed for FoxP3 analysis (B) or restimulated by PMA/ionomycin in the presence of Brefeldin A for 6 hrs to determine intracellular IL-10 (A). Data of one representative experiment out of three is shown.



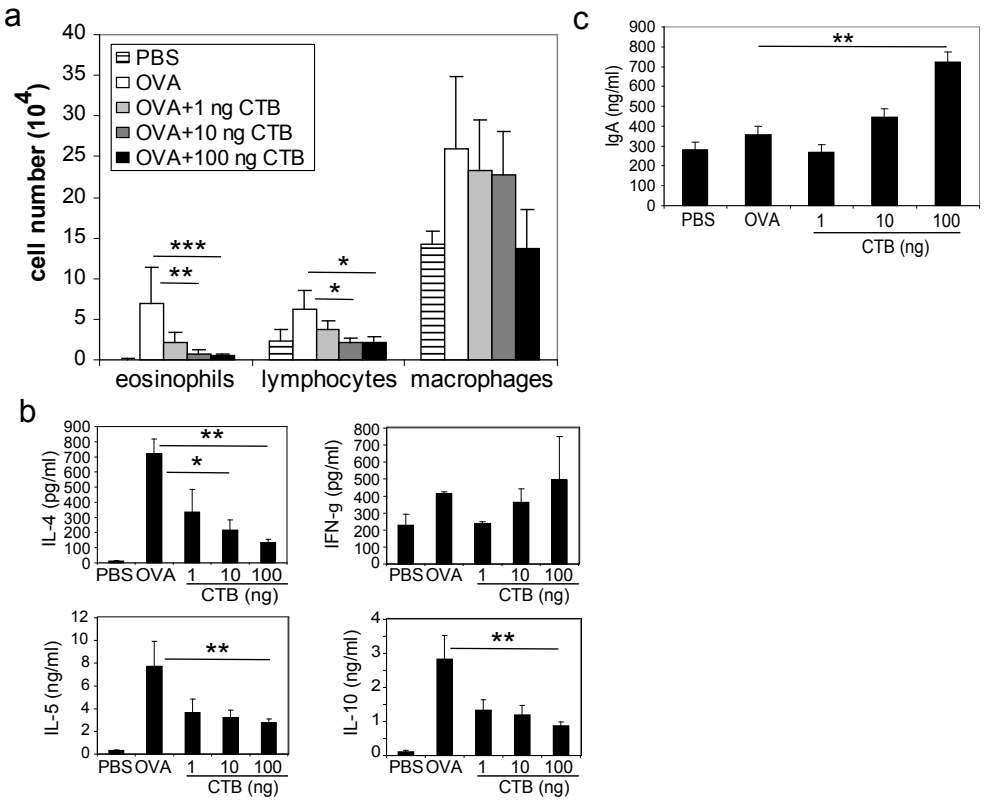
Supplementary Figure 4: T cells from OVA+CTB DC immunized mice show an ex vivo inhibitory activity and express enhanced levels of IL-10 and TGF-β. Mice were treated as described in the legend to figure 1. (A) After challenge, MLN T cells were co-cultured with CFSE-labeled OVA-specific TCR transgenic T cells ($1*10^6$ cells/well) and APCs. At day 4, CFSE dilution in the OVA-specific TCR transgenic T cells was determined by flowcytometry and expressed as a percentage T cell proliferation compared to co-cultures with OVA pulsed DCs. Data are mean +/- s.e.m, n=4-6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of three are shown. (B) Lungs were flushed and digested using collagenase/DNAse. RNA was isolated from sorted $CD3^+CD4^+$ T cells and RT-PCR for IL-10 and TGF-β was performed. Data of one representative experiment out of two is shown.



Supplementary Figure 5: Role of IL-10 and TGF-β in reducing eosinophilic airway inflammation by OVA+CTB pulsed DC. Mice were treated as described in the legend of figure 1. (A) IL-10R-Ab (250 µg) or rat IgG1 (isotype control) was administered i.p. one day before DC instillation. Mice were challenged followed by lung lavages. The cellular composition of the BAL was determined by flowcytometry. (B) At day 0, DC ($1*10^6$ cells) were instilled in the airways and IL-10R-Ab (250 µg) or rat IgG1 (isotype control) was administered i.p. one day before the challenge. One after challenge, lung lavages were taken and the cellular composition of the BAL was determined by flowcytometry. (C) At day 0, DC ($1*10^6$ cells) were instilled in the airways. Anti-TGF-β antibodies (11B1; 20 µg) or mouse IgG (isotype control) was administered i.p. 4 times starting one day before challenge. One after challenge, lung lavages were taken and the cellular composition of the BAL was determined by flowcytometry. (D) After challenge, MLN $CD4^+$ T cells ($5*10^6$ cells) were isolated and injected i.v. into recipient OVA-sensitized mice. Two days after injection, the recipient mice were challenged and lung lavages were taken to determine the cellular composition by flowcytometry. Data are mean +/- s.e.m, n=4-6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of three are shown.



Supplementary Figure 6: OVA+CTB pulsed DC preferentially drive IgA isotype switch in splenic $CD19^+$ B cells *in vitro.* Bone marrow-derived DC were cultured and pulsed as described in the legend of figure 1. One million DC were co-cultured with $1*10^6$ $CD19^+$ spleen B cells and anti-IgM Fab-fragments (10 µg/ml). After 7 days, supernatants were harvested and IgG1, IgG2a, IgA and IgE levels were determined by ELISA. Data of one representative experiment out of three are shown * P<0.05, ** P<0.01, *** P<0.001.



Supplementary Figure 7: CTB prevents allergic respiratory sensitization when given directly to the airways. Naïve mice were injected i.p. with anti-Gr-1 (to deplete pDCs) or isotype control antibodies from day -2 to day 1, injected i.t. with PBS or OVA in the presence or absence of CTB on day 0 and exposed to three OVA aerosols 10 d later. (A) BAL cell differential counts were determined by flowcytometry, (B) MLN cells were cultured with OVA (10 mg/ml) for 4 days. Cytokine production was determined by ELISA. (C) CD19+ lung B cells were sorted. B cells were stimulated by LPS (10 mg/ml) for 7 days. Total IgA was determined by ELISA. Data are mean +/- s.e.m, n=4-6 mice in each group. * P<0.05, ** P<0.01, *** P<0.001. Data from one representative experiment out of three are shown.

3

CHOLERA TOXIN B PRIMES NON-MUCOSAL DENDRITIC CELLS TO PROMOTE IGA PRODUCTION VIA RETINOIC ACID AND TGF-β

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Manuscript submitted, pending revisions

Abstract

It is currently unknown how mucosal adjuvants cause induction of secretory immunoglobulin A (IgA), and how T cell-dependent (TD) or -independent (TI) pathways might be involved. Mucosal dendritic cells (DCs) are the primary antigen presenting cells driving TI IgA synthesis, by producing a proliferation-inducing ligand (APRIL), B cell activating factor (BAFF), Retinoic Acid (RA), TGF-β or nitric oxide (NO). We hypothesized that the mucosal adjuvant Cholera Toxin subunit B (CTB) could imprint non-mucosal DCs to induce IgA synthesis, and studied the mechanism of its induction.

In vitro, CTB-treated bone marrow derived DCs primed for IgA production by B cells without the help of T cells, yet required co-signaling by different Toll-like receptor (TLR) ligands acting via the MyD88 pathway. CTB-DCs induced IgA production was blocked *in vitro* or *in vivo* when RA receptor antagonist, TGF-β signaling inhibitor or neutralizing anti-TGF-β was added, demonstrating the involvement of RA and TGF-β in promoting IgA responses. There was no major involvement for BAFF, APRIL or NO.

This study highlights that synergism between CTB and MyD88-dependent TLR signals selectively imprints a TI IgA-inducing phenotype in non-mucosal DCs, explaining how CTB acts as a mucosal IgA adjuvant.

Introduction

Secretory immunoglobulin A (SIgA) is abundantly present at mucosal surfaces of the gastrointestinal and respiratory tract. Here, SIgA prevents pathogens and commensal bacteria from binding to epithelial cells, it prevents ingested or inhaled allergens to cause immunopathology and it neutralizes toxins, thus broadly acting to maintain homeostasis in the gut and lung (1-4). Inducing IgA synthesis might be beneficial in a number of immune-mediated mucosal diseases like asthma. Interestingly, lack of IgA is associated with increased rates of sensitization to inhaled and ingested allergens (5,6), whereas adoptive transfer of allergen-specific IgA or IgA producing B cells in mice can protect from allergic disease (7,8). If we are to exploit the full potential of IgA as an immunomodulatory immunoglobulin in mucosal diseases, we need to understand better how IgA is induced and how we can promote the synthesis of IgA through the use of adjuvants.

IgA synthesis is induced by both T cell-dependent (TD) and T cell-independent (TI) pathways. In TD IgA synthesis, antigen specific naïve B cells differentiate into IgA^+-committed B cells upon stimulation with CD40L on activated T cells and TGF-β expressed by multiple cell types. Alternatively, TI IgA synthesis is induced in polyclonal naïve B cells by dendritic cell (DC)- and epithelial cell- derived molecules, such as proliferation-inducing ligand (APRIL), B cell activating factor (BAFF), retinoic acid (RA), TGF-β or nitric oxide (NO) (9-11). Mucosal DCs, from Peyer's Patches (PP), lamina propria or lungs (12), are the primary antigen presenting cells that can drive TI (canonical) IgA class switching. Importantly, mucosal conditioning of DCs occurs via tissue-derived factors, such as RA and TGF-β, but also by (commensal) bacteria expressing Toll-like receptor (TLR) ligands (13-15).

We hypothesized that there might exist mucosal adjuvants that imprint a mucosal phenotype of DCs, thereby stimulating humoral IgA responses completely independent of residence in the mucosal environment. We focused on the TLR-independent molecule Cholera Toxin subunit B (CTB), produced by the bacterium *Vibrio cholerae.* Cholera toxin (CT) contains a toxic ADP-ribosyltransferase subunit A, linked to a pentamer of non-toxic B carrier subunits. CTB was shown to bind specifically to GM1-ganglioside (GM1), a receptor found in common on the membrane of most types of epithelial cells, but is also expressed on various hematopoietic cells. CTB is widely used as a mucosal adjuvant, stimulating tolerance to co-administered antigen (16,17). In a mouse model, CTB enhanced IgA responses against inhaled allergens (8). Here we studied whether CTB can mimic mucosal conditioning and prime non-mucosal DCs to induce IgA production, and whether similar molecular signals are involved in the cellular communication between DCs and B cells as described for TI IgA synthesis induced by mucosal PP DCs.

Materials and methods

Ethics statement

Mice were housed under SPF-conditions at the animal facilities of the Leiden University Medical Center in Leiden, the Netherlands. All animal studies were performed according to the institutional guidelines and the experimental protocols described in DEC-09028 were approved by the Ethics Committee for Animal Experimentation of the University of Leiden, the Netherlands.

Animals

Female Balb/c and C57BL/6 mice (6-8 wks) were purchased at Harlan, the Netherlands. MyD88-deficient mice were bred in animal facility (UZ Ghent, Belgium). iNOS-deficient mice were kindly provided by Dr. A Cauwels (VIB Ghent, Belgium).

Cell preparation and *in vitro* co-culture

BM-DCs were generated from bone marrow cells, as described before (8), and cultured in RPMI 1640 medium containing glutamax (GIBCO), 50 μM 2-mercaptopethanol Signa-Aldrich), 50 μg/ml gentamicin Invitrogen) or sodium-penicillin (Astellas Pharma B.V), streptomycin (0,1 ug/ml, Sigma-Aldrich), and 5% fetal bovine serum (TCM), supplemented with 20 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor (rmGM-CSF, (a gift from K. Thielemans, Vrije Universiteit Brussel, Brussels, Belgium). After 8 days, DCs were pulsed overnight with PBS or LPS (1 or 100 ng/ml) with or without CTB (10 μg/ml, Sigma-Aldrich, St. Louis, MO). In specific experiments, BM-DCs were pulsed with different TLR ligands (Poly I:C 25 μg/ml, LPS 100 ng/ml, Flagellin 1 μg/ml, FLS1 10 μg/ml, Cl97 1 μg/ml, CpG 2.5 μg/ml) or C-type lectin receptor ligands (CTL) (Zymozan 10 μg/ml, Curdlan 150 μg/ml) with and without CTB. To isolate PP DCs, lymph nodes were collected 10-12 days after i.p. injection with 6 million B16 Flt3-L secreting melanoma cells (18) and digested by collagenase (type 3 filtered, Worthington) and DNAse I (Sigma-Aldrich). DCs were isolated using CD11c microbeads (Miltenyi), with a purity of >85 % as confirmed by flowcytometry.

After overnight stimulation, these DCs ($1*10^5$) were washed and co-cultured with B cells (1:1 ratio) and anti-mouse IgM $F(ab')_2$ (10 μg/ml; Jackson Immunoresearch, West Grove, PA) (adapted from (19)) for 7 days. Murine B cells were isolated from spleens using anti-CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a purity of around 95% as confirmed by flowcytometry. For some experiments, chemical inhibitors were added: RARβ receptor antagonist LE135 (1 μM in DMSO, Tocris Bioscience, Ellisville, MO), or TGF-ß signaling inhibitor (5 μM, SB-431542, Sigma-Aldrich). Supernatant was collected and immunoglobulin levels determined by ELISA.

***In vivo* mouse models**

OVA (800μg/mouse) +/- CTB (0.001 or 0.1μg/mouse, Sigma-Aldrich, St. Louis, MO) intratracheal administration was followed 10 days later by OVA aerosol challenge for 3 consecutive days. One day after the last challenge, lungs were digested with collagenase/DNAse, B cells isolated (CD19+ MACS isolation), and restimulated with LPS (10μg/ml) for 5 days. Supernatant was collected and IgA production analysed by ELISA. Or AF488-labeled recombinant CTB (10 μg per mouse, Molecular Probes, Eugene, OR) or CTB (0.1μg/mouse, Sigma-Aldrich, St. Louis, MO) +/- OVA (800 μg/mouse) was administered into the airways. The lung draining lymph nodes and lungs were removed after 36 hrs and digested using collagenase/DNAse (8); AF488-fluorescence of several cell types or aldehyde dehydrogenase (ALDH) activity (using ALDEFLUOR assay kit, Stemcell Technologies, as described (20)) of DC subsets was studied by flowcytometric staining.

Unpulsed, OVA- (100 μg/ml, containing LPS, Worthington Biochemical Corp., Lakewood, NJ) or OVA+CTB (10 μg/ml) -pulsed BM-DCs ($1*10^6$ cells) were administered into the airways, as described (21), while (1 day before until 3 days after) TGF-β and retinoic acid activity was neutralized by i.p injection of LE135 and aTGF-β antibody (or vehicle/isotype control Ab). This was followed 10 days later by OVA aerosol challenge on three consecutive days. One day after the last challenge lungs were isolated, digested using collagenase/DNAse and analysed for presence of IgA positive B cells by flowcytometry.

RNA extraction and RT-PCR

BM-DCs were pulsed for 24 hrs and cell pellets were snap-frozen. Subsequently, total RNA was isolated, followed by cDNA synthesis, as described (8). Quantitative real-time RT-PCR was performed using Brilliant SYBR Green (Stratagene, Santa Clara, CA) in an ABI 7500 RT-PCR machine (Applied Biosystems). Expression was normalized to the housekeeping gene GAPDH, and presented relative to conventional unpulsed BM-DCs. Sequences of primers were used as described (14). In addition, RALDH2 expression was studied using the following primer sequences: 5'-AGC CCA TTG GAG TGT GT-3' and 5'-CCA GCC TCC TTG ATG AG-3'.

Immunoglobulin measurements

Ig levels (IgA/IgG1/IgG2a/IgE/IgM) were measured by ELISA (BD Biosciences). Detection limit was 2 ng/ml for all Igs.

Statistical analysis

To study whether there is a general difference between 3 or more groups, first ANOVA test was performed, followed by Bonferoni post hoc test to compare specific groups or the Mann-Whitney U t-test was used to compare two individual groups. P values less than 0.05 were considered significant. P-values less than 0.05, 0.01 or 0.001 are indicated by one, two or three asterisks, respectively.

Results

CTB+LPS-primed bone marrow derived DCs promote IgA production *in vitro*

To study whether CTB could prime non-mucosal DCs to induce IgA production, we designed an *in vitro* co-culture system with LPS maturated bone marrow derived (BM)-DCs co-primed with or without CTB and splenic B cells (Balb/C background), in a one-to-one ratio for seven days (adapted from (19)). Significant levels of polyclonal IgA (~200 ng/ml) were measured in supernatant when B cells were co-cultured with LPS (1 ng/ml)+CTB-pulsed DCs, compared to low levels of IgA (<80 ng/ml) in the control conditions CTB- or LPS-primed DCs. Interestingly, although BM-DCs primed with 100 times more LPS (100 ng/ml; LPS^{hi}) were able to induce a significant IgA production, the addition of CTB during priming dramatically further enhanced the IgA production by co-cultured B cells (**Figure 1b**). Surface IgA staining confirmed the generation of IgA positive splenic B cells (~3-fold increase for LPS+CTB condition) (data not shown). IgA induction by non-mucosal BM-DCs was compared to bona fide mucosal PP-DCs, representing the natural IgA inducing capacity of cells specialized in promoting this response: the priming of BM-DC by LPS+CTB yielded IgA-inducing BM-DCs that were as potent as that of *ex vivo* PP-DCs (**Figure 1a**). The difference in IgA levels (200 vs. 60 ng/ml for LPS+CTB condition) can be explained, as in this experiment the PP-DCs were isolated from C57/BL6 mice, not Balb/C, because of the use of subcutaneously injected B16 Flt3-L secreting melanoma cells (22) to enrich for DCs. In general, low levels of other immunoglobulins (IgG1, IgG2a, IgE) were detected in all conditions (**Figure 1c**), showing that CTB has a predominant IgA promoting effect. Addition of an adenylate cyclase inhibitor did not affect the induction of IgA by LPS+CTB-pulsed DCs, excluding the contribution of Cholera Toxin subunit A traces in this process (data not shown). Importantly, B cells exposed to CTB or LPS+CTB (either or not followed by culture with mature DCs) did not produce IgA, indicating that CTB did not act on B cells directly (data not shown).

These results were reproducible in both C57/Bl6-derived or Balb/C-derived cells, although IgA levels were generally (>3 fold) lower in C57/Bl6 cells (**Figure 1a vs 1b**). Throughout the paper, cultures were done with cells isolated from Balb/C mice, except for cultures with KO cells as these mice are of C57/Bl6 background.

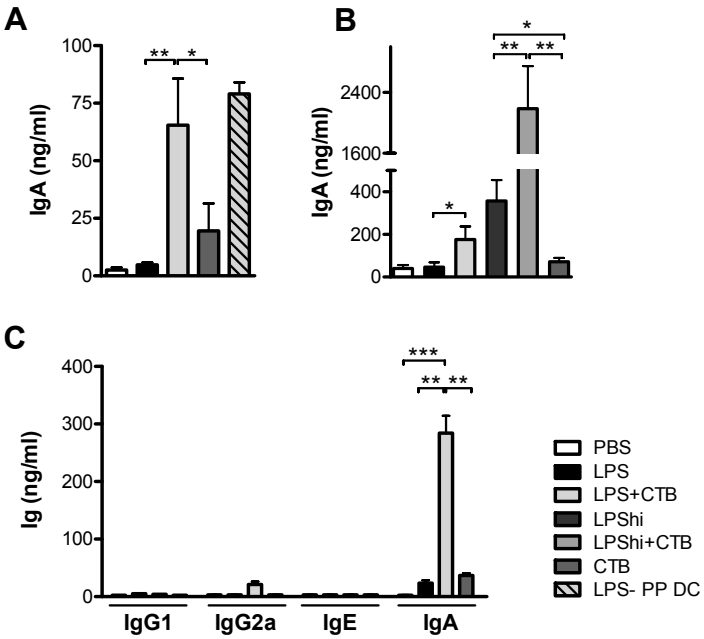


Figure 1. CTB/LPS-primed BM-DCs promote IgA production *in vitro*.
BM-derived DCs were cultured for 8 days with GMCSF, pulsed overnight with PBS, LPS (1 ng/ml) +/- CTB (10 μg/ml) or CTB alone, and then co-cultured with splenic B cells (ratio 1:1) and anti-IgM Fab-fragments (10 μg/ml). After 7 days, IgA levels were determined by ELISA. (A) BM-DC and B cells of Balb/c background were used for the co-culture. In addition to the conditions described, a high concentration (100 ng/ml) of LPS was used to pulse the BM-DCs (B) Besides BM-DCs, PP-DCs were used to put in co-coculture with B cells (all of C57/Bl6 background) (C) In addition to IgA, also other Immunoglobulin isotypes were measured in supernatant of cocultures (Balb/c) by ELISA. Mean + sem of at least 5 individual experiments are shown. * P<0.05, ** P<0.01, *** P<0.001.

MyD88 driven pathways synergize with CTB priming to drive IgA promoting DCs.

The fact that CTB-primed DCs do not induce IgA production by co-cultured B cells and CTB+LPS-primed DCs do, suggests a synergistic effect of CTB- and LPS activated pathways. LPS activates mainly the TLR4 pathway, which includes the downstream adaptor molecule MyD88. The IgA promoting effect of LPS+CTB primed DCs was abrogated when wild type B cells and MyD88-deficient DCs (C57/Bl6 background) were cultured together, even when the high concentration LPS (100 ng/ml) was used, pointing at a crucial involvement of MyD88 (**Figure 2a**).

Next we questioned whether co-activation of the DCs via other Pattern Recognition Receptor (PRR) pathways was also able to promote CTB-driven IgA induction by DCs. Therefore DCs were stimulated with different TLR ligands (FLS1 (TLR2/6), poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), CL97 (TLR7/8), or CpG (TLR9)), or non-TLR PRRs such as C-type lectin ligands (CTLs) Zymosan (TLR2/Dectin) and Curdlan (Dectin-1) with or without CTB, followed by co-cultures with splenic B cells (Balb/C). Interestingly, high level microbial priming of BM-DCs by several TLR ligands alone, e.g.

LPS (high dose, 100 ng/ml)), FLS1, CL97 and CpG, was sufficient to induce IgA production (**Figure 2b**, black bars). Importantly, CTB strongly further enhanced the IgA induction by DCs primed with MyD88-dependent TLRs; but only slightly or not at all for DCs primed with the MyD88-independent TLR ligand (poly I:C) or the non-TLR-dependent CTLs (Zymozan and Curdlan) (**Figure 2b)**. Intriguingly, the IgA inducing capacity of DCs primed by CpG was not further enhanced by CTB, although TLR9 activity is also MyD88-dependent.

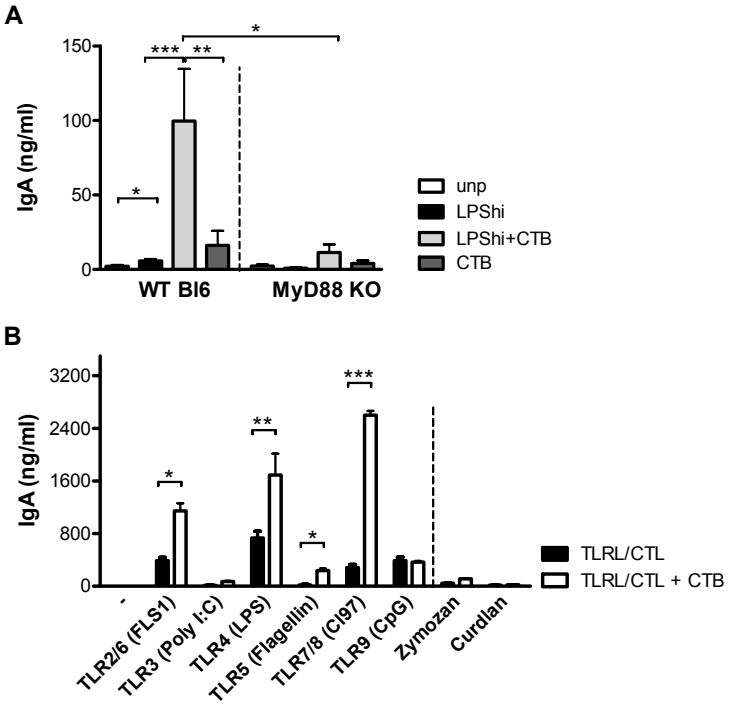


Figure 2. MyD88 driven pathways synergize with CTB priming to drive IgA promoting.
DCs. (A) BM-DCs from WT or MyD88$^{-/-}$ mice (C57/Bl6 background) were pulsed with PBS, LPShi (100 ng/ml), LPShi+ CTB (10 μg/ml) or CTB alone and co-cultured as described at Figure 1. (B) BM-DCs were pulsed with PBS, or different TLR ligands (Poly I:C 25 μg/ml, LPS 100 ng/ml, Flagellin 1 μg/ml, FLS1 10 μg/ml, Cl97 1 μg/ml, CpG 2.5 μg/ml) or CTLs (Zymozan 10 μg/ml, Curdlan 150 μg/ml) with and without CTB, followed by co-cultures with splenic B cells. Data of one representative experiment out of three is shown. * P<0.05, ** P<0.01, *** P<0.001.

Critical involvement of DC-derived RA and TGF-β

Priming of non-mucosal BM-DCs with CTB in synergy with MyD88-dependent TLR ligands leads to IgA-inducing DCs. Several factors, including NO, RA, TGF-β, APRIL and BAFF can promote IgA production at the mucosa (10). To elucidate whether the IgA inducing capacity of CTB-primed BM-DCs was associated with one of these factors, we analyzed gene expression profiles of

RA synthesizing enzymes retinal dehydrogenase 1 (RALDH1) and 2 (RALDH2), inducible NO Synthetase (iNOS), TGF-β, BAFF and APRIL in 24 hrs conditioned DCs by PCR from 4 independent Balb/C DC cultures. RALDH1, RALDH2 and iNOS gene expression was upregulated in LPS-primed DCs 24 hrs post-pulsing, compared to unpulsed DC (**Figure 3**). Interestingly, LPS+CTB-primed DCs which support IgA responses, express highly elevated levels of RALDH1 and iNOS compared to the other conditions, while BAFF, APRIL and TGF-β gene expression was equally expressed in all conditions (**Figure 3**). Gene expression of TGF-β was not enhanced, but regulation may occur at the posttranscriptional level. Furthermore, our previous studies point towards an important role for TGF-β in CTB-driven IgA-dependent protective responses against AAI (8). Subsequent protein expression of TGF-β (measured by, yet inactive, LAP) was slightly but consequently increased in CTB-primed DCs, compared to control DCs (supplementary Figure 1).

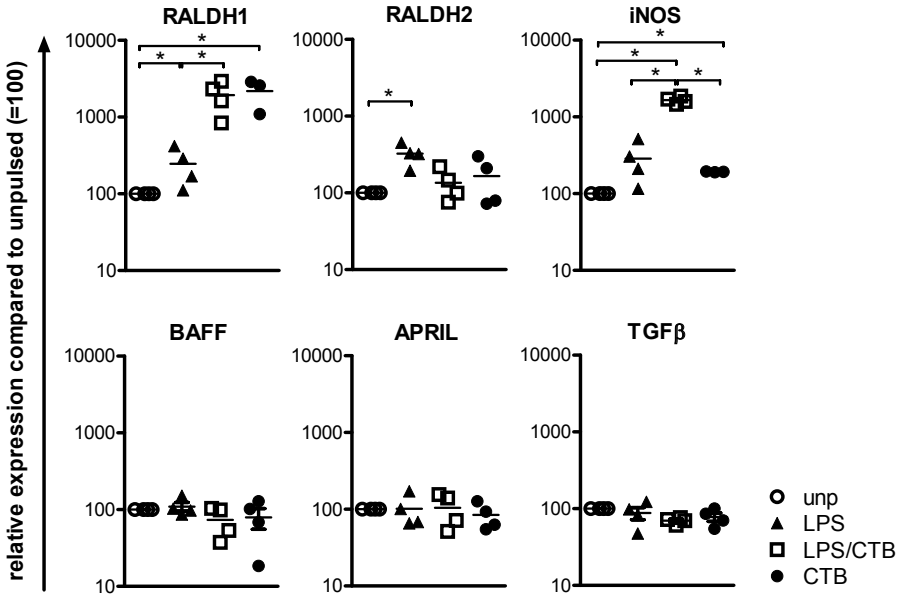


Figure 3. Expression of candidate IgA inducing molecules of BM-DCs.
BM-DCs were cultured as described previously and pulsed with PBS, LPS (1 ng/ml) +/- CTB (10 μg/ml) or CTB alone. After 24 hrs the following gene expression was analyzed (A) RALDH1, RALDH2, iNOS, BAFF, APRIL and TGF-β. Expression is normalized against the house keeping gene GAPDH and displayed as relative to conventional unpulsed BM-DCs. Mean + sem of 4 indvidual BM-DC cultures are shown * P<0.05, ** P<0.01, *** P<0.001.

To study the functional involvement of these molecules, *in vitro* experiments were performed in which either the cells were deficient for certain genes or chemical inhibitors were added. When the RA receptor was blocked by the antagonist LE135, we consistently observed a partial

inhibition of IgA production by B cells cultured with LPS+CTB-DC (**Figure 4a**). RA receptor inhibitor LE540 or Citral, which block RA production by enzyme blockade, resulted in similar inhibition (data not shown). Blocking TGF-β signaling, using SB-431542, significantly decreased (2-4 fold) IgA production by LPS+CTB-primed DCs (**Figure 4b**). Importantly, when both TGF-β signaling and signaling via RA were blocked, the IgA response was completely abrogated (**Figure 4b**). Equal proliferation of CFSE-labeled B cells and similar IgM levels were found for all conditions (data not shown) excluding cytotoxic effects of the inhibitors.

Although mRNA expression for iNOS was strongly elevated in LPS+CTB-primed DCs, blocking NO synthesis by the inhibitor L-NIL only had a marginal effect on IgA production (data not shown). Furthermore, co-cultures of $iNOS^{-/-}$ BM-DCs and B cells resulted in similar IgA levels as in WT cultures (**Figure 4c**), indicating that iNOS is not involved in CTB-induced IgA production in our model. Blocking either BAFF and/or BAFFR3 did not affect CTB-induced IgA production (supplementary Figure 2a), together with the gene expression data, not favoring a role for BAFF. APRIL gene expression was not induced in LPS+CTB-primed DCs, however APRIL may also be regulated during translation (23). Therefore, we investigated a possible role for APRIL in mice lacking both receptors for APRIL, the Transmembrane activator and CAML-interactor (TACI) and B cell maturation antigen (BCMA) double deficient mice. Of note, TACI and BCMA are also receptors for BAFF, but BAFF can still signal through a third receptor, the BAFF3 receptor. Surprisingly, co-cultures of $TACI/BCMA^{-/-}$ B cells and LPS/CTB-primed DCs resulted in strongly elevated levels of IgA (> 1000 ng/ml) compared to WT control cultures (supplementary Figure 2b). These results may suggest that signaling via the receptors TACI and BCMA has a negative influence rather than a promoting function. Alternatively, these results may be explained by highly proliferating cells in which the process of class switching was prevented.

In summary, these data support a dominant role for both TGF-β and RA in driving CTB-induced IgA responses *in vitro*.

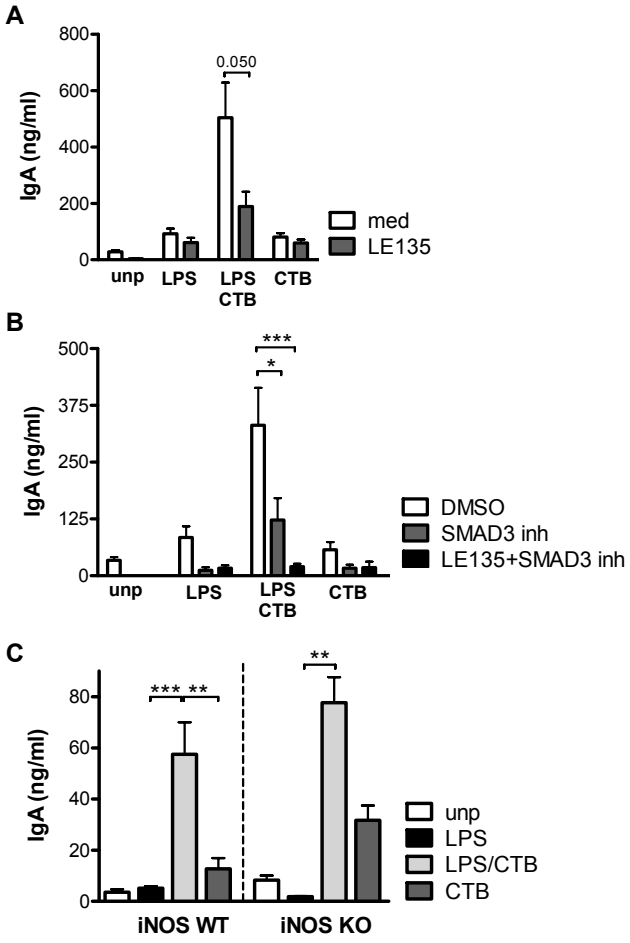


Figure 4. *In vitro*, RA and TGF-β are responsible for IgA induction by CTB-primed DCs.
BM-DCs were pulsed with PBS, LPS (100 ng/ml), LPS+ CTB (10 μg/ml) or CTB alone and co-cultured as described at Figure 1. (A) During the co-culture the RA receptor was blocked by LE135 (1 μM). After 7 days, IgA levels were measured in supernatant. (B) Similar as in A, but here in the presence of SB-431542 (5 μM) (or vehicle) alone to inhibit TGFβ-specific signaling or together with LE135. After 7 days, IgA levels were measured in supernatant. (C) BM-DCs and B cells were co-cultured as described, however the DCs were WT or deficient for iNOS (C57/Bl6 background). Data shown are mean +/- s.e.m from at least 3 pooled experiments * P<0.05, ** P<0.01, *** P<0.001.

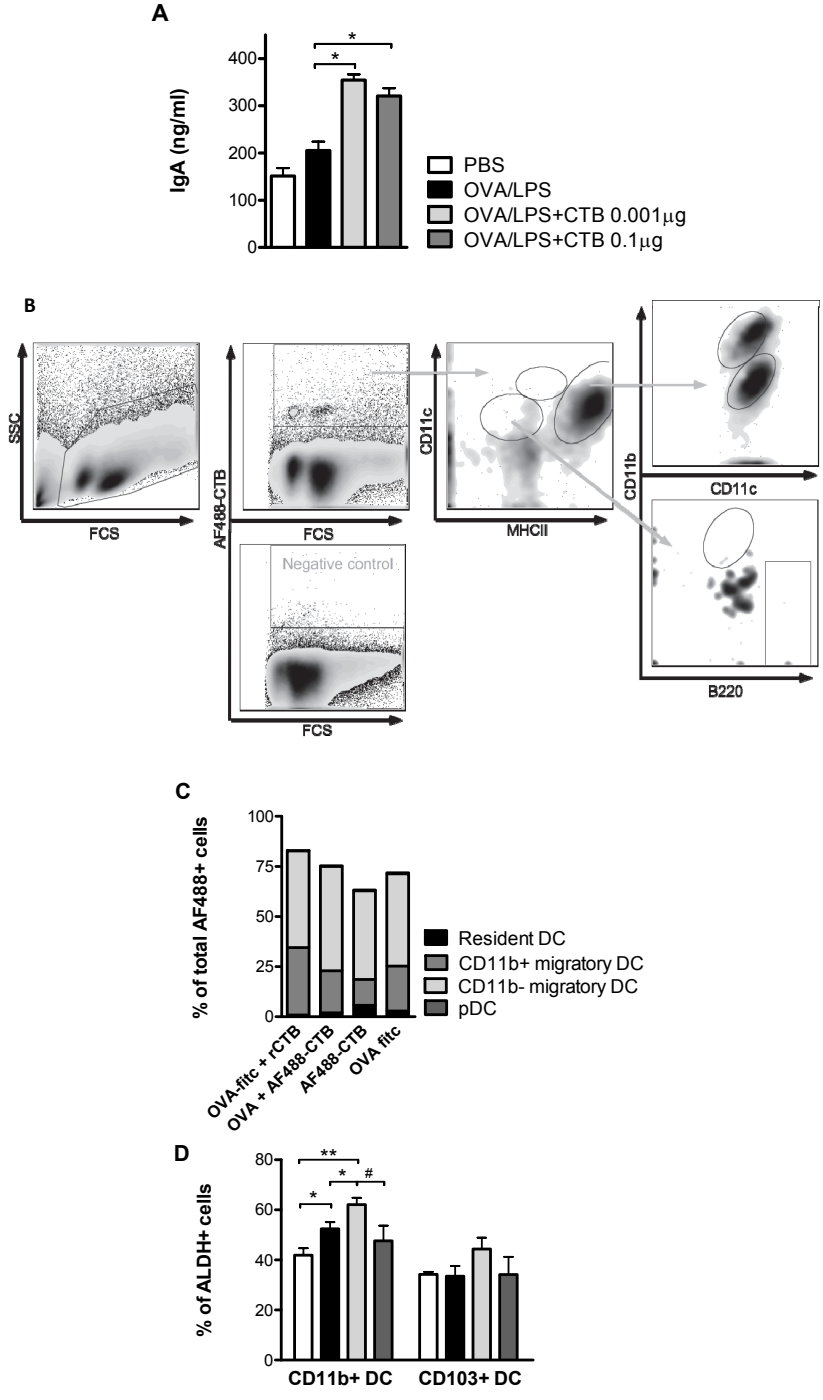
***Ex vivo* CTB-pulsed DCs promote IgA responses in the lungs via RA and TGF-β**

In vitro, synergistic signaling of CTB and MyD88 condition non-mucosal DCs to promote IgA responses, via TGF-β and RA. We wondered whether it was possible to prime DCs *in vivo,* and stimulate IgA responses. Indeed a single injection of CTB along with the OVA into the airways resulted in increased IgA production by *ex vivo* restimulated lung B cells that where isolated one day after the last OVA challenge (**Figure 5a**). Previously, in a paper focusing on OVA-induced experimental asthma, we published that adoptive transfer of the *in vitro* generated OVA/CTB-pulsed BM-DCs results in increased IgA responses *in vivo* (8). The OVA (Worthington) used as a model antigen here, contains a sufficient amount of LPS to provide for the necessary MyD88 signaling (supplementary Figure 3).

Furthermore, we studied whether DCs are prominently targeted by CTB when administered in the airways, as many hematopoietic cells express GM1 (16). AF488-labeled CTB with or without OVA was administered into the airways of naïve mice, after 36 hrs the AF488^{hi} cells present in the lung-draining lymph nodes were analyzed for different DC subsets as depicted in **Figure 5b.** The vast majority of the AF488^{hi} cells (almost 80%) consisted of DCs; both CD11b^{+} and CD11b^{-} migratory DCs (MHCII^{hi}CD11c^{hi}), resident DCs (MHCII^{int}CD11c^{hi}), and some pDCs (MHC^{lo}CD11c^{int}B220^{+}CD11b^{-}) (**Figure 5c**). Some B cells (MHCII^{hi}B220^{+}, ~10%) and only a few T cells (CD3^{+}) and macrophages (<1%, MHCII^{lo}CD11c^{int}CD11b^{+}B220^{-}) had taken up the labeled CTB (data not shown). The remaining non-migrated AF-488^{hi} cells left behind in the lungs also mainly consisted of DCs, containing equal percentages of resident CD11b^{+} and CD11b^{-} DCs (MHCII^{+}CD11c^{+}) (data not shown).

As it was clear now that also *in vivo* in the lungs CTB predominantly targets to DCs and leads to increased IgA, we subsequently aimed to investigate whether CTB could equally affect DC function *in vivo* as was observed in our *in vitro* assays. Therefore, CTB +/- OVA was instilled in the airways of mice and the presence of DCs endowed with RA producing capacity was studied at locations of potential interaction between DCs and B cells, i.e. the lung draining Mediastinal LN (MedLN) and in the lungs itself. Aldehyde dehydrogenase (ALDH, catalyzes oxidation of retinal into RA) activity of DCs was determined using ALDEFluor staining kit. Both CD103^{+} and CD11b^{+} migratory DC subsets found in the MedLN showed ALDH enzyme activity, ~35% and ~45% of the DC subsets respectively. But upon OVA+CTB instillation, a significantly higher percentage of migratory CD11b+ DCs (>60%) were positive in the ALDEFluor assay compared to CD11b+ DCs (<50%) from control mice not treated with OVA+CTB (**Figure 5d**). In contrast, there were considerably less lung-remaining DCs expressing ALDH: 10-20% positive cells for both CD103^{+} and CD11b^{+} DCs, but no differences were observed between the groups (data not shown).

The findings that CTB targets mainly DCs and induces increased IgA via upregulating the expression of the RA synthesizing enzyme on CD11b^{+} DCs *in vivo,* directed us to further study the role of RA and TGF-β in the *in vivo* induction of IgA in CD11b^{+} DCs. However, the numbers of CD11b^{+} DCs in lung draining LNs are very low, and to prevent use of unethical numbers of mice to generate sufficient numbers of DCs, BM-DCs which are mainly of the CD11b^{+} type are used for *in vivo* experiments. As published previously, DCs exposed ex-vivo to OVA+CTB and injected into the airways of naïve mice resulted in increased IgA levels in BAL fluid, increased IgA production by *ex vivo* restimulated lung B cells, and increased number of IgA^{+} cells in lung tissue, compared to mice injected with unpulsed, OVA-pulsed or CTB-pulsed DCs (8). Here, we now confirmed the involvement of both TGF-β and RA in CTB-driven IgA production, by blocking the function of these factors *in vivo*. One day before until 3 days after intratracheal administration of OVA +/- CTB-pulsed DCs, anti-TGF-β antibody and RA receptor blocking agent LE135 were administered. After OVA aerosol challenge both percentage and the absolute number of IgA^{+} cells in the lung were reduced in lung tissue of mice treated with LE135 and anti-TGF-β compared to control mice (**Figure 5e)**.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OVA/LPS
OVA/LPS+CTB 0.001μg
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AF488-CTB
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Negative control
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CD11c
MHCII
CD11b
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B220
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% of total AF488+ cells
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Resident DC
CD11b+ migratory DC
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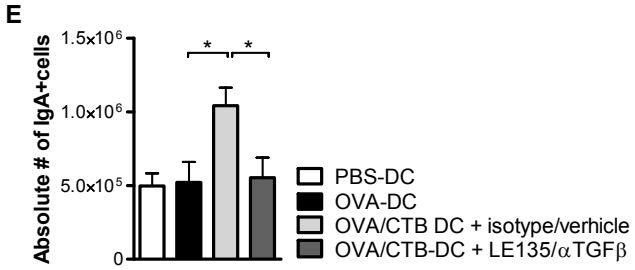

Figure 5. *Ex vivo* CTB pulsed DCs promote IgA responses in the lungs via RA and TGF-β. PBS, OVA (800 μg/80 μl/mouse), OVA + CTB (0.1 or 0.001 μg/80 μl) or CTB alone were instilled in lungs of naïve mice. (A) After OVA challenge, lung B cells were isolated, cultured and restimulated by LPS (10 μg/ml) for 7 days and IgA levels were determined (B) AF488-labeled CTB (and as a control Fitc-labeled OVA) was used. After 36 hrs, MedLN and lung tissue were studied for the presence of AF488-positive cell populations: Dead cells are excluded based on FSc and SSc; AF488 positive cells selected; migratory cDCs ($MHCII^{hi}$) subdivided into CD11b+ and CD11b- subsets; resident DCs ($MHCII^{int}$); $CD11c^{lo}MHCII^{lo}$ cells, subdivided into pDCs (B220+) and alveolar macrophages (CD11b+) (C) As in B. Percentage of AF488-positive DC subsets total AF488+ cells in MedLN (D) After 36 hrs, MedLN were isolated. ALDH enzyme activity of different DC subsets was determined using ALDEFLUOR assay, by flowcytometry. (E) BM-DCs were generated as described and pulsed with PBS, OVA (100 μg/ml), OVA+CTB (10 μg/ml) or CTB only (10 μg/ml) and the next day intratracheally injected in naïve mice. One day before until 3 days after DC instillation, mice were treated i.p with LE135 and anti-TGFβ antibody (or isotype Ab and DMSO). After one week, the mice were challenged by OVA for 3 consecutive days. One day after challenge, the number of IgA+ cells in digested lungs was determined by flowcytometry. Data are mean +/- s.e.m, of at least 2 individual experiments with 4 mice per group (for D: 12 mice per group, pooled per 3 mice, resulting in 4 datapoints per group). # p=0.10 * P<0.05, ** P<0.01, *** P<0.001.

Discussion

This study shows a novel function for the mucosal adjuvant CTB in promoting IgA responses by directly targeting DCs and mimicking signals from a mucosal environment. For optimal CTB driven IgA induction by non-mucosal DCs, MyD88-dependent TLR co-signaling in DCs is essential, resulting in enhanced expression of RALDH1 and TGF-β.

DC-driven IgA induction has been demonstrated to be a unique feature of mucosal DCs such as lamina propria or PP DCs in the gut (24,25), expressing several IgA inducing factors, including RA, TGF-β, iNOS, APRIL and/or BAFF (15). This property was not shared with peripheral DCs. Here we demonstrate that CTB, in conjunction with MyD88-dependent TLR ligands, can imprint non-mucosal BM-DCs to induce comparable IgA responses as mucosal PP DCs do (figure 1b). This involves partly similar IgA-inducing factors as described for mucosal DCs, as we identified a dominant positive role for RALDH1 and TGF-β, but not for iNOS, BAFF and APRIL. It has been shown, that also the mucosal factor RA can enhance the IgA inducing capacity of non-mucosal BM-DC, by increasing RALDH2 expression and TGF-β production and being amplified by MyD88 signaling, similar to what we here observe for CTB (22,26,27). An interesting possibility might be that the working mechanism of CTB can be explained via the induction of enhanced RA expression by DCs which may affect DC function in an autocrine loop.

Despite the widespread availability of retinol, RALDH expression is limited to certain cell types. DCs that are located in gut-associated lymphoid tissue (GALT) and express RALDH2 are well known for their RA production and IgA inducing ability. But other DC subsets with RA producing capacity are described and expression of one of the aldehyde dehydrogenases, RALDH1, 2 or 3, is essential for a cell to be able to catalyze the oxidation of retinal into RA (13,28,29). In lung DCs, expression of RALDH1 is more prominent, like in our CTB-treated BM-DCs (30). The fact that we do not find differences in RALDH2 expression of cultured CTB exposed BM-DCs, could be due to the GMCSF which is used to generate the BM-DCs *in vitro*. Yokota et al showed that GMCSF is sufficient to markedly induce RALDH2 expression in cultured BMDCs, and this could overrule the effect of CTB in our system (31).

Interestingly, CTB either partly mimics the effects of RA by inducing similar mucosa-associated factors in non-mucosal DCs or acts via the induction of RA itself. Thus, the mechanism of DC priming may be independent of the presence of specific mucosal factors, and the use of CTB as an adjuvant to induce IgA responses may not be limited to the mucosal route.

CTB binds to GM-1 ganglioside, which is present on many different hematopoietic and structural cell types (16). Despite this wide expression, we showed that CTB administered into the airways mainly targeted DCs and that CTB-loaded DCs migrated to the draining LNs (figure 5b). This finding is supported by other studies in the skin, showing a specific recruitment of DCs towards areas of CT administration (32,33). In the lung of mice, several DC subsets have been described: e.g. two major subsets of conventional DCs have been defined based on their CD103 and CD11b expression (34). Very recently, it was published that *in vitro* mouse $CD11b^{high}$ lung DCs induce IgA more efficiently, than $CD103^+$ lung DCs (35). However, it was not clear by what mechanisms this was induced, as the expression of BAFF, APRIL or RALDH1 was not different between the two lung DC subsets. In the gut, specialized DC subsets inducing TI IgA synthesis are $iNOS^+TNF^+$ DCs (tipDCs) (36), and the recently described $CD11b^+$ DCs expressing TLR5 and not TLR4 (37). Moreover, $CD103^+$ DCs, by producing RA and TGF-β, are responsible for imprinting gut-homing molecules on B cells, and support IgA synthesis (13,28,38,39). Administration of CTB in the airways results in increased IgA responses, and increased RA-producing capacity as measured by higher ALDH enzyme activity of DCs, compared to control conditions. Although in the MedLN a larger proportion of CTB-loaded DCs were $CD11b^-CD103^+$ than $CD11b^+$ (figure 5b), expression of ALDH was higher in $CD11b^+$ DCs compared to in $CD103^+$ DCs after CTB administration (figure 5c). This in contrast to the gut, where the $CD103^+$ DCs are the ones that express higher levels of RALDH, and not the $CD11b^+$ DCs. Interestingly, and in line with our study, Guilliams et al recently described that in the skin RA production and tolerogenic functions were restricted to $CD103^-$ DCs (20). Furthermore, in the lung, it was suggested recently that CD11b+ DCs are more potent in their capacity to induce IgA responses than the CD103+ subset (35). Thus, CD103 or CD11b may not be specific markers for the identification of specialized DCs subsets with IgA promoting capacity. Although, in our model the increased ALDH activity on the $CD11b^+$ DC subset, and the

increased IgA after transfer of ($CD11b^+$) BM-DCs, could suggest that this subset is more important in driving CTB induced IgA responses in the airways, we cannot rule out involvement of $CD103^+$ DCs nor other DC subsets. The role of different lung DC subsets and their mechanism of driving humoral IgA responses needs to be further investigated.

CTB can enhance phosphorylation of multiple signaling molecules downstream of TLRs (40), but requires a MyD88-dependent co-activation signal for its IgA inducing capacity. MyD88 is an adaptor molecule downstream of many TLRs (41). In the gut, DCs are constantly exposed to TLR ligands derived from commensal bacteria, and these signals play a major role in driving IgA responses (2,9,14). Indeed *in vitro* priming of DCs by different MyD88-dependent TLR ligands enhanced IgA production by B cells, but not by MyD88-independent PRR-ligands, such as Poly I:C, Zymosan and Curdlan (figure 2b). Although, CpG DCs induce some IgA response by B cells, CTB was not able to enhance this effect. This might be explained by the endosomal localization of TLR9, although this is also the case for TLR 7 and 8 (ligated by CL97) for which we did observe CTB-enhanced IgA induction. Therefore an alternative explanation might be the negative charge of CpG that may influence the IgA-inducing instructions of CTB to the DCs.

Importantly, CTB does not only enhance IgA induction by high dose TLR-ligand primed DCs, but also initiates IgA production in case of low dose exposure to MyD88-activating ligands. This is particularly interesting, considering the thought that decreased or altered microbial exposure associated with affluent life style allows uncontrolled inflammatory responses against either innocuous or self-antigens (42). Indeed, Hilty et al compared the airway microbiota of patients with asthma and controls, and found disturbed microbiota in asthmatic airways (43). Therefore CTB may have potential in the clinic, by translating insufficient microbial signals into enhanced, and putative protective, IgA levels. In a mouse model, CTB was able to enhance protective mucosal sIgA responses against aero-allergens (8). Furthermore, in some models CTB can also induce $FoxP3^+$ regulatory T cell responses *in vivo* when coupled to specific antigens (44). Interestingly, oral CTB has already been applied for treatment of Crohn's disease, reporting decreased disease activity scores (45), which shows the potential use of CTB for hyperinflammatory diseases.

Our study contributes to a better understanding of how CTB acts as a mucosal IgA inducing adjuvant, by showing that CTB specifically targets to DCs, promotes DC-driven IgA production and identifies co-factors necessary. Future studies on CTB are needed to investigate whether CTB equally influences DC driven IgA responses in humans.

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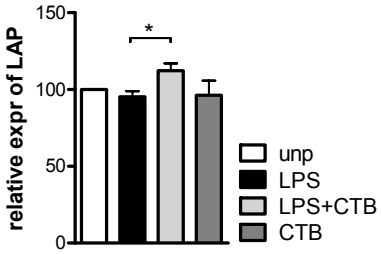
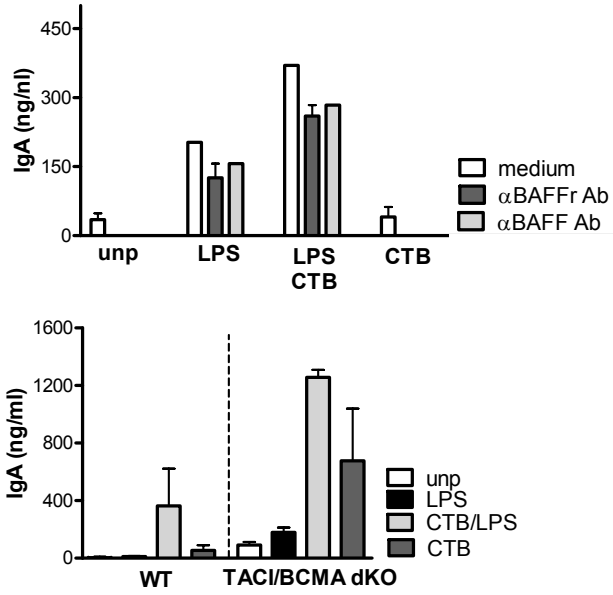
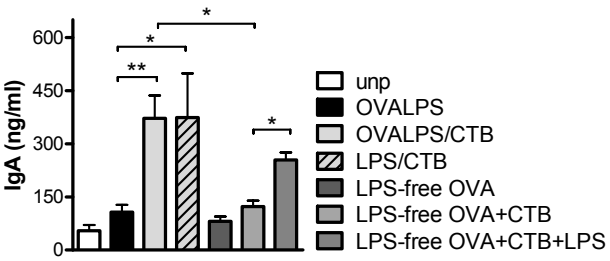
Supplementary figures



Supplementary figure 1: Increased LAP expression on LPS+CTB treated BM-DCs. BM-derived DCs were cultured for 8 days with GMCSF, pulsed overnight with PBS, LPS (1 ng/ml) +/- CTB (10 μg/ml) or CTB alone. LAP expression of PFA fixed and Brefeldin A treated pulsed DCs, by FACS. Geomean is displayed, relative to the expression of unpulsed BM-DCs.
Mean + sem of 4 individual experiments are shown * P<0.05, ** P<0.01, *** P<0.001.



Supplementary figure 2: Role of BAFF and APRIL in IgA induction by CTB-primed DCs. BM-derived DCs were cultured and pulsed as described in the legend of figure 1, and then co-cultured with splenic CD19+ B cells (ratio 1:1) and anti-IgM Fab-fragments (10 μg/ml). After 7 days, IgA levels were determined by ELISA. (A) During co-culture either blocking antibodies against BAFF (0.2 μg/ml, R&D systems), BAFFR3 (2 μg/ml, R&D systems), or isotype controls were added. (B) BM-DCs from B6129S2F1 mice were generated, pulsed and co-cultured with splenic TACI/BCMA$^{-/-}$ B cells (on a B6129S2F1 background) as described.
Data from one representative experiment out of 4 are shown. * P<0.05, ** P<0.01, *** P<0.001.



Supplementary figure 3: OVA Worthington contains sufficient LPS to induce IgA in synergy with CTB. BM-derived DCs were cultured, and pulsed overnight with PBS, OVA (100 μg/ml, containing LPS), LPS free OVA (100 μg/ml, Seikagaku [de Heer, J ex Med 2004]) or LPS (1 ng/ml), either or not in combination with CTB (10 μg/ml) or CTB alone, thereafter cultured with B cells for 7 days as described. Supernatant was collected and IgA production measured by ELISA.
Data from one representative experiment out of 2 are shown. * P<0.05, ** P<0.01, *** P<0.001.

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CHOLERA TOXIN B INDUCED PROTECTION AGAINST BIRCH-POLLEN DRIVEN ALLERGIC AIRWAY INFLAMMATION IN V10YEN MICE WITH A RESTRICTED B CELL REPERTOIRE

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Abstract

Reduced mucosal IgA responses are associated with the development of asthma or other allergic diseases. Mouse studies supplementing IgA or artificially inducing IgA could prevent the onset of allergic airway inflammation (AAI). Considering the therapeutic application of IgA-inducing agents, such as Cholera Toxin B (CTB), we questioned whether protection against AAI requires allergen-specific IgA antibodies or whether enhanced levels of random and/or polyreactive IgA are sufficient.

In a Birch Pollen Extract (BPE)- driven AAI model, we studied if induction of IgA by CTB was able to induce protection in transgenic (Tg) mice with a restricted BCR repertoire, biased for recognizing the irrelevant Vascular Stomatitis Virus (VSV) antigen (V10YEN). As shown previously for OVA-driven AAI, CTB treatment of BPE-pulsed bone marrow-derived dendritic cells (BMDCs) abrogated their ability to induce AAI, and increased total IgA levels in lungs of both WT and Tg mice. WT lung lavages contained BetV1 (the major antigen of BPE)- specific IgA, which was increased upon treatment with CTB, while VSV-specific IgA was undetectable. Surprisingly, the Tg plasma cells did not exclusively produce IgA directed against VSV, but also BetV1-specific IgA was found in Tg lung lavages. In contrast to WT mice, CTB induced protection against BP-driven AAI in Tg mice was correlated with total IgA in BAL fluid, but not with allergen-specific IgA.

Taken together, CTB works as a general mucosal adjuvant, inducing a diverse IgA response. However, due to the presence of both allergen-specific and random (VSV specific) IgA at Tg lung mucosa, we cannot exclude nor confirm a generalized role for random non-specific IgA in CTB induced protection against AAI.

Introduction

At the mucosa, such as gastrointestinal and respiratory tract, secretory immunoglobulin A (SIgA) plays a major role in mediating immune exclusion ('antigen avoidance') of luminal antigens and homeostasis with the commensal flora as well as the protection against invading pathogens (1,2). As these areas are exposed to a broad range of environmental particles, this requires a very broad reactivity profile of the IgA response. Conversely, elaborated on in the general introduction (Chapter 1), various studies in young children have suggested that an insufficient development of humoral IgA responses is associated with the development of allergy, including asthma (3-5).

Only few IgA plasma cells are present in neonates and in germ-free mice, but their cell numbers rapidly increase upon colonization with commensal microbacteria, concomitantly leading to the development of local and systemic IgA responses (6-10). Interestingly, Benckert studied the reactivity profile of plasmablasts from the human terminal ileum, and found that approximately 25% of intestinal IgA and IgG plasmablast antibodies were polyreactive/cross-reactive against diverse self and non-self antigens, including bacteria; whereas the majority (~75%) were antigen-specific and exclusively directed against pathogenic microbes, commensal flora or self antigens (11). It was shown already in neonates that SIgA limits penetration of commensal intestinal bacteria through the intestinal epithelium, suggesting that this is a primitive and basic process that does not require antigen induced diversification of the primary natural antibody repertoire (12). Likewise, in a helminth infection model it was shown that in addition to parasite-specific antibodies also antibodies with (polyreactive) random specificities contributed to protection against enteric helminth (re)infection (13). This indicates that for commensal bacteria and intestinal pathogens like certain parasites, also antibodies with irrelevant specificity and/or a polyreactive profile contribute to the process of immune exclusion and this does not require mono-reactive or antigen-specific antibodies per sé. It is not clear, however, whether this also applies for other types of environmental foreign particles, such as allergens.

In healthy individuals allergens do not induce inflammatory reactions and mouse studies have suggested that IgA at mucosal sites plays a protective role. Indeed, treating mice with allergen-specific IgA or IgA-inducing agents, like Cholera Toxin B (CTB), protects against allergic airway inflammation (AAI) (14,15). Furthermore, in house dust mite (HDM) allergic children, less HDM-specific IgA antibodies in salivary and serum are found than in non-allergics (16). Furthermore, sensitized infants with higher salivary levels of specific IgA antibodies (against the most common food or inhalant cat allergen) are associated with significantly less late-onset wheezing (17). These studies suggest that allergen-specific IgA is crucial to prevent the development of allergy. Although many studies have reported an association between early (microbial-induced) maturation of mucosal and/or serum IgA in young children and protection against development of allergy and asthma, usually these studies were restricted to total IgA responses and did not address the issue of allergen-specificity (3,4,8). The relative contribution of polyreactive and random IgA versus IgA antibodies with a mono-specificity for individual allergens may have important consequences

for the therapeutic application of IgA-inducing agents against allergic diseases, and the possible need for allergen-specific IgA responses to prevent unwanted responses to other antigens.

First, we set up a birch pollen- specific AAI model. Birch pollen is an allergen that is more clinically relevant for human allergies, whereas the commonly used OVA is not. As there is much experience with immunotherapy for birch pollen in humans, this model may allow the evaluation of supplemented IgA-inducing molecules in future immunotherapy protocols. Next, to get more insight in the nature of protective IgA responses against allergens, i.e. whether allergen-specific IgA or enhanced levels of IgA of random (polyreactive) specificities are sufficient to reduce allergic airway inflammation (AAI), we used a transgenic mouse model (V10YEN) with a restricted B cell receptor (BCR) repertoire, recognizing the - in our AAI model irrelevant - Vascular Stomatitis Virus (VSV) antigen. These V10YEN mice were previously generated by crossing a B cell receptor transgenic strain expressing the VSV-specific κ light chain (YEN) to mice with a VSV-specific recombined V_HDJ_H heavy chain variable region inserted 5' of the intronic enhancer at the correct physiological position in the heavy chain locus (i.e. knockin mice, allowing correct somatic hypermutation, class switch recombination to all isotypes and receptor editing). The VSV-specific heavy and light chains of the individual mouse strains were derived from a hybridoma secreting a VSV neutralizing antibody (VI10), to which also the anti-idiotypic antibody 35.61 was available (18). The B cells of the V10YEN mice have a restricted BCR repertoire, biased towards production of VSV-neutralizing antibodies, as > 93% of B cells could be recognized by the 35.61 antibody.

Using this novel model of birch pollen- induced AAI together with the BCR Tg mouse model, we found that CTB is still able to suppress allergy in mice with a restricted B cell repertoire.

Materials and Methods

Mice

Female Balb/c mice were purchased from Harlan, the Netherlands. These mice were housed under SPF-conditions according to the guidelines of the animal ethics and welfare committee of the Leiden University Medical Center. Female and male TgH(V10)xYEN (C57/Bl6 background) mice were bred and maintained at the University of Zürich and Bio-support, Zürich.

Bone marrow- derived dendritic cell culture

Bone marrow-derived dendritic cells (BMDCs) were generated from BM cells cultured in DC medium (DC-TCM: RPMI 1640 medium containing glutamax (GIBCO), 50 μM 2-mercaptopethanol (Sigma-Aldrich), 50 μg/ml gentamicin (Invitrogen) or sodium-penicillin (Astellas Pharma B.V), 0.1 ug/ml streptomycin (Sigma-Aldrich), and 5% fetal bovine serum) supplemented with 20 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor (rmGM-CSF, a gift from K. Thielemans, Vrije Universiteit Brussel, Brussels, Belgium). After 8 days, DCs were pulsed overnight with PBS, Ovalbumin (OVA, 100μg/ml Worthington Biochemical Corp., Lakewood,

NJ) or Birch Pollen extract (BPE, 10, 50 or 100μg/ml, provided by Prof U. Wiedermann, Medical University Vienna (19) with or without Cholera Toxin B (CTB, 10 μg/ml, Sigma-Aldrich, St. Louis, MO). Supernatant was harvested for ELISA and cells were analyzed by flowcytometry.

***In vivo* mouse models**

Unpulsed, OVA (100μg/ml)-, OVA+CTB (10μg/ml)-, BPE (100μg/ml)- or BPE+CTB (10μg/ml)-pulsed BMDCs ($1*10^6$) were administered into the airways of naïve WT and V10YEN Tg mice as described before (20), followed 10 days later by OVA or BPE (50 μg/ml) intranasal challenge for 3 consecutive days. One day after the last challenge, mice were sacrificed: Broncho-alveolar lavages (BAL) were preformed (15), cells from lung draining mediastinal lymph nodes (MLNs) were cultured (200,000 cells/well) and restimulated with 10μg/ml OVA or 25μg/ml BPE for 4 days. Supernatant was collected and cytokine or Ig production analyzed by ELISA.

TLR-transfected HEK cell stimulation

HEK-293-CD14/TLR4 cells (a gift from Dr. E. Latz, University of Massachusets) were maintained in DMEM culture medium, supplemented with 10% FCS, 10μg/ml ciprofloxacin and 5μg/ml puromycin. For stimulation experiments, cells were seeded 3.5 x104 cells/well in 96-well flatbottom plates and were stimulated the next day. Supernatant of MD-2 transfected cells was added (12.5%). IL-8 production was measured in supernatant after 22 hours using a commercial kit (Sanquin, Amsterdam, The Netherlands), by following the manufacturer's recommendations.

Flowcytometry

BMDC phenotype was determined by staining for co-stimulatory molecules CD40 (eBioscience), CD80, and CD86 (both from BD Biosciences). Cells present in BAL fluid were stained for CD3, CD19 (both from BD Biosciences), CD11c (eBioscience), MHC class II (MHC-II) (BD Biosciences), and CCR3 (R&D systems). Acquisition of four-color samples was done on a FACS calibur. Numbers of eosinophils ($CD3/CD19^-CCR3^+$), B cells (CD19+MHCII+), total T cells (CD3+MHCII-), macrophages (remainder) were analyzed using FlowJo software (21).

Cytokine and Immunoglobulin measurements

IL-4, IL-5, IL-10, IFNγ (all from BD Biosciences) and IL-13 (R&D systems) levels in MLN cell culture supernatant were determined by ELISA. IgA and IgE levels, total and specific for BetV1a (Biomay AG) and VSV (using anti-VI10 mAb (clone 35.61)), were measured in BAL fluid by ELISA (BD Biosciences). Detection limits of ELISA-kits were in general 5 – 10 pg/ml for cytokines, and 0.2 ng/ml for immunoglobulins.

Statistical analysis

To study whether there were differences between 3 or more groups, first an ANOVA test was performed, followed by Mann-Whitney U test to compare specific groups. P values less than 0.05 were considered significant. P-values less than 0.05, 0.01 or 0.001 are indicated by one, two or three asterisks, respectively.

Results

BPE upregulates co-stimulatory molecules on BMDCs in vitro

In our previous studies (Chapter 2 and 3 of this thesis), we observed an essential role for CTB to enhance mucosal SIgA responses via DCs. Therefore, to set up a birch pollen-specific AAI model, we made use of a well-known adoptive transfer model of BMDCs, as described previously for OVA-driven AAI (20).

To evaluate the effect of birch pollen extract (BPE) on DC maturation, BMDCs were pulsed overnight (~24hrs) with different concentrations of BPE at day 8 after generation in culture medium with GM-CSF. Exposure of the BMDCs to 10, 50, and 100 μg/ml BPE induced strong maturation and the cells expressed high levels of CD40, CD80 and CD86 in a dose-dependent manner compared to unpulsed BMDCs. Surface expression of MHCII was already high at day 8 BMDCs and did not change upon exposure to BPE (**Figure 1**).

Exposure of TLR4-transfected HEK cells to 100μg/ml BPE induced an amount of IL-8 production, equivalent to a dose of 1 to 10 ng/ml LPS (data not shown), indicating a certain degree of LPS contamination of the BP extract. Normally, 1 to 10 ng/ml LPS is capable of inducing a partial DC maturation and therefore it is not clear at this stage whether the degree of DC maturation observed in Figure 1 is attributed merely to LPS or LPS and/or BPE. For this study, the presence of LPS in the BPE is beneficial for IgA induction, as we have showed that CTB requires a MyD88 activating signal, such as LPS, to qualify DCs to promote IgA responses (Chapter 3 of this thesis).

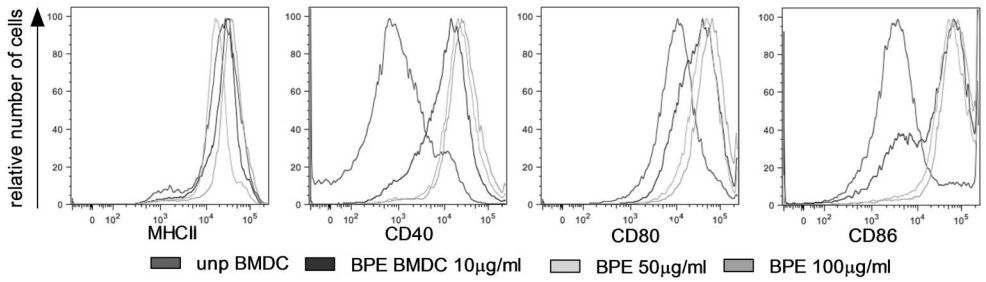

Figure 1 BPE upregulates co-stimulatory molecules on BMDCs *in vitro*. BMDCs were generated from bone marrow cells by culture in DC medium including GM-SCF for 8 days. Subsequently, the BMDCs were pulsed overnight with Birch Pollen extract (BPE, 10, 50 or 100μg/ml). Cells were harvested and expression of MHCII and costimulatory molecules CD40, CD80 and CD86 was determined by flowcytometry. Data from one representative experiment out of 3 are shown.

CTB inhibits the development of AAI by modulating BPE-pulsed BMDC function and increases IgA responses *in vivo*

To investigate the Th2-inducing capacities of BPE-pulsed BMDCs and compare this to OVA-pulsed BMDCs, pulsed BMDCs were injected in the airways of naïve Balb/C mice, followed by OVA or BPE challenge. Total cell numbers in BAL fluid were increased in all mouse groups except the group sensitized with PBS-pulsed DCs (**Figure 2A**). As with OVA-pulsed DCs, BAL fluid of mice receiving BPE-pulsed DCs contained of high number of eosinophils and lymphocytes compared to sensitization with unpulsed DCs, after challenge with BPE (**Figure 2B**). Furthermore, cultures of MLN cells of mice sensitized with BPE-pulsed DCs, showed the presence of high levels of Th2 cytokines, i.e. IL-4, IL-5, IL-10, IL-13, and hardly any IFNγ (**Figure 2C**). The treatment of BPE-pulsed DCs with CTB before transfer into the airways altered their pro-inflammatory potential, preventing eosinophilia and BPE-specific MLN Th2 cytokine production upon challenge (**Figure 2B and C**). At the same time, there was a large increase in the amount of IgA detected in the BAL fluid of the BPE/CTB-BMDC treated mice, comparable to the level in the OVA-model (**Figure 2D**). Thus, CTB protects against the development of both OVA- and BPE-induced AAI by modulating DC function, while increasing local IgA responses. This suggests that the mechanism of protection by CTB described for the OVA-AAI model (15) also applies for the BPE-DC AAI model.

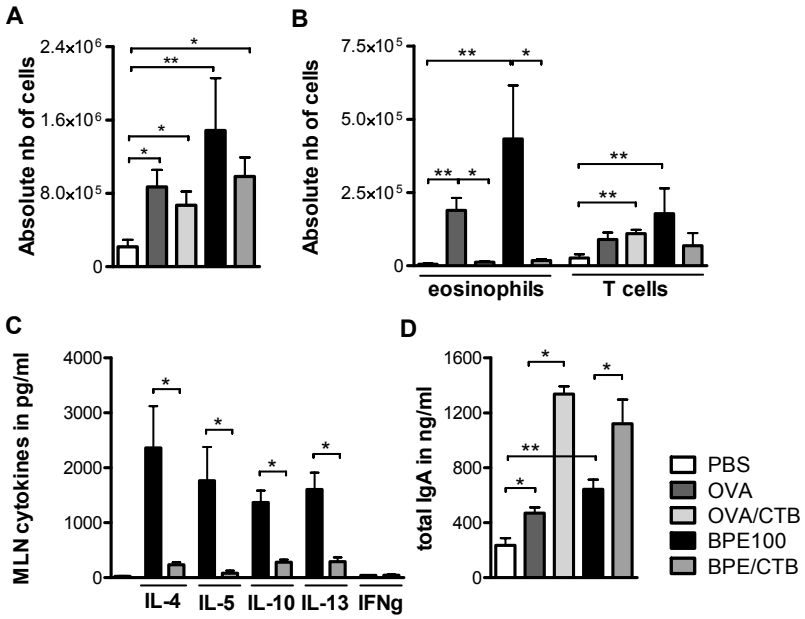

Figure 2 CTB inhibits the development of AAI by modulating BPE-pulsed BMDC function and increases IgA *in vivo*. BMDCs were generated as described, pulsed with PBS, OVA (100 μg/ml) or BPE (100 μg/ml) with or without CTB (10μg/ml) and instilled into the airway of naïve mice before allergen challenge. One day after the last challenge the mice were sacrificed. A) Absolute number of total cells present in BAL fluid, and B) Absolute number of different cell types present in BAL fluid, as determined by flowcytometry. C) Level of BPE specific MLN cytokine production in pg/ml and D) Level of total IgA in BAL fluid, measured by ELISA. Data are represented as mean + SEM, n=4 mice per group. * P< 0.05, ** P=< 0.01, *** P<0.001.

CTB/BPE-pulsed BMDCs protect against AAI in the V10YEN mouse model, and increase total IgA levels

Next, we aimed to study whether CTB-induced allergen-specific IgA is responsible for the protection against AAI or that SIgA of random specificities are sufficient. Therefore we made use of TgH(V10)xYEN (C57/Bl6 background) mice (18). We hypothesized that stimulating IgA responses with CTB (via BPE-exposed BMDCs) in these mice would result in the production of VSV-specific IgA antibodies, an irrelevant specificity when applying the BPE specific AAI model.

Both in WT and in Tg mice, sensitization with BPE- pulsed and BPE/CTB-pulsed BMDCs resulted in recruitment of immune cells to the airways, as seen by the significant increase in the total number of leukocytes present in the lung lavage (**Figure 3A**). However, when WT or Tg mice were sensitized with BPE/CTB-BMDCs, there was a clear reduction in the number of eosinophils present in the BAL fluid upon allergen challenge, compared to sensitization with BPE-BMDCs (**Figure 3B**). In accordance, Th2 cytokine production in cultures of MLN cells restimulated with BPE were also significantly reduced in CTB-BMDC treated WT and Tg mice (data not shown). Furthermore, the decrease in airway inflammation in CTB-treated WT and Tg mice was accompanied by a lower level of IgE in BAL fluid (**Figure 3C**). Interestingly, in BAL fluid of Tg mice similar increased levels of total IgA were observed as in WT mice (**Figure 3D**). Thus, the CTB driven decrease in airway inflammation was accompanied by increase in local IgA in both WT and V10YEN Tg mice. Although VSV was not present to drive Ab production by Tg B cells, we found enhanced levels of IgA in the conditions with CTB, emphasizing that CTB is a general IgA-inducing adjuvant. Based on these data we would conclude that random (in this case VSV-specific) IgA can prevent the development of inflammatory responses to BPE, and that CTB induced random (allergen-non specific) IgA is sufficient for protection against AAI.

Specificity of CTB induced IgA antibodies in V10YEN Tg mice

To investigate the specificity of IgA induced in the BAL fluid of Tg and WT mice, VSV and BetV1 (major allergen of BPE)-specific ELISA was performed. As expected, in WT BAL fluid no or background levels of VSV- specific IgA were found. In Tg BAL fluid of mice receiving BPE- or BPE/CTB- pulsed BMDCs the levels of VSV-specific IgA were equally induced (**Figure 4A**).

In contrast, a significant increase in BetV1-specific IgA was observed in CTB-treated WT mice compared with allergic WT mice (**Figure 4B**). Surprisingly, BetV1-specific IgA was detected in the BAL fluid of all three Tg mouse groups, whereby the levels were similar in the PBS-, BPE and BPE/CTB mouse groups (**Figure 4B**). Of note, BetV1-specific IgA levels in BAL fluid of Tg mice instilled with PBS-DCs were elevated compared to WT mice (**Figure 4**). These data show that plasma cells of the Tg mice did not exclusively produce IgA directed against VSV, but rather that substantial levels of IgA antibodies that bind BetV1 were also present in BAL fluid. Taken together, CTB- BMDC treated WT mice have increased total and allergen-specific (BetV1) IgA in BAL fluid compared to BPE-BMDC exposed allergic control mice. In contrast, in Tg mice the CTB induced protection against BP-driven AAI is correlated with increased total IgA but not with allergen-specific IgA in BAL.

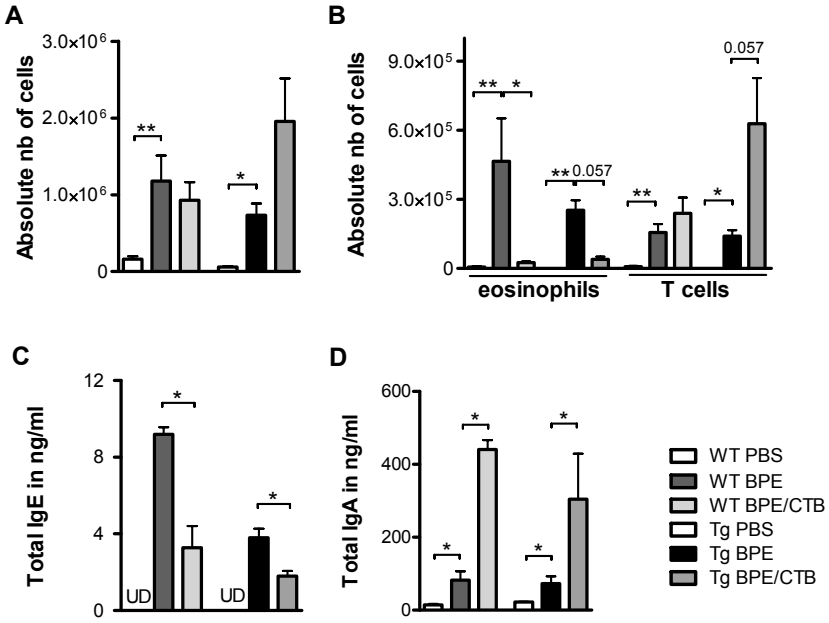

Figure 3 CTB/BPE-DCs protect against the development of AAI in the V10YEN mouse model, and increase total IgA levels. WT and V10YEN Tg mice were sensitized with BPE- or BPE/CTB- pulsed BMDCs, followed 10 days later by intranasal challenge on 3 consecutive days. One day after the last challenge mice were sacrificed. A) Total number of cells in BAL fluid, and B) Absolute number of different cells types present in BAL fluid, determined by flowcytometry. C) Level of total IgE in BAL fluid in ng/ml and D) Level of total IgA in BAL fluid in ng/ml, measured by ELISA. UD = undetectable. Data are represented as mean + SEM, n=4 mice per group. * P< 0.05, ** P=< 0.01, *** P<0.001.

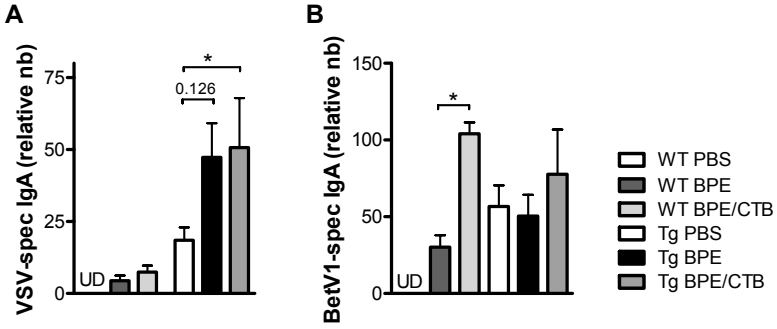

Figure 4 Specificity of CTB induced IgA antibodies in V10YEN Tg mice. WT and Tg mice were treated as described in figure 2. A) Level of VSV-specific IgA in BAL fluid in relative amount and B) Level of BetV1-specific IgA in BAL fluid in relative amount, by ELISA. UD = undetectable. Data are represented as mean + SEM, n=4 mice per group. * P< 0.05, ** P=< 0.01, *** P<0.001.

Discussion

CTB treatment of BPE-pulsed BMDCs abrogated their ability to induce AAI, and increased total IgA levels in lungs of mice. It is tempting to speculate that the mechanism of protection against BP driven AAI by CTB predominantly involves increased IgA responses, as demonstrated for the OVA model ((15), chapter 2). Using Tg mice with a restricted B cell receptor (BCR) repertoire, recognizing the irrelevant VSV antigen (V10YEN), we showed that CTB still protects against AAI and that it works as a general mucosal adjuvant, inducing a diverse IgA response. However, due to the presence of both allergen-specific and random IgA at Tg lung mucosa, we cannot exclude nor confirm a generalized role for random non-specific IgA in CTB induced protection against AAI.

It was shown that plasma cells of the Tg mice did not exclusively produce IgA directed against VSV, and that substantial levels of IgA antibodies that bind BetV1 were also produced. It may be that natural polyreactive antibodies are present or that cross-reactive antibodies have developed in Tg mice which bind to other antigens than VSV, such as BetV1. In addition, 93 to 98 % of the B cells of the V10YEN mice expressed the complete VSV restricted BCR (data not shown). It is possible that the remaining proportion of B cells of the Tg mice without the restricted VSV-specific BCR expanded upon recognition of their cognate antigen (in this case Bet V1) and/or upon activation by CTB, and produced significant amounts of antibody with a different specificity than VSV. Therefore, in addition to VSV- and BetV1-specific IgA, IgA of other undetermined specificities may also be present. In this study, we did not have an internationally recognized standard for BetV1 or VSV-specific IgA, and only measured relative levels and not absolute protein amounts of specific IgA. This means that the exact fraction of BetV1-specific IgA (or other specificities) within the total IgA pool and their possible contribution in the protection against AAI remains uncertain. By backcrossing V10YEN Tg mice to RAG KO mice, all B cells would express the complete VSV restricted BCR, and not just 93-98%, however SHM will still alter the BCR specificity of activated B cells in Germinal Center reactions, although these mutations are expected to further increase the affinity for VSV. A practically very challenging model could be to adoptively transfer IgA expressing B cells that are exclusively directed against a known antigen (e.g from V10YEN Tg mice), using B cell deficient recipient mice. Taken together, it is very complex to develop a model with mono-reactive B cells, and in which the development of random IgA specificities is completely eliminated. Nevertheless, CTB-BMDC exposed Tg mice do have increased total IgA and, although present, no increased allergen-specific (BetV1) IgA compared to allergic control Tg mice, suggesting that the allergen-specific IgA cannot be responsible for preventing AAI against BPE. However, because of its presence and unknown reactivity, a role for allergen-specific IgA in the CTB-induced protection against AAI cannot be excluded.

Furthermore, it is possible that the protection by CTB in VI10YEN mice was due to other mechanisms than the ones operating in WT mice with a polyclonal B cell repertoire. Indeed, close inspection of the BAL fluid cellular composition of Tg mice revealed that there was a dramatic increase in number of T cells in CTB-BMDC treated Tg mice compared to the allergic Tg mice,

a finding not observed in WT mice (**Figure 3A/B**). It has been shown in other experimental models that mucosal administration of CTB coupled to an antigen can stimulate antigen-specific regulatory T cells, and thereby suppress the development of different immunopathological reactions such as autoimmune diseases (22,23). Although, we could not demonstrate a role for CTB-induced Treg cells in the OVA-model in WT mice (15), we cannot exclude that in VI10YEN Tg mice a different (compensatory) mechanism is responsible for the suppression of inflammation by CTB, and that e.g. Treg cells are induced, bypassing IgA-dependent protection. In this scenario, the presence of BPE-specific IgA molecules in VI10YEN mice could just be an epiphenomenon. Therefore, the phenotype and function of the T cells in the BAL of Tg mice exposed to BPE/CTB-BMDC must be studied in more detail.

It is hypothesized that a reduced infectious pressure of Westernized countries results in insufficient maturation of the regulatory arm, including less mucosal IgA, allowing uncontrolled inflammatory responses against innocuous antigens later in life. A general IgA stimulating adjuvant, such as CTB, could partly compensate this by stimulating the development of enhanced mucosal IgA with a broad reactivity repertoire. This may form 'protective' IgA and help to prevent responses to self antigens, allergic responses or respiratory infections. These arguments would favor a general protective effect of enhanced random polyreactive mucosal IgA above allergen-specific IgA molecules. Moreover, therapeutic approaches focusing on the generation of allergen-specific IgA molecules only, calls for much more sophisticated approaches, i.e. antigens coupled to appropriate adjuvants without losing their boosting activity. This will be much more demanding to develop instead of applying a general IgA inducer.

In addition to the reactivity or specificity of an antibody to an antigen/allergen, the binding affinity to its Fc-receptor is also important for the type of signaling that is induced as this can mediate both anti- and proinflammatory functions of IgA. Monomeric IgA only binds with low affinity to the FcαRI and activates the ITAMi which does not lead to cell activation or degranulation/oxidative burst (in the case of granulocytes) (24). In contrast, IgA immune complexes show a stronger binding and subsequent activating signal, resulting in cell activation (25,26). This would even argue that the presence of allergen-specific, high affinity IgA molecules could in fact stimulate inflammation and increase allergy severity.

CTB works as a general mucosal adjuvant, enhancing a diverse IgA response, and it is tempting to speculate that enhanced levels of SIgA of random (polyreactive) specificities are sufficient to reduce AAI. However, the data shown here cannot firmly exclude nor confirm a generalized role for non-specific IgA in CTB induced protection against AAI. No information is available yet on the level, the reactivity or affinity of allergen-specific mucosal IgA molecules in patients with allergic diseases, like allergic asthma. This would help to better understand the disease etiology and to develop new treatments based on IgA inducing mucosal adjuvants of allergies and asthma.

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5

MODULATION OF IGA RESPONSES BY BLOOD DENDRITIC CELL SUBSETS IN ADULT HOUSE DUST MITE ALLERGIC ASTHMATIC PATIENTS

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Abstract

A protective role for mucosal immunoglobulin A (IgA) in allergic inflammation has been suggested by a number of studies and dendritic cells have been shown to play an important role in the induction of IgA by their interaction with B cells. Here, we compared the capacity of blood DC subsets of 14 adult house dust mite (HDM) allergic asthmatic (AA) patients and age/sex-matched non-allergic (NA) controls to induce IgA responses and studied the modulation of this process by the TLR7/8 ligand CL97.

AA patients had less circulating plasmacytoid DCs (pDCs) compared to NA controls but similar levels of myeloid DCs (mDCs). AA pDCs expressed less CD86 compared to NA pDCs, but LAP and PDL-1 expression were similar. Anti-IgM/IgG-stimulated B cells produced less IgA when cultured with AA pDCs compared to NA pDCs, while production of the other Ig subclasses was not different. Although CL97 priming did not change the IgA-inducing capacity of both pDC and mDC, it did increase the percentage of $TNF\alpha^+$ B cells, but this effect was similar between the two groups. No differences between AA and NA were observed for mDCs or Ig production in mDC/B cell co-cultures. Equal level of total IgA and higher level of HDM-specific IgA were found in nasal lavage fluid of AA compared to NA individuals. It is therefore unclear as yet whether the small differences observed in pDC frequency and function with respect to IgA induction have consequences for mucosal IgA responses *in vivo* and local immune responses to allergens.

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Introduction

Allergic asthma is defined as a chronic inflammatory disorder of the airways and is characterized by airway inflammation, bronchial hyperresponsiveness and a variable degree of airflow obstruction, leading to episodes of wheezing and breathlessness (1). Interestingly, individuals with immunoglobulin A (IgA) deficiency are at increased risk of developing autoimmune and allergic diseases, including asthma (2). Many papers report an inverse relationship between mucosal IgA and asthma symptoms (3). For example, Balzar et al found lower IgA levels in brochoalveolar lavages of severe asthmatics compared to healthy subjects, which showed a reversed correlation with lung function and asthma symptoms (4). In sensitized Swedish children, slow maturation of the secretory IgA system was associated with induction of allergic symptoms and was suggested to be related to a lower microbial pressure (5). Conversely, high levels of specific IgA antibodies in salivary of sensitized infants were associated with significantly less late-onset wheezing (6). Indeed, treating mice with allergen-specific IgA or IgA-inducing agents, like Cholera Toxin B (CTB), protect against experimental allergic airway inflammation (AAI) (7,8). Thus, it is suggested that IgA contributes to the maintenance of mucosal tolerance by dampening immune responses (9). As a consequence, IgA may have a role in preventing the development of hyperinflammatory responses towards environmental allergens, such as house dust mite (HDM), that otherwise would cause allergic inflammation in susceptible individuals.

Dendritic cells (DCs) form a important cell type in the differentiation of IgA producing B cells by producing several B cell stimulating and antibody-promoting molecules, like TGF-β, IL-6, IL-10, proliferation-inducing ligand (APRIL), and B cell activating factor (BAFF) (9). The IgA inducing capacity of DCs can be enhanced by both local factors produced by mucosal tissues (e.g. retinoic acid) and/or by microbial products ligating to various Toll-like Receptors (TLRs) (10-12). Furthermore, we have shown that CTB can condition murine bone marrow-derived DCs to promote IgA responses in synergy with MyD88-dependent TLR ligands (Chapter 3 of this thesis). The two distinct DC subsets, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), have unique response patterns to TLR ligands and may therefore differentially modulate B cell activity. For example, TLR7/8 ligand CL97 is able to stimulate both pDCs and mDCs, and induces enhanced production of IFNα and IL-12, respectively (13). Human TLR7/8 stimulated blood pDCs supported B cell proliferation and differentiation into IgM-, IgG- and IgA-producing cells by the production of type 1 interferon (IFN) (14,15). Although mouse gut lymph node-derived pDCs as well as mDCs were able to support IgA production *in vitro*, pDCs were more superior and their activity was dependent on type 1 IFN induced APRIL and BAFF expression (16). Nonetheless, several mucosal mDC subsets in the mouse are specialized to drive enhanced IgA responses (17-19). Thus depending on local factors, both mDC and pDC may promote Ig responses by different mechanisms.

It has been reported that DCs from allergic (asthma) patients and healthy controls behave differently. For example, mDCs from patients with atopic dermatitis and asthma produce less IL-

12, resulting in the development of effector T cells that produce only little IFNγ (20,21). pDCs of atopic children or adults produce less IFNα in response to virus infection (22) or TLR9 stimulation (23) compared to controls. In response to the major allergen of HDM, Der p 1, mDCs from allergic donors favored a Th1 profile ($IFN\gamma^{high}/IL\text{-}4^{low}$) while Der p 1-pDCs promoted a more Th2 profile ($IFN\gamma^{low}/IL\text{-}4^{high}$) in allogeneic $CD4^+$ T cells from a healthy donor (24). Furthermore, it was suggested that differential expression of costimulatory molecules, such as CD80 and CD86, on the DCs of allergic individuals contributed to the differences observed in Th polarization (24-26). Thus, alterations in mDCs and pDCs function may play a role in the Th2 biased immune responses to allergens in asthmatic individuals. Until now, no data are available on the role of blood DC subsets to induce or support humoral protective IgA responses to allergens and whether this is different in patients with allergic asthma.

Here, we aimed to investigate the capacity of blood DC subsets from allergic asthma (AA) patients and non-allergic (NA) controls to prime for IgA production by B cells and how this is influenced by the TLR 7/8 Ligand CL97.

Materials and methods

Subjects

Non-smoking (or ex-smoking for more than one year, less than 10 pack/years) house dust mite (HDM) AA patients and controls (n=14; age: 18-46 yrs; Table I) were characterized using asthma control questionnaires (ACQ; based on (27)), spirometry, airway hyperreactivity (AHR) tests in response to metacholine (PC_{20}metacholine); skin prick tests (SPT) for HDM, tree and grass pollen, cat, dog and aspergillus. NO levels were analyzed in exhaled breath, total serum IgE and allergen-specific IgE for HDM, tree and grass pollen, cat, dog, horse and Aspergillus (Phadiatop) were measured in serum. Inclusion criteria for healthy controls: Total IgE <30 IU/ml and/or PC_{20} >19 mg/ml and negative SPT for all antigens. Inclusion criteria for AA patients: Detectable specific IgE (>0.7 kU/l) for at least HDM or grass/tree and/or PC_{20} <9.6 mg/ml and a positive SPT (wheal >5 mm). AA patients (8 out of 14) using inhaled corticosteroids (ICS) or combination therapy were asked to cease their medication two weeks before blood drawing. All subjects gave informed consent before participation. Ethical approval for the study was provided by the Medical Ethical Committee of the Leiden University Medical Center, Leiden, the Netherlands.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient over Ficoll-Hypaque, and $1*10^6$ PBMC were fixed (1.9% PFA) and cryopreserved. After depletion of B and T lymphocytes with anti-CD19 and anti-CD3 microbeads respectively, mDCs were magnetically sorted using anti-BDCA1 beads, followed by negative selection for pDCs using a non-pDC antibody cocktail according to the guidelines of the manufacturer (Miltenyi Biotec.).

Isolated DC subsets were stained for CD123, CD11c, BDCA-1, CD4, CD8, HLA-DR, and CD20 to determine the purity of the populations. Fixed PBMC were stained for CD11c, HLA-DR, BDCA-1, CD80, CD86, CD123, LAP, and PDL-1 to analyze the frequency and phenotype of the two DC subtypes in blood.

DC stimulation and co-cultures

Blood mDC (purity >80%) and pDC (purity >90%) were cultured in RPMI 1640 medium (Invitrogen; 42401-042) supplemented with 10% FCS (Greiner Bio-one), 100 U/ml Penicillin, 100 μg/ml Streptomycin, and the amino acids pyruvate (1mM) and glutamate (2mM), containing 10 ng/ml recombinant human GM-CSF (Bioscource) or 10 ng/ml recombinant IL3 (Strathmann), respectively. Blood mDC ($1x10^4$) and pDC ($5x10^3$) were stimulated with medium, or CL97 (1μg/ml, Invivogen), and after 20 h, supernatant was harvested. After thorough washing, the DCs were cultured with allogenic CD19+ B cells ($1x10^5$; isolated from a buffy coat) and affinipure F(ab') 2 goat anti human IgG+IgM (10μg/ml, Jackson Lab.). After 6 days of co-culture, supernatant was harvested and immunoglobulin or cytokine levels were determined. The cells in co-culture were counted, restimulated for 6 hrs with phorbol myristate acetate (PMA, 20 ng/ml), ionomycin (2 μg/ml), and for the last 4 hours in presence of Brefeldin A (10 μg/ml, Sigma-Aldrich), then fixed (1.9% PFA) and cryopreserved. After thawing, the cells were stained for CD20, TNFα and IL-10 to study cytokine production in the B cells.

Nasal lavage

Nasal lavages were obtained using a syringe connected to a catheter equipped with an inflatable balloon. The balloon was placed into the *vestibulum nasi*, and gently inflated. In a sitting position, subjects extend their neck 30° from the horizontal (writing position), and 10ml of sterile phosphate-buffered saline (PBS, 37°C) was instilled by slowly compressing the syringe. After 5 min, the nasal device was removed and the fluid was collected in a plastic petridish [modified from (28)]. This procedure was carried out in each nostril. Recovered volume varied from 5.0 - 12.0 ml (50%–120% by volume), was filtered over a cell strainer (100μm), centrifuged and stored at -20°C until further analysis (29).

Cytokine and immunoglobulin measurements

The cytokines IFNα, IL-1β, IL-6, IL-10, IL-12p70, and TNFα were measured in DC supernatant by Luminex (Bio-Rad Laboratories). Immunoglobulins IgA, IgE, IgG1, IgG2, IgG3, IgG4, IgM were measured in DC/B cell co-culture supernatant using the Human Immunoglobulin Isotyping Assay (Bio-Rad Laboratories). Luminex assays were performed according to the manufacturer's description. Detection limits ranged between 3 and 12 pg/ml for the DC cytokines and between 0.3 and 10ng/ml for the immunoglobulins, except for IgG1, IgG2 and IgG3 with lower limits between 24 and 63 ng/ml. In the nasal lavage fluid, HDM-specific IgA and IgE were determined using ImmunoCAP according to the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden).

Statistical analysis

Differences between the two patient groups were tested using the non-parametric Mann Whitney U test. The paired Wilcoxon signed R test was used to compare different priming conditions. Data are represented as dotplots with the mean. Probability values less than 0.05 were considered significant.

Results

Patient characteristics

To study the capacity of human peripheral blood DCs to promote IgA production in AA patients, 14 adult non-smoking patients and 14 age/sex-matched NA control subjects were included. Their characteristics with respect to lung function, bronchial hyperreactivity, allergen skin prick test reactivity and serum IgE levels are described in **Table 1**. All patients were allergic to HDM, except for one. But as this person was allergic to all other allergens tested (tree, grass, dog and cat) and matched the lung function and hyperresponsiveness inclusion criteria for allergic asthma, we kept this person in the analysis. Individual values for this person were not different from the other subjects in the AA group. Blood samples of patients and of their matched controls were processed and analyzed simultaneously. PBMC counts per ml blood were similar between groups (data not shown).

Blood DC frequencies and costimulatory marker expression in AA patients and NA controls

Isolated PBMC of NA and AA individuals were stained for different DC subsets and costimulatory markers and analyzed by flowcytometry. The percentage of pDCs ($CD11c^{-}MHCII^{+}CD123^{+}$) was significantly lower in AA compared to NA donors, while there was no difference in percentage of mDCs ($CD11c^{+}MHCII^{+}BDCA1^{+}$) (**Figure 1A**). This was also reflected in the absolute numbers of mDCs and pDCs isolated from PBMC (data not shown), and resulted in a significantly increased mDC/pDC ratio in AA compared to NA (**Figure 1B**).

Furthermore, AA pDCs showed a slightly lower CD86 expression than NA pDCs, while there were no differences in CD80 expression. Comparing the two groups, we observed a trend towards lower HLA-DR expression in AA patients for both pDCs and mDCs (p=0.068) (**Figure 2A**). In addition, we measured Latency Associated Peptide (LAP), which associates with mature TGF-β prior to secretion, and PDL-1 expression on the DC subsets, as these markers are associated with induction of IgA and immune regulation (9,30). LAP expression was only observed in mDCs (~10% of mDCs), but not in pDCs (**Figure 2B**), while mDCs express less PDL-1 than pDCs (**figure 2C**). For both markers, no differences (in % nor in GM) were observed between NA and AA (**Figure 2B/C**, supplementary figure 1).

Table 1 Clinical characteristics of house dust mite allergic asthmatic adult patients and control subjects.

	AA (n=14)	**NA** (n=14)	p-value
Age (yrs)	26 (19-44)	24 (21-45)	0.6424
BMI	23 (19-35)	23 (18-25)	0.1949
ICS	8	-	-
NO	26 (13-80)	13 (5-31)	0.0004
ACQ	4 (0-12)	0 (0-1)	-
FEV1-%predicted	101 (91-118)	111 (95-128)	0.0095
FEV1/FVC ratio	0.80 (0.64-1.18)	0.87 (0.77-0.97)	0.1457
PC20-methacholine	1.3 (0.1-9.6)	>19	-
SPT (> 5mm)			
HDM	13	-	-
Grass/Tree	11	-	-
Cat/Dog	9	-	-
Total IgE (IU/ml)	113 (30-307)	7 (2-31)	<0.0001
Spec. IgE (IU/ml)			
HDM	13 (0.11-51)	-	-
grass	4.5 (0.03-38)	-	-
tree	0.18 (0.02-10)	-	-
Cat	0.52 (0.01-12)	-	-
Dog	0.56 (0.10-2.7)	-	-

Allergic Asthma patients (AA); Healthy Controls (NA); years (yrs); Body mass Index (BMI); inhalation corticosteroids (ICS); Exhaled nitric oxide (NO); Asthma Control Questionnaire (ASQ); Forced expiration volume in 1 second (FEV_1); Forced Vital Capacity (FVC), provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}); Skin Prick Test (SPT); House dust mite (HDM). P-values are based on the Mann-whitney U test.

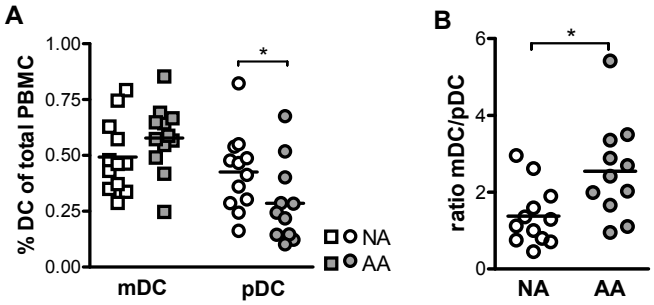

Figure 1 Percentage of DCs in PBMC from blood of NA (white) and AA (grey), determined by Flowcytometry. A) % of mDCs (squares) and pDCs (circles), B) Ratio of mDC/ pDC. P-values are based on the Mann Whitney U test, * P< 0.05, ** P< 0.01, *** P<0.001.

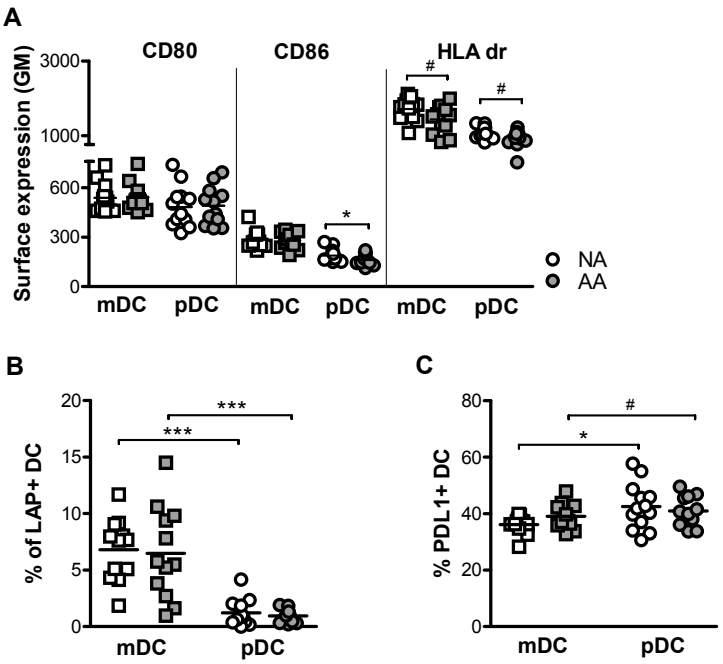

Figure 2 Marker expression on blood DC subtypes of NA (white) and AA (grey), measured by Flowcytometry of fixed PBMC. A) Expression of costimulatory molecules CD80, CD86 and HLA-DR (GM = Geomean), B) Percentage of mDCs (squares) and pDCs (circles) expressing Latency Associated Peptide (LAP), and C) Percentage of DCs expressing PDL-1. P-values are based on the Mann Whitney U test # P<0.070, * P< 0.05, ** P< 0.01, *** P<0.001.

Influence of blood DC subsets on B cell Ig production in AA patients and NA controls

Next, we were interested whether DC subsets of AA vs NA had a different capacity to support the production of immunoglobulines, like IgA, and how TLR ligands influences this system. As TLR7 and TLR8 are expressed by both mDC and pDC we focused on CL97, which is a ligand for TLR7/8. The two DC subsets were primed overnight by medium or CL97 and washed several times to remove the stimuli before adding allogenic CD19+ B cells isolated from a buffy coat and stimulating antibodies against the B cell receptor (BCR), mimicking BCR triggering. After 6 days different immunoglobulin isotypes were measured in the supernatants. Although B cells from different buffy coats were used, always the same B cell donor was used for matched pairs of AA patients and healthy controls. In unprimed conditions of NA DC subsets, similar IgA levels were found in co-cultures of B cells/pDCs and B cells/mDCs (~ 40 ng/ml), while supernatant of anti-IgM/anti-IgG stimulated B cells without DCs contained less than 10 ng/ml. Interestingly, less IgA was found in AA cultures of B cells/pDCs compared to similar cultures of NA donors (**Figure 3A**). CL97 exposure did not affect IgA levels in pDC and B cell co-cultures compared to unprimed DCs in both NA and AA individuals. No differences between the groups were observed in IgA levels in co-cultures of B cells and mDCs (**Figure 3A**).

In the co-cultures of B cells and pDCs, we found significantly more IgG4 (~70 ng/ml) compared to similar cultures with mDCs (~45 ng/ml) (**Figure 3B**), but there was no difference between the NA and AA individuals. Yet, CL97 priming of NA pDCs pretreatment resulted in a slight increase in IgG4, which was not the case in cultures with AA pDCs. No differences were observed for mDC cultures between NA and AA individuals. Although generally the IgE levels in DC B cell co-cultures were very low (~1ng/ml) (**Figure 3C**), a small increase was observed in CL97-primed AA pDC cultures compared to unprimed AA pDC cultures which was not seen in NA patients (**Figure 3C**). No differences were observed for mDC cultures between NA and AA individuals. Substantial levels of IgM were measured (~20 ng/ml), but the levels were similar in all culture conditions and were not affected by CL97 DC priming (data not shown), indicating that survival or proliferation of B cells was not different between these conditions. Furthermore, there were no significant differences in absolute numbers of cells present after 6 days of co-culture between any of the conditions. This may exclude the possibility that the Ig concentrations in the co-culture were influenced by differences in B cell activation, survival or proliferation, instead of differences on the level of DCs (data not shown).

IgG1, IgG2, and IgG3 levels were below the detection limit of the Luminex kit used (data not shown). Other papers studying the effect of DC/ B cell interactions for immunoglobulin expression found IgG+ cells, but they do not distinguish between different IgG subtypes (14,31). It may be that a more sensitive assay was used to measure detectable levels of isotypes. In addition, class switching and secretion of the various isotypes are differently regulated and some may dependent on signals from T cells (and tissue environment), which were not present in our assay.

Taken together, we observed a weaker capacity of unprimed AA pDCs to induce IgA in B cells, while CL97 priming did not influence the IgA inducing capacity of both NA and AA pDC. In contrast, CL97 DC priming in AA patients seem to promote IgE production. However the total levels in the culture system remain very low, making it difficult to extrapolate these findings towards elevated levels found in vivo. Equal responses were observed for B cell co-cultures with unprimed mDCs from NA versus AA individuals.

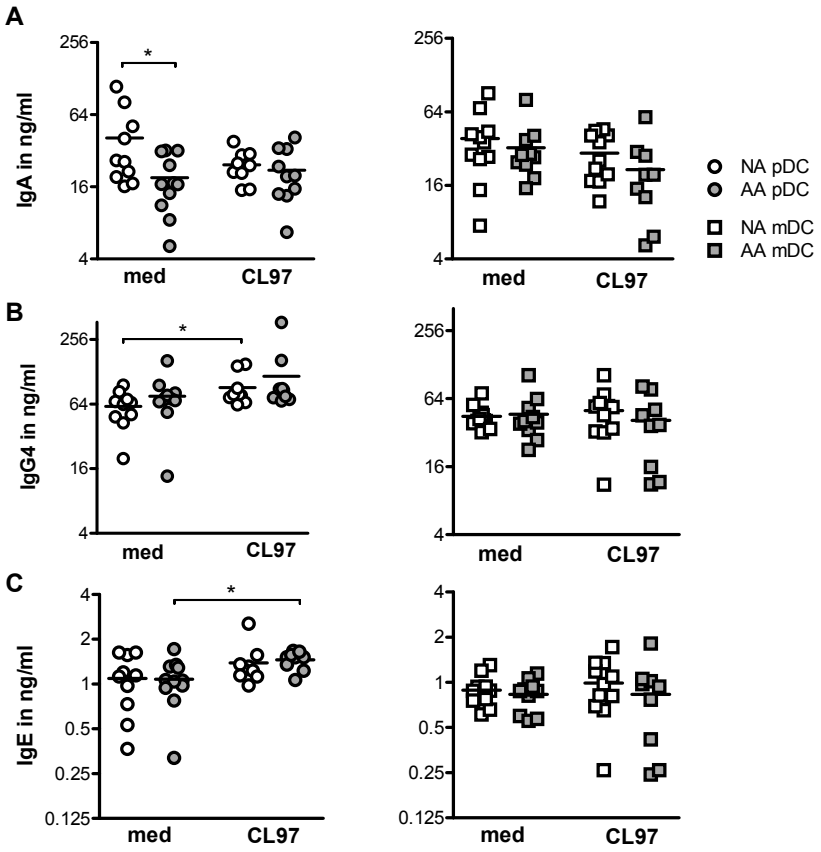

Figure 3 Ig production of B cells co-cultured with medium or Cl97- primed pDC (circles) or mDCs (squares) of NA (white) and AA (grey) for 6 days, measured in supernatant by Luminex assay. A) IgA production, B) IgG4 production, C) IgE production in ng/ml. P-values are based on the Mann Whitney U or Wilcoxon signed rank test * P< 0.05, ** P< 0.01, *** P<0.001.

Influence of blood DC subsets on B cell cytokines in AA patients and NA controls

In addition to immunoglobulins, upon activation B cells can also produce different pro-inflammatory and anti-inflammatory cytokines. Here, we studied the intracellular cytokine expression of IL-10 and TNFα by B cells after a 6 day co-culture with pDCs or mDCs of NA and AA individuals. Interestingly, the percentage of IL-10 positive B cells in co-cultures with pDCs was significantly higher compared to co-cultures with mDCs (2.37% versus 1.63%, p= 0.0418), while TNFα percentages were the same. Nevertheless, this feature was not different between the two donor groups. CL97 priming of pDCs and mDCs did not change the percentage of $IL10^{+}$ B cells (**Figure 4A**), but CL97 priming of mDCs strongly enhanced the percentage of $TNF\alpha^{+}$ B cells in both NA and AA cultures (**Figure 4B**), while CL97 priming of pDCs only significantly induced $TNF\alpha^{+}$ B cells in NA cultures but not AA conditions. B cell survival in the BCR-stimulated samples without

DCs at day six was low and as a consequence hardly any intracellular cytokines were measured upon restimulation (data not shown).

Thus, priming of DCs with TLR7/8 ligand CL97 resulted in an enhanced capacity to induce pro-inflammatory cytokine TNFα in B cells, although this effect was less clear in pDCs from AA patients.

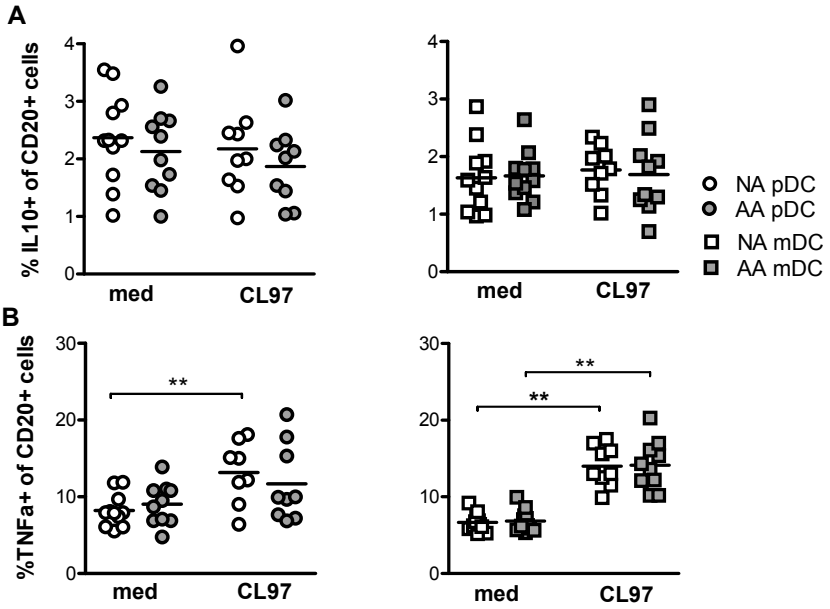

Figure 4 Cytokine expression of B cells co-cultured with medium or CL97- primed pDCs (circles) or mDCs (squares) of NA (white) and AA (grey) for 6 days, in fixed cells by flowcytometry. A) Percentage of IL-10 positive B cells, and B) percentage of TNFα positive B cells of total CD20+ cells. P-values are based on the Mann Whitney U or Wilcoxon signed rank test * P< 0.05, ** P< 0.01, *** P<0.001.

Cytokine production by blood DC subsets from AA and NA individuals

To explain the observations found for Ig and B cell cytokine production in the DC/B cell co-cultures, cytokine production of DCs was determined after overnight stimulation by multiplex analysis. No cytokines were detectable in supernatant of unprimed pDC (data not shown), while only low levels of IL-6 (~30 pg/ml), and TNFα (~30 pg/ml) were found in supernatant of unprimed mDC (**Figure 5A).** In contrast, CL97-primed pDCs produced detectable levels of IL-6 (~10 pg/ml) and TNFα (~100 pg/ml), but no IL-1β, IL-10, IL-12p70 or IFNα was measurable. Detectable levels of IL-1β (~400 pg/ml), IL-6 (~4000 pg/ml), IL-10 (~90 pg/ml), IL-12p70 (~20 pg/ml), and TNFα (~1000 pg/ml) were produced by CL97-primed mDCs (**Figure 5B**). No differences were observed in the analyzed cytokines when comparing NA and AA in individuals, indicating that none of the cytokines measured may explain the differences found with respect to Ig levels and B cell cytokines in AA patients compared to NA individuals.

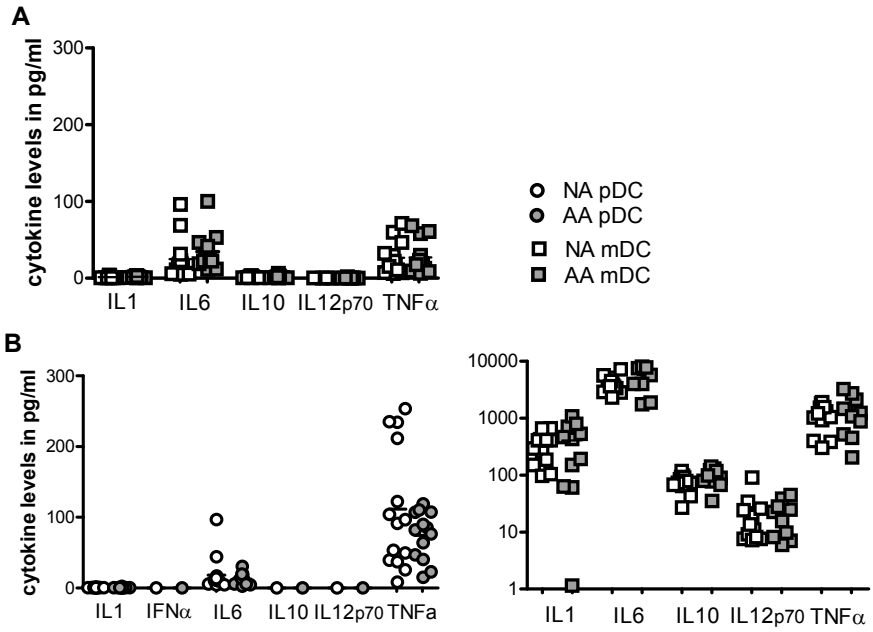

Figure 5 After isolation of the blood pDCs and mDCs from NA (white) and AA (grey), DCs were primed overnight with medium or CL97 and supernatant was collected. IL-1β, IFNα, IL-6, IL-10, IL-12p70, and TNFα levels were determined by Luminex A) Cytokine levels of medium primed mDC. B) Cytokine levels in CL97-primed pDC (circles) and mDC (squares). P-values are based on the Mann Whitney U test * P< 0.05, ** P< 0.01, *** P<0.001.

IgA levels at mucosal surfaces are not lower in AA compared to NA adults

To investigate whether the reduced number and reduced IgA inducing capacity of AA pDCs had any consequences for IgA responses at mucosal surfaces, we analyzed Ig levels in nasal washes. Here, we found significantly higher HDM-specific IgE, but not total IgE, in nasal lavages of AA compared to NA (**Figure 6A and B**). However, we did not find differences in total IgA, IgA1 or IgA2 levels in nasal lavage fluid of AA and NA (**Figure 6C** and Supplementary Figure 2A/B). In contrast to what was expected (3,5,6), HDM-specific IgA levels at mucosal surfaces were higher in AA compared to NA individuals (**Figure 6D**).

Furthermore, as a control for local inflammation, α(2)-macroglobulin was measured in the nasal lavage, which is a marker of mucosal exudation of bulk plasma. However, there were no differences observed in α(2)-macroglobulin levels in AA and NA (Supplementary Figure 2C) and no correlation was detected between α(2)-macroglobuline and IgA. This may indicate that inflammation-induced vascular and epithelial leakiness in AA was not of major influence on the IgA levels found in the nasal fluid of AA compared to NA individuals at the time of collection.

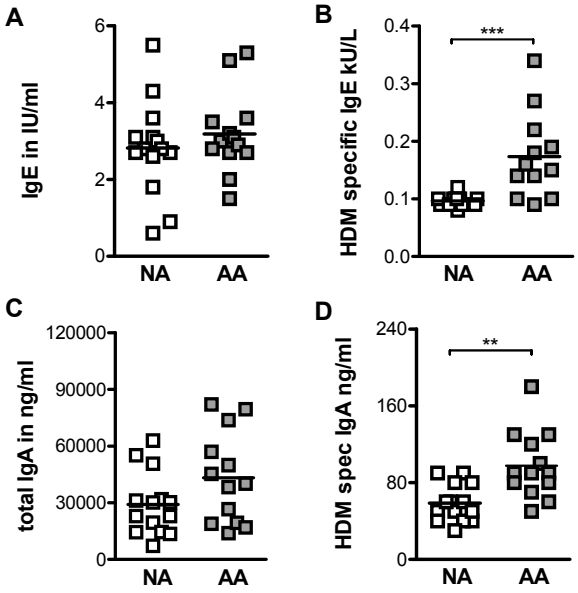

Figure 6 Immunoglobulin levels in nasal lavage fluid of NA (white) and AA (grey), measured by ELISA. A) Total IgE levels in IU/ml, B) HDM-specific IgE levels in kU/L C) Total IgA levels in ng/ml D) HDM-specific IgA levels in ng/ml. P-values are based on the Mann Whitney U test, * P< 0.05, ** P< 0.01, *** P<0.001

Discussion

Here, we have compared the capacity of blood DCs from AA patients and control individuals to promote IgA responses *in vitro*. In AA patients, we observed a reduced number of circulating pDCs combined with slightly, but significantly, lower capacity to drive B cell IgA responses. Cl97 priming of DCs did not change IgA inducing capacity, instead we observed more IgE in AA pDC cultures compared to AA unpulsed pDC cultures, although the levels remained low. No differences were observed in mDC function with respect to priming of IgA responses. At present, it is unclear whether the differences observed in pDC function and frequency have any *in vivo* consequences for mucosal IgA responses, as both total and HDM-specific IgA levels in nasal lavage were not reduced in AA compared to control subjects.

The finding that AA patients have a lower percentage of blood pDCs is interesting as pDCs have been associated with inhalation tolerance and homeostasis in the lung and the reduced numbers may point at an intrinsic defect in AA patients (32,33). At present, it is unclear whether this is the result of increased trafficking in the airways (34,35) as shown in asthma patients in response to allergen challenge (36-38) or because of a reduced recruitment of newly generated pDCs from the bone marrow (39,40). Alternatively, it has been reported that corticosteroids can reduce circulating pDC numbers (41,42) and this may form a confounding factor. However, we

did not observe differences in pDC or mDC numbers, marker expression or cytokine production between patients who did not use ICS and patients who temporarily stopped ICS (8/14) (data not shown). Therefore, it is not expected that the reduced numbers of circulating pDCs in AA are the result of corticosteroid use.

We studied the function of the two blood DC subsets to induce IgA responses. Interestingly, pDCs, but not mDCs, of AA patients were weaker in promoting IgA responses, as we found lower IgA levels in co-cultures with peripheral B cells compared to cultures from NA donors. Other Igs and B cell cytokines studied were equal to pDC/B cell cultures from NA donors, suggesting a selective effect. Our findings are in line with various other studies suggesting that pDC are also qualified to induce humoral responses, including IgA, like DCs derived from mucosal tissues such as the gut (14,16). To explain the functional differences between NA and AA pDCs with respect to their IgA instructing capacities, we studied costimulatory marker expression of ex vivo pDCs. We found a reduced CD86 expression on AA pDCs compared to NA pDCs, while other markers were similar. Until now CD28 and CTLA-4 (CD152) have been identified as ligands of CD86 (B7-2). While the engagement of CD28 induces T cell activation by DCs (43), that of CTLA-4 results in a negative signal that is essential for immune homeostasis (44,45). Interestingly, CTLA-4 (in addition to other inhibitory receptors) is also expressed on B cells (46,47), and therefore would allow for interactions with CD86 expressed on DCs, possibly interfering with the regulation of B cell responses including Ig responses. Engagement of the B cell CTLA-4 by immobilized mAb inhibits IgG1 and IgE production (48). However, up to now, there is not much known about the regulation and the function of CTLA-4 on B cells, and it would be interesting to study their role in the context of allergic diseases.

It is reported, that TLR priming of DCs can influence their IgA inducing capacity (14,15). Here we used the TLR7/8 ligand CL97, which is able to stimulate both pDCs and mDCs and induces enhanced production of IFNα and IL-12, respectively (13). Although we did not observe an effect of CL97 priming of DCs on IgA production, we found substantial levels of TNFα and IL-6 in cultures of both pDC and mDC in response to CL97, but IFNα was not detectable and IL-12p70 was very low. This might be explained by the low cell numbers used, resulting in insufficient concentrations of IL-10, IFNα and IL-12p70 in the supernatant and as a consequence to insufficient instructions for B cell Ig modulation, rationalizing the lack of any further IgA induction following CL97 priming.

Another explanation might be found in the experimental set-up, as some studies added and stimulated DCs and B cells simultaneously, while we first primed DCs overnight and only started the co-culture with B cells after extensive washing of the DCs. Our set-up excludes possible direct effects of the DC priming compounds on B cells and allows studying B cell modulation via DCs only. However, we cannot investigate the effect of early DC derived factors (produced in the first 20 hours) on the instruction of B cell Ig production. As we do not find a strong IgA induction after DC priming, this may suggest that the very early signals from recently activated DCs are important in driving Ig production by B cells. Alternatively, in addition to BCR ligation, B cells might need direct signals from TLR ligands for proper B cell responses and/or Ig production, and

this was lacking in the set-up of our co-cultures (31,49,50). The cytokines APRIL and BAFF were not determined, but as these are very important T cell-independent class switch inducing factors produced by epithelial cells and DCs, it would be interesting to investigate their presence and to study whether they are differently produced by NA and AA DCs.

A lower blood pDC frequency together with a weaker function of pDC to prime for IgA responses in AA patients, could lead to diminished mucosal IgA responses *in vivo*. Indeed, several studies have shown reduced mucosal IgA responses in saliva or serum from allergic and asthmatic children compared to healthy children (6,51), although the mechanisms leading to these impaired IgA responses were not studied in much detail. We used nasal lavages to estimate local mucosal IgA responses (28). In contrast, in our cohort of young adults, IgA levels in nasal lavage fluid from AA were not lower than those of control individuals. It is possible that age is an important factor in the observed differences in IgA which were mainly reported from infants whereas our study subjects were young adults. Mucosal IgA production increases with age, reaching adult levels around 5 years (5), and it might be that a delayed or impaired development of mucosal IgA responses allows for sensitization and/or the development of allergic symptoms during the first few years of life. By the time that adulthood is reached this impaired IgA response may have been gradually restored as a consequence of microbial exposure leading to a normally developed IgA repertoire in the end (5). Thus, defects in the mucosal IgA system, possibly as a result of a different DC capacity and which were present early in life, may no longer be detectable at adult age. Future studies need to be conducted in young children to assess whether DC/ B cell interaction is important for mucosal IgA. Collectively, this will lead to a better understanding of the pathogenesis of allergic diseases, and may help to improve existing therapy or develop novel strategies for treatment.

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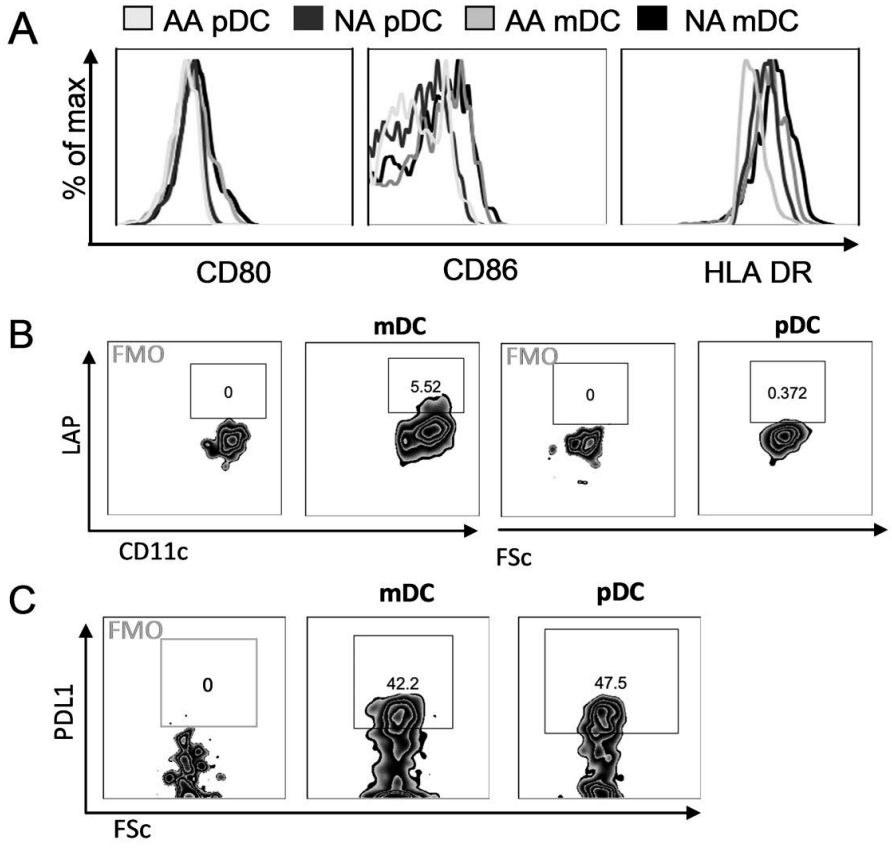
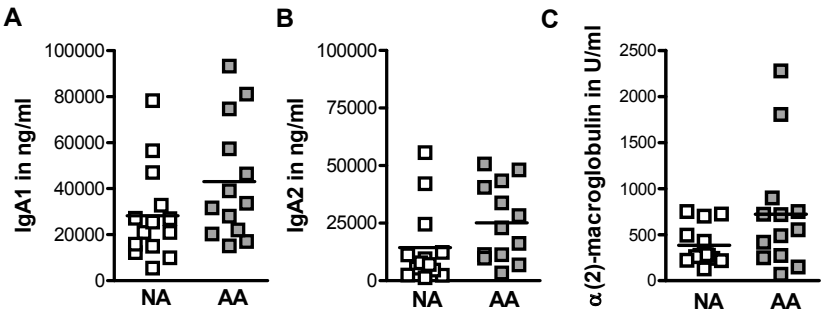
Supplementary figures



Supplementary Figure 1 Marker expression on blood DC subtypes of one representative donor (pair), measured by Flowcytometry of fixed PBMC. A) Histograms showing an overlay of the difference in costimulatory marker expression of CD80, CD86 and HLA-DR, B) FACS plot showing LAP expression, C) FACS plot showing PDL-1 expression. FMO = Fluorescence Minus One control.



Supplementary Figure 2 Immunoglobulin levels in nasal lavage fluid of NA (white) and AA (grey), measured by ELISA. A) Total IgA1, and B) Total IgA2 in ng/ml, and C) α(2)-macroglobulin levels in units/ml. P-values are based on the Mann Whitney U test.

6

SUMMARIZING DISCUSSION

The studies presented in this thesis focus on the regulation and adjuvant stimulation of immunoglobulin A responses, centered around the hypothesis that induction of IgA responses constitutes a novel therapeutic approach against allergic asthma and other allergies.

Mucosal IgA and protection against experimental allergic airway inflammation

Several observational studies have shown a negative association between immunoglobulin A (IgA) and allergy development, suggesting a protective role for IgA against allergies (1-4). Only few studies have been published that focus on a causal relationship between IgA and protection against inflammatory response to allergens. Here, we demonstrated that development of experimental asthma can be suppressed predominantly by inducing secretory IgA (SIgA). Mice were protected against the development of allergic airway inflammation (AAI) by increasing IgA responses via dendritic cells (DCs), while mice deficient for polymeric Ig Receptor (pIgR) and that lack SIgA, were not (**Chapter 2**).

At the mucosa, such as the gastrointestinal and the respiratory tract, SIgA plays a major role in mediating immune exclusion ('antigen avoidance') of luminal antigens and homeostasis with the commensal flora as well as the protection against invading pathogens (5,6). Polyreactive (natural) IgA antibodies or antibodies of irrelevant specificity are suggested to be sufficient for immune exclusion of commensal bacteria and intestinal pathogens, such as certain parasites (7,8), however it is not clear whether this also applies to other environmental particles, such as allergens. In **chapter 4** we aimed to study whether enhanced levels of IgA of random specificity or polyreactive IgA are sufficient to provide protection against immune responses to allergens, or that allergen-specific responses are required. We made use of transgenic mice with a restricted B cell receptor (BCR) repertoire recognizing the – in the context of birch pollen allergy - irrelevant Vascular Stomatitis Virus antigen (V10YEN). In these mice, the elevated total IgA levels were associated with a reduced development of AAI. Even though we used mice that in principle should not recognize the allergen applied, we still found detectable allergen-specific IgA levels in the bronchoalveolar lavage fluid, yet not specifically increased in the protected mice. These allergen-specific antibodies may constitute of natural polyreactive or (mutation induced) cross-reactive antibodies, or may be derived from the small percentage of B cells that did not express the complete VSV-specific BCR. These experiments may suggest that excess random non-specific IgA is sufficient to prevent AAI, however a contributing role for allergen-specific IgA cannot be fully excluded in this model (**Chapter 4**). Due to the complex regulation of BCR expression and antibody formation, it is very difficult to develop a model with mono-reactive B cells in which the development of random IgA specificities is completely eliminated. Nevertheless, these data suggest that CTB works as a general mucosal adjuvant, stimulating a diverse IgA response. It is clear from the literature that for the optimal functionality of mucosal immunity and humoral IgA responses, a diverse spectrum of random (polyreactive) mucosal IgA is desirable to prevent the

entry a large array of pathogens and environmental particles (9). Therefore, we have investigated in this thesis whether stimulating the development of enhanced (secretory) IgA responses against allergens is a suitable approach to protect against the development of allergic diseases including asthma.

IgA inducing adjuvant Cholera Toxin B and the role of dendritic cells

To investigate the role of mucosal IgA in protection against AAI, we focused on adjuvants that were associated with strong mucosal Ig responses, but did not induce strong type 1 inflammation. Induction of a strong Th1 response to inhaled antigens is associated with neutrophilic influx that can damage the lung to the same extent as influx of eosinophils (10). Cholera Toxin, produced by the bacterium *Vibrio Cholerae*, is a very potent adjuvant inducing strong mucosal IgA and serum IgG plus IgE responses, and drives a mixed Th1 and Th2 cell response (11). The choice of the non-toxic subunit of CT (CTB) as a mucosal adjuvant was not random as it has the potential to mimic some of the aspects of the immune response to allergens in healthy individuals. Indeed, CTB is especially associated with immunoregulatory events, such as the induction of Treg cells in response to orally co-administered antigen (12) and it was suggested also to stimulate the formation of IgA responses (13). Here, we demonstrated that CTB administration stimulates local IgA responses *in vivo* using different models. When CTB was administered directly in the airways, it was found to target mainly to DCs and to increase IgA levels (**Chapter 3**). Adoptive transfer of *in vitro* generated CTB treated DCs was sufficient to enhance IgA responses in mouse lungs (**Chapter 2**). Furthermore, *in vitro* co-cultures of CTB exposed bone marrow-derived DCs and B cells also resulted in the induction of IgA production (**Chapter 3**). Based on these results, we conclude that CTB promotes IgA responses by modulating the function of DCs.

DCs have an important role in the regulation of IgA responses, both via T cell-dependent (TD) and T cell-independent (TI) pathways. They not only provide signals enhancing B cell survival, plasma cell differentiation and IgA secretion, but they also provide IgA class-switch signals in case of TI IgA synthesis (6,14). To better understand how adjuvants promote the synthesis of IgA, we studied the interaction of CTB-DCs and B cells. *In vitro* it was shown that synergism between CTB and MyD88-dependent TLR signals selectively imprints an IgA inducing phenotype in DCs, characterized by RALDH1 and TGF-β expression (**Chapter 3**). The role of these DC derived factors was confirmed *in vivo* (**Chapter 3**). It was long thought that only DCs that differentiated in the specialized microenvironment of the gut mucosa were capable of driving IgA synthesis, whereas DCs derived from the bone marrow or other central lymphoid organs were unable to do so. It was thought that conditioning by microbial-flora derived TLR ligands and special mucosal tissue derived factors, such as retinoic acid, TGF-β, BAFF and APRIL, were necessary for this imprinting effect. In the gut lamina propria, several specialized DC subsets have been described, such as TLR5 expressing $CD11c^{hi}CD11b^{hi}$ DC, TNFα/iNOS-producing DCs (tipDCs) and retinoic acid-producing

CD103+ DCs (14-16). During the period that different specialized lamina propria DC subsets were identified, Naito et al published that also DCs derived from the lung mucosa were able to induce IgA production (17). Further studies revealed that lung CD11b^{hi} DCs from mice more efficiently induced IgA *in vitro* compared to CD103^{+} DCs (18). Interestingly, we now show that CTB by itself can condition non-mucosal, bone marrow-derived DCs to promote IgA responses both *in vitro* and *in vivo* (**Chapter 2 and 3**), most likely explaining why CTB is acting as a "mucosal adjuvant" when given at different sites. For optimal CTB driven conditioning of non-mucosal DCs, MyD88-dependent TLR co-signalling is essential (**Chapter 3**), but it is independent of special mucosal factors, such as retinoic acid.

In addition to the conventional mouse DC subsets which drive IgA synthesis and are portrayed in the previous paragraph, also a plasmacytoid DC subset has been described (19). These plasmacytoid DCs (pDCs) differ from conventional DCs (cDCs) in expressing lower amounts of CD11c, yet produce very high amounts of IFNα. Although both mouse gut lymph node-derived pDCs and cDCs were able to support B cell IgA production, pDCs were more superior *in vitro* due to an enhanced type 1 IFN production (20). Also in humans, IFNα producing pDCs seem to be more advanced in supporting B cell proliferation and differentiation into antibody producing cells, including IgA, compared to myeloid DCs (mDCs, grossly equivalent to the mouse cDC subset) (21,22). In our study, human blood pDCs and (BDCA1^{+}) mDCs were equal in supporting IgA responses, but pDCs were better in stimulating IgG4 and IL-10 cytokine production by B cells than mDCs (**Chapter 5**). Therefore it can be concluded that both cDCs and pDCs can promote Ig responses, but by different mechanisms, depending on local factors. Upon *in vivo* administration, CTB targeted mainly to cDCs, and not so much to pDCs (**Chapter 3**), and ALDH activity was especially increased in the CD11b^{+} lung DC subset (**Chapter 3**). The use of CD11b as a marker for lung cDCs is however confusing, as CD11b is not only found on a subset of cDCs, but also on the population of monocyte-derived DCs (moDCs), that are recruited to the lungs at times of inflammation. Interestingly, the bone marrow-derived DCs differentiated in GM-CSF are a good model for CD11b^{+} moDCs and were used for adoptive transfer and our *in vitro* studies. These experiments suggest that (CD11b+) moDCs could be responsible for CTB induced IgA responses in the mouse airways. This is certainly a possibility as CTB seems to work best at inducing IgA responses when accompanied by some degree of LPS, e.g. like found in the OVA antigen. LPS is a known trigger of moDCs recruitment (23). Until we have more specific depleting antibodies or transgenic mouse strains to selectively deplete moDCs, we can however only speculate at this stage whether this is true. The effect of CTB on pDCs function *in vivo* was not specifically studied here because CTB did not target to pDCs *in vivo* in our experiments.

If we are to exploit the full potential of IgA as an immunomodulatory immunoglobulin in allergic asthma and other immune mediated diseases, the role of different DC subsets in the regulation of IgA responses and modulation by adjuvants should be studied in more detail.

IgA and dendritic cells in allergic asthmatic patients

IgA levels are lower in the circulation and at the mucosa of asthmatics than in healthy control children, and negatively correlated with lung functions and allergic symptoms (1,2,4,24). Additionally, successful immunotherapy for asthma is associated with induction of IgA (and IgG4) responses at the airway mucosa (25-28). Therefore, we wondered whether the IgA-priming capacity of blood DC subsets of allergic asthmatic individuals is different from non-allergic control individuals. Here we observed that in allergic asthmatic patients, blood pDCs, unlike mDCs, were reduced in number and weaker in stimulating B cell IgA responses compared to control individuals (**Chapter 5**). This was a selective effect, as other Igs and B cell cytokines studied were equal to co-cultures of non-allergic donors. This also included the isotype IgG4. We were interested in this isotype as it is generally considered as an anti-inflammatory Ig, and may contribute to tolerance-inducing or tolerance-maintaining mechanisms. The Th2 cytokines IL-4 and IL-13 are involved in class switching to both IgG4 and IgE however the additional presence of IL-10 may shift the balance towards the IgG4 ('modified Th2 response') instead of to the pro-allergic IgE (29,30). In the context of IgE-mediated allergy the appearance of IgG4 antibodies is usually associated with a decrease in symptoms (31). Supportive in this context might be the observation that human pDCs were strong in stimulating both IL-10 and IgG4 production by B cells. However, this capacity was similar between patients and controls (**Chapter 5**). In mice, the IgG4 isotype does not exist and its role in the development of allergic asthma was not studied in our experimental setting.

In view of the promising data from the mouse studies in this thesis (**Chapter 2 and 4**), it would be interesting to study whether CTB could increase the capacity of human blood DCs to stimulate IgA responses and whether a putative impairment in IgA-induction by those DCs could be restored. Indeed, previous studies have shown that CTB may affect human DC function: CTB/antigen conjugates suppress maturation and function of (human umbilical cord) blood DC precursors (moDCs) based on the increase of IL-10 and/or decrease in IL-12 and IL-6 production (32-34). Furthermore, we performed pilot experiments with human blood DCs and observed enhanced cytokine production in response to CTB, including TNFα and IL-10 production, by both mDCs and pDCs (unpublished). Further experiments are required to study how CTB modulates human pDC and mDC function, what the involvement of different co-factors like TLR ligands or allergens is, and to learn how this affects their Ig stimulating capacity.

In case of pDC-B cell interactions, IFNα appears to be the main IgA-inducing factor. It is questionable though, whether the approach of stimulating IFNα by pDCs to enhance mucosal IgA responses is suitable for therapeutic application of allergic asthma. Until now, IFNα production by pDCs is mostly induced by strong inflammatory stimuli, such as viral RNA/DNA via TLR-7/8/9 (35). Furthermore, in humans elevated IFNα levels are associated with development of several severe autoimmune diseases, such as lupus and psoriasis (36,37). Following this line, the approach of promoting IFNα-producing pDCs to increase local IgA responses, may concomitantly result in inflammatory processes also affecting other cell systems. This is in great contrast with the

tolerogenic pDCs in the airways which suppress the cardinal features of asthma independent of IFNα, and drive Treg cell development (38,39). Furthermore, mucosal IgA production stimulated by DCs, as described for different specialized mouse gut cDC subsets, appears to occur under mostly tolerant conditions in which the development of Treg cells is also stimulated. In this environment factors like TGF-β and retinoid acid are dominantly present, and they are involved in regulatory processes driving IgA induction and Treg cell development, thus preventing local inflammation. As in the mouse CTB induces IgA mainly via modulated cDCs and/or moDCs expressing TGF-β and retinoic acid (**Chapter 3**), the strategy of modulating human mDCs, and not pDCs, function seems to be a more feasible approach, despite the superior IgA-inducing ability of IFNα-producing pDCs and its weaker function in adult AA patients. Based on this, it would be very interesting to further investigate whether adjuvants like CTB could stimulate IgA responses via modulation of mDC function and whether this would be suitable for therapeutic application in human allergic diseases.

Strategy for allergy intervention via induction of IgA

Interestingly, CTB is already registered and used as a mucosal vaccine, i.e. the oral cholera vaccine (Dukoral®). Many studies have shown that this vaccine is safe in both children and adults (11). Moreover, oral CTB has already been applied for treatment of mild and moderate Crohn's disease (40). Here, we have shown the potential of CTB against the development of allergic asthma, and investigated the mode of action which was mainly dependent on DCs. There may be several therapeutic strategies against allergies, in which an IgA inducing adjuvant like CTB could contribute.

Treatment. Allergen-specific immunotherapy (SIT) represents the only curative treatment of allergic diseases currently available, and involves the incremental delivery of the allergen to which the individual is sensitive. This activates various regulatory responses, including Treg cells and humoral responses dominated by IgA and IgG4 (25-28), and effectively suppresses the clinical symptoms of allergy (41). In its current form, SIT has major drawbacks and cannot compete with treatment on the basis of symptom relief (anti-histamines, corticosteroids) for many asthma patients. High concentrations of allergen extract need to be administered on a long term (~5 years) and regular basis. This introduces a risk of potentially life-threatening allergic reactions (42). Given the data on the mechanism of CTB as a mucosal IgA-inducing adjuvant in the mouse studies here (**Chapter 2-4**) and as a putative Treg cell inducer in the literature (12), this may be an attractive compound to improve efficacy and safety of SIT. For example the allergen, when coupled to CTB, will be efficiently targeted to the DCs (**Chapter 3**), allowing the use of lower allergen doses and decreasing the risk of anaphylactic shocks. Furthermore, as CTB was found to boost the development of regulatory IgA and Treg cell responses, application of CTB during SIT may strongly enhance the efficacy of the treatment reaching its peak of immunoregulatory

responses more quickly, possibly reducing the length of treatment. Future experiments in mouse models for true allergens, like birch pollen or house dust mite, experimental SIT models and (cells from) allergic patients will need to point out the usefulness of its application in current SIT protocols. The birch pollen-driven allergic airway inflammation model, which was set-up and described in **chapter 4**, will facilitate the evaluation of the IgA-inducing adjuvant CTB in future immunotherapy protocols.

Prevention. The establishment of commensal flora in the intestine and respiratory tract starts at birth and is considered to be crucial for stimulating and directing the development of the host immune system, including the mucosal IgA response (43-45). In our *in vitro* co-culture system, we confirmed the role for microbial derived TLR ligands in the conditioning of DCs for stimulating IgA responses (**Chapter 3**). Interestingly, CTB does not only enhance IgA induction by TLR-ligand primed DCs, but also initiates IgA production in the case of low dose exposure to MyD88-activating signals which are insufficient to induce IgA on their own (**Chapter 3**). This is interesting considering the hypothesis that decreased or altered microbial exposure associated with an affluent life style is contributing to the increase in asthma prevalence during the last decades. Only recently we have started to appreciate the importance of the microbiota on human health, and restoring or manipulating disrupted host-microbiota relationship has become a potent strategy for treating inflammatory diseases, including asthma (46). CTB could contribute to broad antibody repertoire and sufficient mucosal IgA levels in people with impaired or delayed IgA synthesis, by reducing the threshold for microbial signals or providing the necessary co-signals, to maintain mucosal immunity and local homeostasis.

It was shown that children who developed allergy had less diverse (gut and airway) microbiota (47) and decreased serum or mucosal IgA responses (2,24,48) compared to healthy controls. In our cohort of adult allergic asthmatic patients, we did not find reduced (secretory) IgA levels in nasal washes compared to non-allergic controls, despite differences in pDCs frequency and function with respect to stimulating IgA responses (**Chapter 5**). Studies that measured IgA levels at different time points showed an increase over time which may be due to microbial exposure and microbiota development (24,48). Especially during the first months and year, crucial events such as mode of birth delivery, type of 'first' milk (breast- versus formula-milk) and microbial exposure will determine the composition of microbiota (44). This suggests that particularly during this early period in life impaired IgA responses may allow for sensitization and/or development of allergic symptoms. However, by the time that allergic (asthma) patients have reached adulthood, impaired IgA responses may be restored to normal levels, but the 'damage' has already occurred and allergen-specific inflammatory responses have developed. Alternatively, in a fraction of the allergic infants with a slowly developing mucosal IgA repertoire, allergy symptoms may relieve together with the establishment of a fully developed IgA response. Either way, the association between IgA and allergy will be misinterpreted using an adult cohort.

In addition to the level and Ag-reactivity/specificity of an antibody as studied in this thesis, the binding affinity of an antibody to its Fc-receptor and the receptor expression will determine

the effector function of IgA. In human serum IgA occurs mainly in the monomeric form, while in mice polymeric IgA is the main isotype in serum. In tissues primarily dimeric or polymeric IgA is present, which will bind to the polymeric immunoglobulin receptor (pIgR) at the apical side of the epithelium to be transported to the luminal and mucosal side. All forms of IgA are capable to bind the receptor FcαRI (CD89) but their binding capacities differ, at least in humans. In mice this is unknown, as the receptor for IgA has not been identified in mice. Monomeric IgA only binds with low affinity to the FcαRI and activates the inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) which does not lead to cell activation or degranulation/oxidative burst (in the case of granulocytes) (49). In contrast, IgA immune complexes bind with stronger affinity, subsequently resulting in cell activation and elimination of the pathogen (50,51). In certain cases IgA complexes can even cause severe inflammation and pathology, like in immune complex-glomerulonephritis (52,53). Furthermore, eosinophils and neutrophils express receptors for IgA that can activate the cells upon binding of IgA immune complexes (49,54). For example, human eosinophils express FcαRI (CD89), transferring receptor (TfR or CD71), pIgR, asialoglycoprotein receptor (ASGPR), and a special receptor for secretory component (SCR), and *in vitro* cross-linking of these receptors is shown to result in activation and/or degranulation of the cell (55,56). Therefore, also in severe asthma, where in addition to eosinophils also neutrophils are important mediators, IgA may aggravate the inflammation instead of promoting tolerance. Interestingly, both in patients with IgA nephropathy and in patients with asthma, abnormal glycosylation of the IgA antibody or the FcαRI receptor was found, which may allow exacerbated immune responses and disease development (57,58). Thus, the immunological effect of IgA/FcαRI interaction is determined by several characteristics of the different IgA forms (mono- versus dimeric and the secretory forms) and is influenced by local inflammatory factors.

In conclusion, to evaluate the strategies for IgA based treatment against allergic diseases, mucosal IgA responses in health and disease, including aspects such as the level, affinity and reactivity of the antibodies, need to be further studied. However, based on the dynamics of the development of IgA responses in life and the functional duality of the IgA-receptor interaction, it seems essential to stimulate IgA responses under non-inflammatory conditions, thus early intervention preventing the development of allergic symptoms is recommended.

Conclusion

The studies in this thesis have addressed several aspects of the induction of IgA responses as a therapeutic approach for allergic asthma. By immunizing mice with CTB and OVA-pulsed DCs, we could inhibit airway inflammation upon secondary challenge with OVA antigen, and this effect was mediated via B cells, not T cells. We have provided clear proof that IgA, secreted via the pIgR receptor, is mediating the protective effect of B cells against allergen challenge. We have next elucidated the molecular mechanisms by which CTB affects DC function, and found that

it acts as an inducer of TGF-β and retinoic acid, and thus fully imprints a “mucosal phenotype” in GM-CSF cultured DCs. The experiments as to whether the induced IgA is antigen specific or not are inconclusive at this stage, yet this question needs further study. Modulation of the mDC function by adjuvants, such as CTB, forms a successful strategy to affectively enhance mucosal IgA responses and protects against allergic airway inflammation. Although pDCs of asthmatic individuals are weaker in driving IgA responses, it is not clear whether these cells should be targeted to enhance IgA responses in patients. Previous studies have shown that pDCs mostly promote IgA production by the expression of IFNα and stimulating this function in patients may have adverse effects. Instead, we hypothesized that the development of protective mucosal IgA responses will occur best in the context of a homeostatic environment, through the activation of dedicated mDCs. Although local IgA induction during specific-immunotherapy may have potential to improve treatment of allergies, it is recommended to apply this form of intervention as early as possible, aiming at the induction of a fully developed mucosal IgA repertoire in time and preventing the development of inflammatory responses to allergens.

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NEDERLANDSE SAMENVATTING

Allergisch astma is een chronische ontsteking van de longen die ontstaat door een overdreven afweerreactie tegen ingeademde onschuldige stoffen, zoals huisstofmijt, graspollen en/of huidschilfers van dieren. Herhaalde blootstelling aan deze stoffen kan leiden tot vernauwing van de luchtwegen en slijmproductie, waardoor hoestprikkels en benauwdheid ontstaan.

Het afweersysteem

Het afweersysteem beschermt het menselijk lichaam tegen allerlei schadelijke indringers van buiten, zoals bacteriën en virussen. Het bestaat uit twee delen: het aangeboren of aspecifieke afweersysteem en het verworven of specifieke afweersysteem. Het aspecifieke afweersysteem vormt een snelle, algemene verdediging tegen binnendringende ziekteverwekkers, terwijl het specifieke afweersysteem meer tijd nodig heeft om zich te ontwikkelen en specifiek gericht is tegen de ziekteverwekker die de infectie veroorzaakt. Voor de specifieke afweerreactie zijn bepaalde typen witte bloedcellen, zoals de T cellen en B cellen, erg belangrijk. Er zijn verschillende soorten T cellen die ieder door het uitscheiden van signaalstoffen een bepaald type infectie helpen bestrijden. Zo produceren bijvoorbeeld T helper (Th)1 cellen grote hoeveelheden interferon (IFN) γ en beschermen tegen virussen. Th2 cellen produceren vooral de signaalstoffen interleukine (IL)-4, IL-5, en IL-13 en bieden bescherming tegen worminfectie. B cellen helpen bij het bestrijden van infecties door antistoffen (ook wel immunoglobulinen genoemd) te produceren. Er zijn verschillende typen antistoffen, zoals IgE en IgG, die binden aan ziekteverwekkers waardoor deze herkend en opgeruimd kunnen worden.

De cellen van het afweersysteem die belangrijk zijn voor het aansturen van Th cellen en B cellen na infectie, zijn dendritische cellen. Dendritische cellen komen vooral voor in weefsels die in contact staan met de buitenwereld en waar ziekteverwekkers kunnen binnendringen, zoals de huid, darmen en longen. Deze cellen zijn in staat om bepaalde structuren van de verschillende ziekteverwekkers te herkennen via bepaalde antennes op hun celoppervlakte. De dendritische cellen nemen delen van de ziekteverwekker in zich op en samen met signalen uit de omgeving (bijvoorbeeld van het longweefsel) worden ze op een bepaalde manier geactiveerd. Daardoor kunnen ze precies die typen T en B cellen stimuleren die geschikt zijn om de betreffende ziekteverwekker te bestrijden. Daarnaast kunnen dendritische cellen ook regulatoire T cellen activeren. Deze regulatoire T cellen vormen een veiligheidsmechanisme, waardoor een te sterke afweerreactie kan worden onderdrukt die potentieel schadelijk is voor de gastheer. Ook kunnen zij ervoor zorgen dat geen afweerreactie ontstaat tegen lichaamseigen structuren of tegen onschuldige stoffen, waardoor respectievelijk auto-immuun ziekten en allergieën, zoals allergisch astma, kunnen ontstaan.

Allergisch astma en Immunoglobuline A

Allergisch astma ontstaat door een afweerreactie tegen ingeademde onschuldige stoffen (allergenen). Belangrijke cellen van deze afweerreactie zijn Th2 cellen en B cellen die veel van het antistof IgE tegen de ingeademde stof maken. Het IgE bindt aan speciale witte bloedcellen,

die daardoor de signaalstof histamine uitscheiden. Histamine zorgt ervoor dat spieren zich samentrekken en dat bloedvaten zich verwijden. De signaalstoffen geproduceerd door de Th2 cellen (IL-4, IL-5, IL-9, IL-13) trekken verschillende afweercellen aan en zorgen voor activatie van slijm-producerende cellen. Tezamen resulteert dit in vernauwing van de luchtwegen, overmatige slijmproductie, irritatie van het weefsel en ophoping van ontstekingscellen die de reactie verder versterken. Naast deze cellulaire processen, wordt ook gedacht dat structurele veranderingen in het longweefsel bijdragen aan het ontstaan van allergisch astma, bijvoorbeeld een verdikte spierlaag of een stuggere luchtwegwand.

Regulatoire T cellen zijn vaak wel aanwezig in patiënten met allergisch astma, maar zij lijken niet (optimaal) te functioneren waardoor de allergische afweerreactie tegen het allergeen niet wordt onderdrukt. In tegenstelling tot de regulatoire T cellen, krijgt een speciaal soort antistof, het IgA, relatief weinig aandacht in relatie met allergisch astma. IgA komt voor in de circulatie, maar is veruit de meest geproduceerde antistof op lichaamsoppervlakten (slijmvliezen), zoals in de darmen, neusholten en de longen. Dit zijn de gebieden waar ziekteverwekkers of onschuldige stoffen het lichaam zouden kunnen binnendringen. In verschillende studies worden IgA antistoffen geassocieerd met bescherming tegen allergische afweerreacties. Bijvoorbeeld, jonge kinderen met hoge hoeveelheden IgA in hun speeksel, ontwikkelden minder vaak allergische symptomen na blootstelling aan allergische stoffen dan kinderen met lagere hoeveelheid IgA. Omgekeerd is beschreven dat longspoelingen van een groep ernstig astma patiënten minder IgA bevat dan in die van gezonde controle personen. Door de locatie van IgA op de lichaamsoppervlakten, kunnen de antistoffen werken als een soort pantser. Dit doet IgA door te binden aan indringers van buiten waardoor deze het lichaam niet kunnen binnenkomen. Daarnaast, kan IgA ook de functie van verschillende cellen van het afweersysteem beïnvloeden. Op deze manieren zou IgA een afweerreactie tegen onschuldige stoffen, en dus de symptomen van allergie en astma, kunnen voorkomen of remmen (**Hoofdstuk 1**). Het onderzoek beschreven in mijn proefschrift is dan ook gericht op verschillende aspecten van het stimuleren van IgA productie, met als doel allergische afweerreacties te voorkomen en/of te verminderen.

Onderzoek beschreven in dit proefschrift

De microbiële stof Cholera toxine wordt geproduceerd door de bacterie *Vibrio Cholerae* en bestaat uit een A en een B deel. Cholera Toxine B (CTB) is, in tegenstelling tot Cholera toxine A, niet ziekteverwekkend en juist interessant omdat het is geassocieerd met de ontwikkeling van IgA antistoffen. In **hoofdstuk 2** is bestudeerd of IgA een rol zou kunnen spelen bij het onderdrukken van een allergische afweerreactie. Hiervoor hebben we gebruik gemaakt van een muizenmodel voor allergisch astma. Wanneer muizen via de longen werden blootgesteld aan CTB gelijktijdig met de allergische stof ovalbumine (OVA), resulteerde dat in een lokale toename in IgA en de muizen bleken beschermd tegen de ontwikkeling van allergisch astma. Het IgA was noodzakelijk voor het voorkomen van een allergische afweerreactie tegen ingeademd OVA. Hiermee hebben we een directe relatie tussen verhoogd IgA en bescherming tegen allergisch astma aangetoond. Vervolgens is het mechanisme waarmee CTB de productie van IgA door B cellen stimuleert

onderzocht. Resultaten van verschillende experimenten in muizen suggereerden dat CTB vooral effect heeft op de dendritische cellen. Om dit te bevestigen en de relatie tussen de dendritische cellen en de IgA producerende B cellen goed te kunnen bestuderen, is gebruik gemaakt van in het laboratorium gekweekte dendritische cellen en B cellen. Uit deze experimenten bleek dat dendritische cellen na blootstelling aan CTB de signaalstoffen Transforming Growth Factor-beta (TGF-beta) en Retinoic Acid (RA) uitscheiden. In reactie op deze signaalstoffen produceerden B cellen IgA antistoffen. Bovendien bleek dat de dendritische cellen voor optimale aansturing van B cellen tot IgA productie, behalve door CTB, ook moesten worden geactiveerd via een specifiek soort antennes of receptoren op hun celoppervlakte. Deze receptoren heten MyD88-afhankelijke receptoren (**Hoofdstuk 3**).

Met het oog op een eventuele therapeutische toepassing, is het de vraag of het noodzakelijk is voor het voorkomen van een allergische afweerreactie dat de IgA antistoffen specifiek de allergische stof herkennen en binden, of dat de aanwezigheid van IgA moleculen waarvan de specificiteit niet gedefinieerd is al voldoende is. Hiervoor is poeder van berkenpollen gebruikt als allergische stof, omdat veel mensen hiervoor allergisch zijn en dit daarom meer representatief is voor de ziekte bij de mens. Dit model is toegepast in speciale genetisch gemanipuleerde muizen (V10YEN muizen), die IgA antistoffen produceren die berkenpollen niet kunnen herkennen en binden. Als deze V10YEN muizen beschermd zijn tegen de ontwikkeling van astma door verhoogd IgA als gevolg van toediening van CTB (dit zou dan alleen niet-specifiek IgA zijn), zou dat suggereren dat de IgA antistoffen de allergische stof niet specifiek hoeven te herkennen en binden. Echter, bleek CTB de productie van zowel niet-specifiek als berkenpollen-specifiek IgA te veroorzaken in de V10YEN muizen, waardoor de rol van specifiek IgA niet kon worden uitgesloten. De specificiteit van IgA voor het beschermen tegen het ontstaan van allergisch astma vergt daarom verdere studie (**Hoofdstuk 4**).

Ten slotte is bestudeerd of de resultaten uit de muizen studies betrekking hebben op de mens. In de muis beschermt het stimuleren van IgA antistoffen, via dendritische cellen, tegen de ontwikkeling van een allergische afweerreactie tegen ingeademde stoffen (hoofdstuk 2). Daarom hebben we in **hoofdstuk 5** onderzocht of dendritische cellen van allergisch astma patiënten minder goed in staat zijn om B cellen aan te sturen tot IgA productie dan dendritische cellen van gezonde personen. Hiervoor is bloed afgenomen van volwassen huisstofmijt-allergisch astma patiënten en gezonde controle individuen, om daaruit dendritische cellen te isoleren. Een bepaald type dendritische cel bleek minder aanwezig te zijn in bloed van patiënten. Bovendien, waren ze minder goed in staat om IgA productie door B cellen te bevorderen in vergelijking met hetzelfde type dendritische cellen afkomstig van gezonde controle personen. Het lagere aantal en de verminderde functie van de dendritische cellen in astma patiënten zou kunnen resulteren in minder IgA antistoffen op de lichaamsoppervlakten. Daarom zijn ook de neusholten gewassen (neusspoelingen) om de hoeveelheid IgA dat aanwezig is op de slijmvliezen te bepalen. Echter, de hoeveelheid IgA in neusspoelingen van patiënten was niet lager dan in die van gezonde controle personen (**Hoofdstuk 5**).

De volwassen leeftijd van onze studiepopulatie zou kunnen verklaren dat er, in tegenstelling tot eerder genoemde studies, geen verschil is in hoeveelheid IgA op slijmvliezen van allergische patiënten en controles. Het zou kunnen zijn dat naarmate men ouder wordt de tekortschietende IgA productie zich herstelt tot normale hoeveelheden, maar dat het afweersysteem al geprogrammeerd is om te reageren op onschuldige stoffen waardoor de allergie blijft bestaan. Vervolg studies met (jonge) patiënten moeten uitwijzen of CTB, net als in de muis, geschikt zou zijn voor therapeutische doeleinden. Er zou bijvoorbeeld gedacht kunnen worden aan het inzetten van IgA-stimulerende stoffen, zoals CTB, voor het verbeteren van allergeen-specifieke immunotherapie. Allergeen-specifieke Immunotherapie is de enige succesvolle behandelende therapie voor bepaalde allergieën, maar heeft in de huidige vorm ernstige nadelen, zoals zeer frequente toediening van een oplopende hoeveelheid allergische stof en risico op levensbedreigende allergische reacties. Echter, een betere strategie lijkt vroege interventie met als doel het tijdig stimuleren van de productie van voldoende IgA antistoffen, waardoor de ontwikkeling van allergische reacties wordt voorkomen **(Hoofdstuk 6)**.

Concluderend heeft het onderzoek beschreven in dit proefschrift tot nieuwe inzichten geleid met betrekking tot de regulatie van IgA productie door B cellen en stimulatie van IgA als middel tegen de ontwikkeling van allergische immuunreacties. Dit draagt bij aan de kennis over mechanismen die aan allergieën te grondslag liggen en kan helpen bij het ontwikkelen van nieuwe therapeutische strategieën tegen allergieën, waaronder allergisch astma.

ABOUT THE AUTHOR

Curriculum Vitae

The author of this thesis was born on October 21st 1981 in Heesch (the Netherlands). In 2000 she finished highschool (VWO) at the Maaslandcollege in Oss. The same year she started her study in Biomedical Sciences at the Radboud University Nijmegen. For her minor program Immunology, she studied activation and maturation of dendritic cells by LPS of different *Shigella Flexneri* strains during a research project at the Center for Immunology and Cancer Research at the Princess Alexandria Hospital, Brisbane (Australia). In May 2007 she started her PhD research at the department of Pulmonary Medicine at the Erasmus MC Rotterdam under supervision of Prof.dr. Bart N. Lambrecht, Prof.dr. Rudi W. Hendriks and Dr. Hermelijn H. Smits. Part of her PhD project was spent at the department of Respiratory Diseases, Laboratory of Immunoregulation and Mucosal Immunology, University of Ghent (Belgium) and the department of Parasitology, Cellular Immunolgy of Helminths at the Leiden University Medical Center. The results of the PhD research are described in this thesis.

PhD Portfolio

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Courses

2010	Biomedical English Writing and Communication (EMC)
	Workshop on Bioinformatic analysis, tools and services (LUMC)
	Advances in biology of APCs & T cells, Advanced Immunology (VUMC)
2009	Transgenesis, gene targeting and in vivo imaging (LUMC)
	Basic Methods and reasoning In Biostatistics (LUMC)
2008	Molecular Immunology (PhD course) (EMC)
	Workshop Molecular microbiology of infectious diseases (EMC)
2007-2012	Weekly presentations at the Department
	Journal Club presentations

(Inter) national scientific presentations

2011	Keystone Immunity in the Respiratory Tract (poster presentation)
2010	NVVI- Dutch association of Immunology, Noordwijkerhout, the Netherlands (oral presentation)
	World Immune Regulation Meeting, Davos, Switzerland (oral presentation)
	EAACI GA2LEN Immunology Winterschool, Grainau, Switzerland (oral presentation)
	EMIG Mucosal Immunology in health and disease, Amsterdam, the Netherlands (poster presentation)
2009	Allergy & asthma: bridging innate and adaptive immunity, Bruges Belgium (poster presentation)
	Keystone Dendritic cells, Banff, Canada (oral presentation)
2008	NVVI- Dutch association of Immunology, Noordwijkerhout, the Netherlands (oral presentation)
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Student coaching and teaching

2010	Supervising the research project (7 months) of a master of science- student, at the department of Parasitology CIH, LUMC
	Supervising and teaching students during their FOS course 'Host-pathogen interaction', organized by department of Parasitology CIH, LUMC
2009	Supervising and teaching students during their FOS course 'Infection and Immunity in practice', organized by department of Parasitology CIH, LUMC

Awards

2010	Selected as a participant for EAACI GA2LEN Immunology Winterschool, Grainau, Switzerland
2009	NVVI grant for Working visits
2008	EAACI GA2LEN short term fellowship award

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