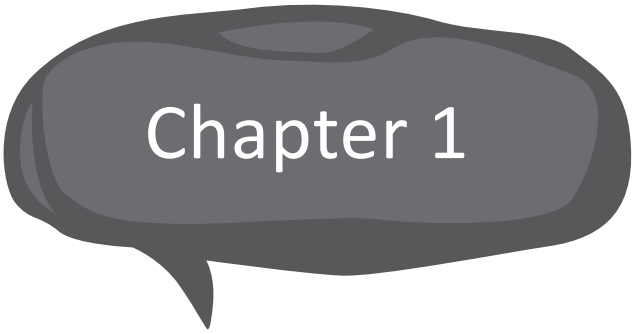

General introduction

Modified from:
Regulatory B cells - Implications in Autoimmune and Allergic Disorders

Luciën E.P.M. van der Vlugt[2]*, Susanne Sattler[1]*, Leonie Hussaarts[2], Hermelijn H. Smits[2] and Fang-Ping Huang[1]

[1] Imperial College London, UK
[2] Leiden University Medical Center, The Netherlands

* These authors contributed equally to this work

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Allergy

Immunity versus tolerance

The human body is continuously exposed to a variety of environmental insults at mucosal surfaces via inhalation or ingestion. The immune system has developed mechanisms to discriminate between danger, such as bacteria or viruses, and self-antigens or harmless substances. At mucosal surfaces, dendritic cells (DCs) are constantly patrolling the environment, looking for danger signals. DCs express evolutionary conserved pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors, or C-type lectin receptors (CLRs), which recognize specific sets of pathogen-associated molecular patterns (PAMPs) on invading pathogens. When activated, DCs take up and process antigens into small peptides and present those via major histocompatibility complexes (MHC) class I and II molecules (1;2). Next, activated DCs migrate towards the draining lymph nodes, where they will present peptides to naive T cells and based on the expression of different sets of polarizing molecules, drive their development into effector T helper cells, such as Th1, Th2, Th17, Th22 or regulatory T (Treg) cells. The T-cell polarizing capacity of the DCs is determined by the type of molecules they have encountered in the peripheral tissues. For example, DC exposure to intracellular microorganisms typically leads to the development of Th1 cells, instrumental in cell-mediated defence against viruses and intracellular bacteria, while exposure to helminth molecules primes DCs to drive polarized Th2 cells, which play a role in immunity against multicellular pathogens such as helminth parasites. Likewise, fungi or certain extracellular bacteria activate DCs and enhance their capacity to drive Th17 or Th22 cells, which contribute to protective immunity against such pathogens (3;4).

An essential part of T-cell-mediated immunity is the development of non-responsiveness toward naturally occurring self-antigens or harmless substances, such as common environmental allergens (e.g. house dust mite (HDM) or pollen), a process which is called immunological tolerance. Tolerance can be divided into central tolerance, which is induced in the thymus and bone marrow and where the immune system learns to discriminate self from non-self, and peripheral tolerance, which occurs in the periphery and prevents over-reactivity of the immune system to environmental entities. DCs play a crucial role in determining the immune outcome after presenting peptides from harmless molecules. In healthy individuals, DCs induce tolerance via induction of anergic T cells, characterized by a hypo-responsive status, or via the induction of Treg cells such as $FoxP3^+$ Treg cells or IL-10 and/or transforming growth factor-β (TGF-β)-producing T cells (Tr1 or Th3 cells) (5). $FoxP3^+$ Treg cells reduce the magnitude of the immune response by suppressing effector T-cell responses as well as the activity of other immune cells by the secretion of elevated levels of anti-inflammatory cytokines and/or contact-mediated suppression. The mechanism of suppressive effects with Tr1 and Th3 cells is only soluble factor-based, namely IL-10 for Tr1 and IL-10 and TGF-β for

Th3 cells (6). In genetically predisposed people, an impaired or weakly developed tolerance may result in responses towards self-antigens and the development of auto-immune diseases, characterised by aberrant Th1 responses or immune responses towards normally harmless allergens, resulting in Th2-polarized allergic inflammation. Well-known allergens are derived from house dust mite, cockroach, grass pollen, animal and fungal molecules. Interestingly, these allergens share common intrinsic biological properties to increase the capacity to penetrate the epithelial barrier, such as serine and cysteine protease enzymatic activity or the capacity to induce the secretion of angiogenic cytokine vascular endothelial growth factor (VEGF). Subsequently, the allergens activate the immune system through interactions between epithelial cells and immune cells (7-9). Allergic inflammation can be found in various mucosal tissues, resulting in different, but related allergic diseases: allergic asthma, atopic dermatitis, allergic rhinitis, toxin and food allergies, in which similar but aberrant immune responses can be found. In this thesis, we focused on respiratory allergic diseases in the upper and lower airways, namely allergic rhinitis and allergic asthma.

Asthma

Asthma is a chronic inflammatory disease in the lower airways of the lungs characterized by bronchial hyper-responsiveness, a variable degree of airway obstruction which is partially reversible with medication and shows symptoms such as shortness of breath, coughing, and wheezing (10). In allergic rhinitis (hay fever), allergen exposure leads to irritation and inflammation of the nasal airways, including excessive mucus production, itching, conjunctival swelling, and eyelid swelling. Allergic rhinitis is very common in patients with allergic asthma. Recent investigations have demonstrated that allergic and non-allergic upper airway disease are both strong risk factors for developing asthma and the current concept is that rhinitis precedes asthma in most patients (11-14). Furthermore, exposure to environmental stimuli, such as cigarette smoke, diesel particles, ozone and (viral) infections are a known risk factor for asthma in genetically susceptible individuals (15). Clinically, at least two forms of the disease can be distinguished, allergic and non-allergic (intrinsic) asthma, of which the latter represents symptomatic asthma in the absence of eosinophilic airway inflammation and is not triggered by allergens, but by other factors such as air pollution or viral infection (16). Here, we will discuss allergic asthma, which is characterized by the presence of IgE antibodies specific for common allergens in the lungs and is mediated for a larger part by Th2 immune responses.

Inflammatory immune responses in asthma

Barrier epithelial cells make up the first line of defence against inhaled pathogens, but also express pattern recognition receptors such as TLRs, CLRs, and protease activated receptors (PARs), which recognize PAMPs, such as microbial motifs and

allergens (17;18). Interestingly, genome-wide association (GWAS) studies have identified some genes involved in regulation of the epithelium barrier function to be linked with a higher susceptibility to develop atopy and asthma (19;20). Related to this, some allergens have the potential to increase the permeability of the epithelium barrier and/or to activate airway epithelial cells to produce cytokines and chemokines that attract and activate innate cells (18;21-25). As a consequence, passage of inhaled allergens into the airway tissue is facilitated, leading to a higher risk of airways sensitization in genetically predisposed individuals, as the allergens are sensed by an elaborate network of DCs underneath the epithelial layer. Importantly, airway epithelial cells (AECs) have been demonstrated to be key modulators of DC behaviour upon allergen exposure via the release of chemokines, cytokines and danger signals (26;27), priming activated DCs to drive the polarization of naive T cells towards effector Th2 cells (26). Next, Th2-cell-associated cytokines orchestrate the allergic inflammatory cascade that occurs in asthma, including promoting immunoglobulin class-switching to the IgE heavy chain, allowing for the production of IgE by B cells (by IL-4 and IL-13), mast cell differentiation and maturation (by IL-3, IL-9 and IL-13), Th2 cell survival (by IL-4), while IL-5 is solely responsible for eosinophil maturation and survival. IgE, once formed and released into the circulation, binds through its Fc portion to high affinity receptors on mast cells and basophils, leaving its allergen-specific receptor site available for future interaction with allergens (28).

Once sensitized, re-exposure to the allergen leads to effector responses, which can be divided into immediate and late phase reaction. The immediate allergic inflammatory reaction is initiated by allergens cross-linking allergen-specific IgE molecules bound to high-affinity IgE Fc-receptors on mast cells and basophils. Consequently, these cells will degranulate and release products such as histamine, leukotriene, and prostaglandins, causing immediate vascular permeability, mucus production, bronchoconstriction, and constriction of airway smooth muscle cells, which are all responsible for the clinical symptoms of allergic responses (10;29;30). The early-phase asthmatic reaction is 4-6 hours later followed by the late-phase asthmatic reaction induced by the recruitment and activation of inflammatory cells such as eosinophils, macrophages or DCs, attracted by products released by airway smooth muscle cells and bronchial epithelial cells in the early phase. The activated eosinophils produce chemokines and inflammatory mediators, which induce substantial damage to the endothelial cells but also lead to further recruitment of eosinophils and Th2 cells to the airway (31;32), responsible for ongoing and chronic inflammatory processes in the lungs. In severe forms of asthma, IL-17-producing T cells are found, which can enhance the Th2-mediated inflammation and recruit neutrophils and other inflammatory leukocytes into the airways (33;34). The interaction of activated effector Th2 and Th17 cells, and structural cells such as AEC and smooth muscle cells will result in extracellular matrix formation, hyperplasia of mucus producing goblet cells, basal membrane thickening and eventually in structural airway remodeling (15).

The 'hygiene hypothesis'

The prevalence and severity of hyper-inflammatory disorders such as allergies, but also auto-immunities has dramatically increased in the Western world over the last 50 years (35;36). Although genetic predisposition is a risk factor for hyper-inflammatory disorders (37-39), only sudden changes in environmental factors may explain the recent rise in these diseases. In 1989, David P. Strachan was one of the first to suggest that infections in early childhood transmitted by unhygienic contact with older siblings or acquired prenatal from a mother infected by contact with her older children may confer protection against hay fever (40). He proposed that the increase in prevalence of allergic disease could be explained by a reduced exposure to infectious agents, which were attributed to declining family sizes, improvements in household amenities, and increased personal hygiene over the past century (40), also known as the 'hygiene hypothesis'. Initially, an insufficient stimulation of Th1 responses due to a limited exposure to bacterial and viral pathogens, which in turn cannot counterbalance the expansion of overactive Th2 responses, was thought to result in predisposition to allergy (41). However, the distorted Th1/Th2 balance hypothesis could not explain the simultaneous increase in several Th1-mediated auto-immune diseases, such as type 1 diabetes (T1D), multiple sclerosis (MS) and inflammatory bowel disease (IBD) together with increases in allergic disorders in the same countries (42;43). Moreover, individuals infected by helminths, which are the most potent natural inducers of Th2 responses, were paradoxically less likely to have allergic disorders, and in some studies anti-helminth treatment even led to increased allergic sensitization (35;44).

The 'Old friends hypothesis'

A more rational explanation for the link between microbial exposure and inflammatory diseases in the hygiene hypothesis was proposed in 2003, based on several epidemiological studies pointing towards a reversed relationship between hyper-inflammatory disorders and vital microbial exposures (35;45). These exposures were not the common childhood- and/or other infections, which have evolved relatively recently over the last 10,000 years, but rather the microbes which were already present in hunter-gatherer times, accompanying mammalian evolution and therefore have been tolerated (46-48). These 'Old Friends' mostly include normal microbiota of the human skin, gut and respiratory tract and that of the animals we are closely living with and certain environmental bacteria and fungi that inhabit our indoor and outdoor environments. For example, various cross-sectional studies demonstrated that children living in farming environments were protected from childhood asthma and allergy and this correlation has been attributed to contact with livestock (49;50), hay and the consumption of raw cow's milk (51-54). In farming environments, both outdoor and indoor microbial exposure are higher and more diverse compared to non-farming environments

(50;55;56). Interestingly, a more detailed analysis of the dust composition showed that a lower risk of asthma was associated with fungi of the *Eurotium* and *Penicillium* species (50). Furthermore, ingestion of orofecal microbes (57) or colonization of certain probiotic bacteria stimulating the gut associated lymphoid tissue (GALT) are also associated with reduced allergic responses or certain auto-immune conditions. An association between the composition of the gastrointestinal microbiome and the prevalence of allergies has been described in several studies, suggesting that *Lactobacilli* and *Bifidobacterium bifidum* may have a protective effect (58;59). In line with these data, changes in faecal microbiota were detected in auto-immune patients suffering from Crohn's disease and ulcerative colitis (60;61). In models of IBD and allergic asthma, germ-free mice were more susceptible to pathology and showed higher inflammatory responses compared to specific pathogen-free mice (62;63). Furthermore, a link between microbiota and dietary fibres was recently described in a HDM allergic airway inflammation model showing that fermentable fibres in the diet promoted the outgrowth of bacteria from the *Bacteroidetes phylum*, and thereby increased levels of circulating short-chain fatty acids, which influenced DC hematopoiesis and functionality in the lungs together with a reduced susceptibility to AAI (64).

Other 'Old Friends' pathogens that are capable of suppressing of Th1 and Th2 responses within the host immune system, are those that establish chronicity, such as hepatitis A virus and helminths. In contrast to the high prevalence in the Western world, it has been documented that children, living in different geographical areas endemic for parasitic infection, show a lower prevalence of allergic symptoms and atopic sensitization (65). For example, chronic infections with intestinal helminths, such as *Ascaris Lumbricoides, Trichuris trichiura* and hookworms, were reported to protect against allergic reactivity in Venezuela (66), Gambia (67), Ethiopia (68), Taiwan (69), and Ecuador (70;71). In addition, individuals from Gabon (72), Brazil (73), Ghana (74) or Indonesia (75) infected with tissue helminths such as schistosomes, filarial worms, and tapeworms, all showed a lower skin prick test (SPT) reactivity to allergens compared to population studies in areas where the prevalence of helminth infections was low or absent (72;76). Strikingly, long-term anti-helminth treatment resulted in increased atopic reactivity to HDM, supporting a direct link between helminth exposure and protection against allergic diseases (44;77). In addition, helminth-infected patients with MS showed better clinical disease outcome compared to control patients with MS (78). A causal relationship between helminth infections and protection against hyper-inflammatory disorders has also been established in various mouse models for food allergy (79), asthma (80-82), T1D (83;84), collagen-induced arthritis (CIA) (85) and experimental auto-immune encephalomyelitis (EAE) (86;87).

Some studies, however, have suggested that infections with helminths show no relationship with the incidence of allergy. A study with school children in Ecuador reported no change in either SPT reactivity to allergens or allergic symptoms after one year of abendazole treatment (88). A study conducted in a

population living in an area of Indonesia where soil transmitted helminths are highly prevalent, showed no statistically significant increase in SPT reactivity after two years of three-monthly albendazole treatment (89). Furthermore, *Trichuris* infection was reported to increase cockroach SPT sensitivity in Ethiopia (90) and *Ascaris lumbricoides* was associated with increased risk of asthma and an increased number of SPT reactivity to aeroallergens in some areas (91). The nematode *Toxocara canis* exacerbates the development of experimental allergic airway inflammation in mice, which is similar to the enhanced allergic responses reported in epidemiological studies in humans infected with *T. canis* (92). Finally, infection with *Trichinella spiralis* and *Ascaris suum* resulted in enhanced allergy in mice (93;94). A possible explanation for this heterogeneity is variation between studies in the species of helminth, the age when infections were acquired, but also the intensity of infection. A systematic review and meta-analysis of 30 cross-sectional studies found an inverse relationship between asthma and hookworm infection, predominantly the *N. americanus* species, which was correlated with infection intensity (95). Smits *et al.* demonstrated that lung lavage eosinophilia, peribronchial inflammation, and OVA-induced allergic airway inflammation were increased during acute *Schistosoma (S.) mansoni* infection, but significantly decreased when infection progressed intro chronicity (80). Furthermore, this suppression was correlated with the intensity of infection, showing the highest suppression in the high-intensity infected group.

Altogether these findings indicate that microbial exposure, especially during early life, seems to be important to prevent hyper-inflammatory conditions. The Th1 versus Th2 imbalance cannot explain the escalation of both allergic and autoimmune disorders and imply common underlying mechanisms in a deregulated immune system that is increased or activated by pathogens. In this thesis, we focussed at immuno-regulatory processes induced during *Schistosoma* helminth infections and the consequences for protection against allergic diseases.

Helminth infections

Immune responses during helminth infections

Schistosomiasis is caused by infection with helminth parasites of the genus trematode, *Schistosoma.* Five species, endemic in different parts of the world, and are responsible for over 200 million infected individuals. Most schistosomiasis is caused by *S. mansoni* (present in Africa, South America, Middle East, and Caribbean), *S. haematobium* (Africa and Middle East), and *S. japonicum* (China and Asia). Less prevalent species are *S. intercalatum* and *S. mekongi*. Depending on the species, different organs are infected, e.g *S. mansoni* and *S. haematobium* infect the venules of the intestinal mesentery or the bladder, respectively.

Field studies in endemic areas and animal experiments, mostly considering *S. mansoni* and *S. haematobium* infections, showed that these schistosomes cause two immunologically distinct phases, namely acute and chronic schistosomiasis.

Acute schistosomiasis is initiated by immune responses primarily directed against worm antigens and mainly Th1 in nature (96). When the first eggs are produced by matured worm pairs 5-6 weeks post-infection, the immune response switches towards strongly polarized Th2 cell responses. This Th2 response is egg antigen-specific, and predominates the worm antigen-specific Th1 response that preceded it (96). Increased numbers of circulating eosinophils, basophils and mast cells, and high plasma levels of IgE are found during acute phase of infection (97;98). The eggs are the major cause of pathology for several reasons, because their released products contain hepatotoxic molecules (e.g. omega-1, and IPSE/alpha-1). However, most morbidity is associated with pathology caused by eggs trapped in the gut and liver when infected with *S. mansoni* or *S. japonicum* or by eggs trapped in the urogenital tract when infected with *S. haematobium*. The induction of type 2 immune responses is an important mechanism that minimizes immunopathology in a setting where the immune response is incapable of clearing the helminth infection. The formation of type 2-mediated granulomatous lesions around the tissue-trapped eggs serves an important host-protective role, preventing the released toxins from reaching surrounding tissue cells (99). In IL-4$^{-/-}$ mice, infection is acutely lethal and accompanied by excessive tissue injury in the liver and intestines (100;101). Type 2 responses during infection promotes wound healing via the IL-4- and IL-13-dependent development of alternatively activated (AA) macrophages, which can inhibit classical pro-inflammatory macrophage activation, responsible for oxidative tissue damage (102;103). Furthermore, arginase 1 (Arg1) is an enzyme produced by AA macrophages that is involved in down-regulation of inflammation and facilitates tissue remodeling (104). Mice lacking Arg1 showed more severe intestinal inflammation following infection with *S. mansoni* (105). Infected RELM-α-deficient mice, another molecule secreted by AA macrophages, showed more severe intestinal inflammation or granulomatous inflammation and fibrosis around schistosome eggs.

AA macrophages also shown a role in resolution of acute inflammation through the production of immunosuppressive cytokines IL-10 and TGF-β, which inhibit IL-17-associated neutrophilia and tissue injury (106-109). Indeed, a correlation was found between a higher frequency of Th17 cells during human schistosomiasis and severe pathology (110;111). When the organ damage is not sufficiently repaired or the inflammation uncontrolled, especially during the latter stage of disease, the granulomatous lesions can lead to gross periportal liver fibrosis and life-threatening hepato-splenomegaly in severe cases in *S. mansoni* infections (112) or cancer of the urinary bladder in *S. haematobium* infections (113).

After reaching the peak of infection at approximately 8 weeks post-infection, a decline, over time, in proliferation and responsiveness of Th2 cells and in the size of granulomas forming around eggs newly deposited in tissues occurs and the infections reaches a phase of chronicity (114). In endemic areas most individuals infected with *Schistosoma* exhibit a chronic, relatively asymptomatic infection. Interestingly, the chronic phase was associated with high circulating

IL-10 levels and regulatory T-cell frequencies forming a regulatory network that can alter immune response to both parasite and harmless antigens (35;115-117).

Regulatory network during chronic helminth infection

Observations that helminths down-modulate inflammation were already described in the 1970s for individuals infected with *S. mansoni* or bancroftian filariasis, showing a general hypo-responsiveness in lymphocyte proliferation (118;119). Three decades later, human and murine studies revealed that helminths are capable of inducing and expanding naturally occurring $CD4^+CD25^+FoxP3^+$ Treg cells (72;120-122), and thereby restraining immune responses against the parasite while at the same time protecting the host against excessive inflammation and tissue damage (123-125). In humans, *S. mansoni*-infected patients with high proportions of naturally regulatory T cells showed a decrease in the frequency of these cells after effective treatment (126). Adoptive transfer experiments demonstrated that $CD4^+CD25^+$ Treg cells markedly protect against exacerbated pathology in murine schistosomiasis (127). Depletion of Treg cells, using anti-CD25 treatment, increased the anti-parasitic responses in murine *Brugia pahangi* (128), *S. mansoni* (127) and *S. japonicum* (129). Evidence that helminth-induced $CD4^+CD25^+$ T cells can induce spill-over suppression against other diseases such as allergies comes from studies showing that depletion of Treg cells, using either anti-CD25 antibodies (121;130) or DEREG mice (131), resulted a higher OVA-induced allergic airway infiltration and eosinophilia in helminth-infected mice.

Next through induction of Treg cells, elevated levels of anti-inflammatory cytokine IL-10 have been reported in many human and murine studies in the context of helminth infections and shows pleiotropic effects in immunoregulation (132). IL-10 seems to be a key immune modulator in the loss of T-cell responsiveness during infection (72;123;133). For example, the loss of IL-10 was shown to exaggerate Th2 responses during the acute phase (109;134), while others studies reported IL-10 was essential for down-modulation during the chronic phase (135). This is however partly debated by a study where they showed that IL-10 was responsible for liver pathology, but not so much for immune hypo-responsiveness (136). Furthermore, IL-10 promoted AA macrophages activation in schistosomiasis, a process which can inhibit Th2 cell proliferation by competing for arginine (137), enhance Treg cell development/activity, but limit Th17 differentiation and dampen antigen-specific T cell proliferation (105). Macrophage maturation and DC differentiation were suppressed in the presence of IL-10, thereby limiting their ability to activate effector T cells (138;139). IL-10 also inhibited the production of pro-inflammatory cytokines, such as TNF-α (138;140).

Some murine experimental models suggested that not only Treg cell-derived IL-10, but also non-T-cell derived IL-10 played an essential part in immune modulation (141;142). Studies in auto-immunity models have indicated that B cells may also form a prominent source of IL-10 (143;144).

Regulatory B cells

The concept of regulatory B cells

B cells represent a major component of the immune system and their best understood effector functions are antibody production, presentation of antigens to T cells and the modulation of immune responses via cytokine production. Although, most of these functions serve to amplify immune responses, B cells with regulatory capacity have become the focus of intense investigations in recent years. However, the general concept that B cells might have the ability to induce tolerance, was introduced already in the 1970s by Katz *et al*., who demonstrated that depletion of B cells from splenocytes abolished their ability to inhibit an inflammatory reaction in a delayed-type hypersensitivity (DTH) model (145;146). More than 20 years later, Janeway and co-workers were the first to demonstrate a role of B cells in protection from auto-immunity, showing that B cell-deficient mice failed to undergo spontaneous remission from EAE (147). The term 'regulatory B cells' was introduced shortly afterwards, by Mizoguchi and Bhan, who identified an IL-10-producing B-cell subset in gut-associated lymphoid tissues (GALT) with up-regulated CD1d expression, which suppressed progression of intestinal inflammation by down-regulating inflammatory cascades (148).

Different regulatory B cell populations in mice and humans

Although nowadays the existence of a regulatory subset of B cells is generally accepted, there is still some controversy concerning their origin and relationship to other B-cell populations (149). In mice, B cells are classified according to their developmental origin, into B1 and B2 cells. B1 are considered an innate type of lymphocytes and arises early in embryonic development and originate from the fetal liver. They produce antibodies with a limited diversity to common pathogens and can respond quickly and independently of T cells (150;151). B1 B cells reside in the peritoneal and pleural cavities and in the spleen in smaller numbers. They are distinguished from the other B cell subsets by expression of CD5, a cell-surface glycoprotein.

B2 lymphocytes on the other hand originate from adult bone marrow and populate secondary lymphoid organs. As immature transitional B cells migrate from the bone marrow, they will enter the spleen and emigrate to the splenic peri-arteriolar lymphatic sheath (PALS). These transitional type I cells (T1) having a $CD21^{neg}CD23^{neg}CD24^{hi}IgM^{hi}IgD^{low}$ phenotype will develop into transitional type 2 cells (T2) with a $CD21^{+}CD23^{+}CD24^{hi}IgM^{hi}IgD^{+}$ phenotype that take residence in the spleen primary follicles. T2 B cells differentiate either into mostly sessile marginal zone (MZ) B cells or into follicular (FO) B cells depending on the B cell receptor (BCR) signal strength. FO B cells represent 60-80% of the B cells and are characterized by a $CD21^{int}$ $CD23^{hi}$ phenotype (152). They reside in follicles of the spleen, circulate between lymphoid organs and populate lymph nodes where they participate in both T-cell dependent and T-cell independent immune responses.

MZ B cells are long-lived cells with a $CD21^{hi}CD23^{-/low}$ phenotype that remain in the marginal zone of the spleen (153). They respond to a wide spectrum of T-cell dependent and T-cell independent antigens, such as blood-borne pathogens, by migrating into the follicle (154) where they activate naive T cells more efficiently than follicular B cells and differentiate into plasma cells (155).

Regulatory B cells or their precursors seem to be able to arise from different subpopulations of both B1 and B2 cells. As shown in Table 1, several Breg cell populations with varying surface phenotypes have been identified in various mouse model systems as well as in different human disease conditions. Some regulatory B-cell populations have also been shown to be induced in diverse disease settings and in response to many different exogenous and endogenous stimuli. Toll-like receptor signalling via TLR-2, -4 and -9 as well as BCR signalling and co-stimulation mediated by CD40, CD80/CD86 or B-cell activating factor (BAFF) has been demonstrated to induce B cells with suppressive activity (144;156-160). One prominent type of 'natural' B cells with regulatory capacity has been isolated from naive mouse spleens and termed B10 cells by reason of their IL-10-dependent suppressive function. Phenotypically, these B cells seem to be predominantly $CD1d^{hi}CD5^{+}$, thus they share surface markers with $CD5^{+}$ B1 cells ($CD21^{hi}CD23^{+}IgM^{hi}CD1d^{hi}Cd93^{int}$), MZ B cells ($CD1d^{hi}CD21^{hi}CD23^{lo}IgM^{hi}$) and transitional 2 (T2)-MZ precursor B cells ($CD1d^{hi}CD21^{hi}CD23^{hi}IgM^{hi}$), but do not exclusively belong to one of these B cell subpopulations (161).

So far most evidence comes from murine models and needs to be confirmed in humans. Nevertheless, the human equivalent to mouse B10 cells has been identified, mainly in a disease setting. A small population within peripheral blood $CD24^{hi}CD38^{hi}$B cells showed impaired IL-10 production and reduced suppression of $CD4^{+}$ T cell cytokine responses in systemic lupus erythematosus (SLE) patients (162) and impaired regulatory function in rheumatoid arthritis patients (163). Additional, in helminth-infected individuals an increased population of $CD1d^{hi}$ B cells was found in peripheral blood, which expressed elevated levels of IL-10 (78). B cells expressing $CD24^{hi}CD27^{+}$ (164) was also associated with IL-10 production and immune regulation, whereas B cells secreting both IgG4 and IL-10 were sharing a $CD25^{+}CD71^{hi}$ phenotype in healthy donors, of which the latter was reduced in frequency in bee venom allergic individuals(165). Next to IL-10-secreting, also an enhanced frequency of $CD5^{+}$ TGF-β-secreting B cells have been identified in response to milk allergens in healthy donors, whereas the frequency was lower in milk-allergic donors (166;167). Although a number of distinct human Breg cell populations have been identified and clear parallels can been drawn in function and phenotype with several mouse Breg subsets, one specific marker that characterizes human Breg cells has not been identified yet.

Because of the variety of Breg cell populations and inducing factors, several models have been proposed that try to explain their origin and development. The first model put forward by Mizoguchi *et al.* states that distinct Breg cell populations are generated from already existing B cell subsets depending on distinct activation processes (143). According to this hypothesis, innate type

regulatory B cells are generated from MZ B cells in the spleen upon stimulation with inflammatory signals such as lipopolysaccharides (LPS) or CpG via toll-like receptors. On the other hand, acquired type regulatory B cells develop from follicular B cells following activation with CD40 ligand and/or B cell receptor (BCR) ligation with self-antigen. A second model proposed by Lampropoulou *et al*. states that all B cells have the capacity to become regulatory B cells due to a hierarchical process of stepwise B cell activation, with TLR ligands initiating the process and BCR and CD40 engagement serving to further reinforce this differentiation (159). A third model, based on shared phenotypic markers between most described IL-10-producing B cell populations, claims that all different B-cell populations contain their own distinct Breg cell precursors, which mature to IL-10-producing cells upon activation (168). Taken together, currently available information suggests, that in addition to distinct 'natural' Breg cell populations arising from specific Breg cell progenitors, members of many B cells subsets potentially are able to

Table 1. B cell populations with regulatory phenotypes in different species.

Species	Phenotype	Initial identification	Organ of origin	Major effector function	Disease condition
Mouse	B10	Yanaba, Bouaziz 2008	Spleen	IL-10	CHS
Mouse	TZ MZ	Carter, Vasconcellos 2011 Evans, Chavez-Rueda 2007	Spleen	IL-10	Arthritis
Mouse	MZ	Gray, Miles 2007	Spleen	IL-10	CIA
Mouse	B1	Nakashima, Hamaguchi 2010	Peritoneum	IL-10	CHS
Mouse	$CD1d^{hi}$	Amu, Saunders 2010 Mizoguchi, Mizoguchi 2010	Spleen	IL-10	AAI, IBD, anaphylaxis
Mouse	$CD23^{+}$	Wilson, Taylor 2010	Mes. LN	?	AAI, EAE
Sheep	$CD21^{+}$ B2	Booth, Griebel 2009	Peyer's patches	IL-10	Healthy
Human	Transitional $CD24^{hi}CD38^{hi}$	Blair, Norena 2010	Blood		SLE
Human	$CD24^{hi}CD27^{+}$	Iwata, Matsushita 2011	Blood	IL-10	Healthy and auto-immune
Human	$CD1d^{hi}$	Correale, Farez 2008	Blood	IL-10	MS
Human	'Br3'	Lotz, Ranheim 1994	Blood	TGF-β	CLL
Human	'Breg'	Noh, Choi 2010	Blood	FoxP3	Healthy
Human	$CD73^{-}$ $CD25^{+}CD71^{+}$	Van de Veen, Stanic 2013	Blood	IL-10, IgG4	Healthy, Bee venom allergics

B cell populations with regulatory capacity have been identified in various different experimental settings or disease conditions in mice, humans and sheep. CHS: contact hypersensitivity, T2-MZ: transitional 2 marginal zone, CIA: collagen induced arthritis, AAI: allergic airway in-flammation, IBD: inflammatory bowel disease, mes.LN: mesenteric lymphnodes, EAE: experimental auto-immune encephalomyelitis, MS: multiple sclerosis, SLE: systemic lupus erythematosus, CLL: chronic lymphocytic leukemia.

acquire suppressive functions as a negative feedback mechanism in response to activation.

Immunological effector functions of regulatory B cells

Breg cells are now considered a key regulatory cell type capable of suppressing effector functions of various target cells including T cells, DCs and macrophages, and can even convert effector T cells into regulatory T cells (169-172). As depicted in Figure 1, many Breg cell functions have been demonstrated to be mediated by the release of immunosuppressive cytokines. IL-10 is the hallmark cytokine of regulatory B cells. It has been shown to be essential for the Breg cell suppressive functions in many auto-immune models. Accordingly, the protective function of Breg cells in CIA, EAE, non-obese diabetes (NOD), spontaneous and induced models of colitis, and IBD is abrogated if B cells are deficient in IL-10 production (144;156;173-176). Breg cells controlling homeostasis were also detected in adipose tissue, showing that B-cell-specific IL-10 deletion enhanced adipose inflammation and insulin resistance in diet-induced obese mice (177). B-cell derived IL-10 efficiently suppresses proliferation and inflammatory cytokine production of T cells (144;156) and can also induce FoxP3^{+} regulatory T cells (157;178). Some of these effects might be indirect and due to the effects of IL-10 on innate cell types, as IL-10 is well known to inhibit antigen presentation and pro-inflammatory cytokine production by DCs, monocytes and macrophages (179).

In addition to IL-10, TGF-β is the second immunosuppressive cytokine found to be secreted by some Breg cell populations to down-regulate inflammatory immune responses (166;169;180;181). Similar to IL-10, TGF-β controls

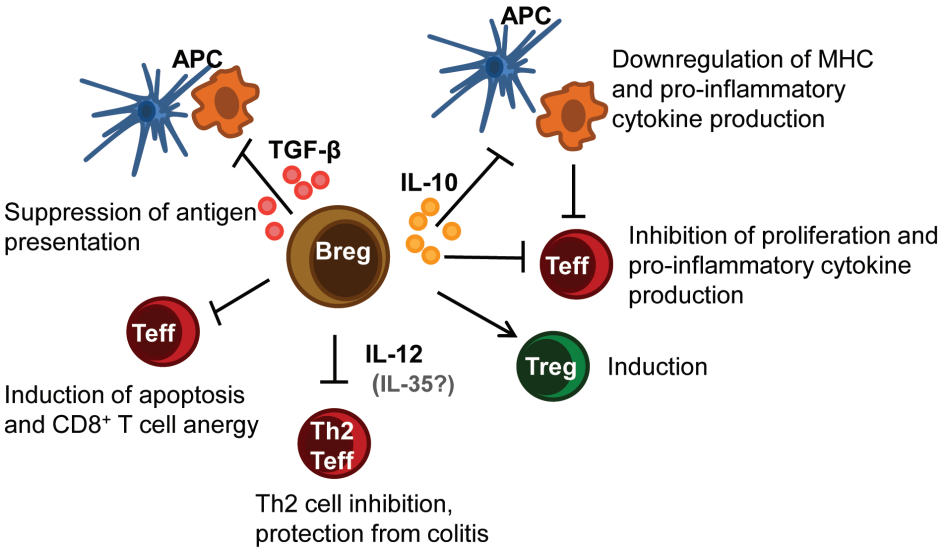

Figure 1. Suppressive functions of Breg cells mediated by the release of cytokines. Breg cells secrete immunosuppressive cytokines causing downregulation of antigen presenting cell function, inhibition of T effector cell function and induction of regulatory T cells. Breg: regulatory B cells, Teff/reg: effector/regulatory T cells, APC: antigen presenting cells.

inflammation via suppression of Th1 and Th2 inflammatory cytokine production, maintenance of Treg cells, and inhibition the function of antigen presenting cells (APC) (182). In addition, TGF-β induces apoptosis in target effector cells and acts as a negative regulator of mucosal immune responses (183).

Interestingly, although not generally considered suppressive, IL-12 production by B cells has also been demonstrated to have immunomodulatory capacity in a T-cell receptor (TCR)α knockout mouse model of Th2-mediated colitis. In this model, IL-10-mediated induction of IL-12-secreting B cells is involved in protection from colitis, as blocking IL-12 using IL-12p35-deficient double knockout mice as well as mice treated with anti-IL-12p40 antibodies, developed a more severe colitis compared to control mice (184).

Independent of cytokine secretion, several B cell surface molecules have been implicated in the suppressive functions of regulatory B cells (Fig. 2). CD1d is not only a major phenotypic marker highly expressed on many Breg cell populations, it has also been suggested to have an active role in Breg cell-mediated suppression. CD1d is a major histocompatibility complex (MHC) class I-like molecule and is responsible for the presentation of lipid antigens to Natural Killer T (NKT) cells (185;186). Mizoguchi *et al.* showed that upregulation of CD1d on B cells is associated with B cell-mediated protection against intestinal mucosal inflammation (148).

As NKT cells had earlier been shown to be protective in mouse models of diabetes (187) and colitis (188), it was feasible to assume that the activation of NKT cells was the underlying mechanism of protection in these models. However,

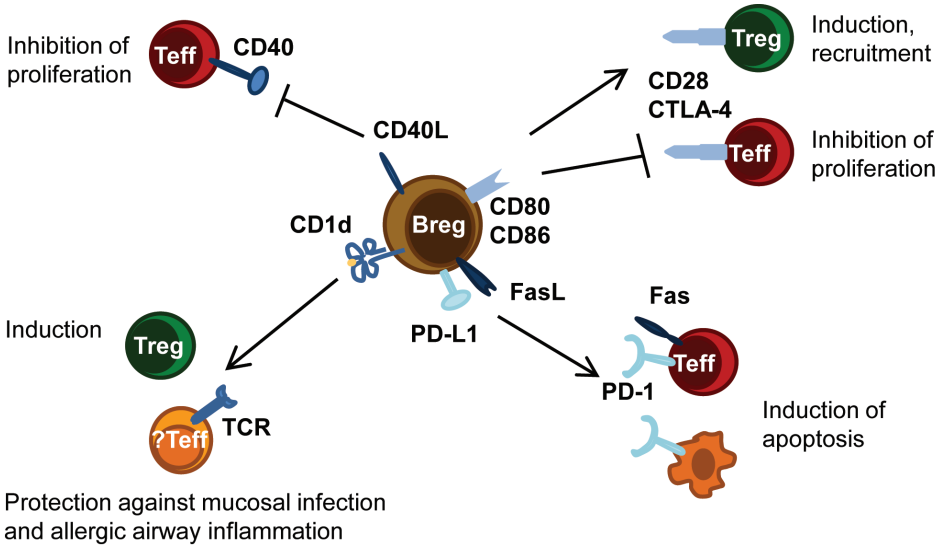

Figure 2. Suppressive functions of Breg cells mediated by cell contact-dependent mechanisms. Breg cells express several cell surface molecules that cause inhibition of T effector cell function, induction of target cell apoptosis and induction of regulatory T cells. Breg: regulatory B cells, Teff/reg: effector/regulatory T cells, TCR: T cell receptor, PD-1: programmed death-1, PD-L1: programmed death-ligand1, FasL: Fas-Ligand, CTLA-4: cytotoxic T-lymphocyte protein 4, CD40L: CD40-Ligand.

as the TCRα knockout mice used in the studies by Mizoguchi *et al.*, do not have NKT cells, the protective effect in this experimental setting has to be mediated by another CD1d responsive cell type. Amu *et al.* later confirmed a CD1d^{high} Breg cell-dependent, but NKT cell-independent mechanism of protection in a model of worm-mediated protection from allergic airway inflammation (81). Another group reported, that CD1d expression on APC and splenic MZ B cells was necessary for efficient generation of regulatory T cells in CD1d-reactive NKT cell-dependent tolerance in immune privileged sites suchsi as the eye (189).

As described earlier, CD40-CD40L interaction seems to play an important role in the differentiation of regulatory B cells. In addition, there are reports indicating that CD40 signalling on target cells might also be involved in the suppressive mechanisms of B cells. Upon activation, B cells express CD40L on their surface (190) and CD40-CD40L interaction has been shown to mediate suppression of colonic inflammation by inhibition of T cells (191). Other co-stimulatory molecules involved in cell contact-dependent suppressive functions of B cells, are the B7 co-stimulatory receptors CD80 and CD86. Interaction of B7 surface receptors with their inhibitory ligands cytotoxic T-lymphocyte protein

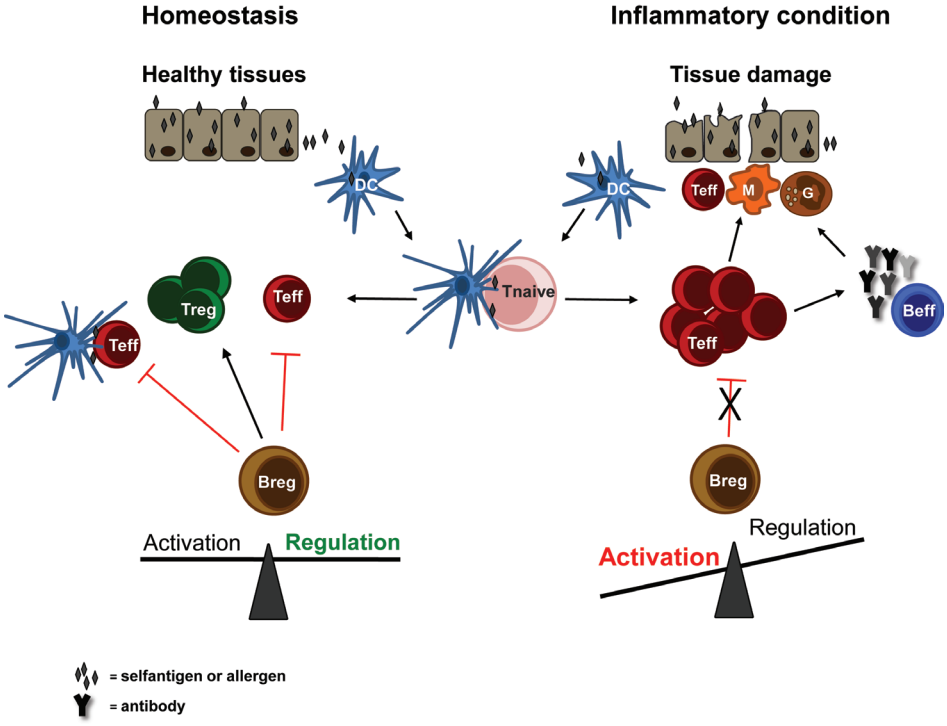

Figure 3. Regulatory B cells in homeostasis and disease. Under normal conditions regulatory B cells control T effector cell activation and proliferation in response to harmless self-antigens and allergens, and induce and activate regulatory T cells. If this Breg cell mediated control fails, effector T cells can proliferate and activate antibody-producing B cells as well as innate immune cell types causing tissue damage. Beff/reg: effector/regulatory B cells, Teff/reg: effector/regulatory T cells, Tnaive: naive T cells, DC: dendritic cells, M: macrophages, G: granulocytes.

4 (CTLA-4) or CD28 on target cells is crucial in regulating T cell activation and peripheral tolerance (192). Expression of B7 molecules has been shown to be essential for recovery from EAE due to B cell-mediated generation and recruitment of Treg cells (193) as well as for the suppression of colonic inflammation through inhibition of effector T cell proliferation (191).

Moreover, evidence exists that Breg cells upregulate surface molecules like Fas ligand (FasL) and programmed death-ligand 1 (PDL-1), which upon interaction with their receptors can directly induce apoptosis or inhibition in target cells, respectively. Lundy and Fox demonstrated that in a mouse model of rheumatoid arthritis, splenic $CD5^+$ B cells express high levels of FasL and that induced T cell apoptosis indeed was due to FasL-mediated direct killing by B cells (194;195). In EAE, Bodhankar *et al.* showed that the well-established protective effect of estrogen is mediated by B cells. The treatment, in addition to increasing the percentage of IL-10-producing regulatory B cells, also induced upregulation of PD-L1 expression on B cells (196). Furthermore, in murine experimental stroke, PD-L1 and PD-L2 expressing B cells were found to be protective due to their capacity to inhibit the activation of inflammatory T cells, macrophages and microglial cells through upregulation of PD-1 expression (197).

The role of regulatory B cells in helminth infections

One of the first observations that helminths, such as *S. mansoni*, could induce suppressive B cells was made in µMT mice, which lack mature B cells. These mice show increased *S.mansoni*-induced tissue pathology compared to infected wild-type mice (198). Subsequent studies with *S. mansoni* demonstrated that B cells isolated from helminth-infected mice could play a protective role in allergy, as transfer of B cells protected recipient mice against systemic fatal anaphylaxis or OVA-induced airway inflammation (81;141;199). Interestingly, these regulatory mechanisms were only active during the chronic phase of infection, where a higher frequency of IL-10 producing B cells was detected (80). Similar results were obtained in *Heligosomoides polygyrus*-infected mice, where $CD19^+CD5^-CD23^{hi}$ B cells isolated from mesenteric lymph nodes of chronically infected mice were able to suppress Derp1-induced airway inflammation, although independently of IL-10 (86). Interestingly, in a worm-only infection of *S. mansoni*, Breg cells also incurred protection against allergic airway inflammation via the induction of regulatory T cells (81). In addition, B cell expressed FasL-mediated apoptosis of $CD4^+$ T cells appeared to be another mechanism used by Breg cells to control inflammation during schistosome infections (200).

Helminth-induced Breg cells also ameliorated symptoms of several auto-immune diseases. Adoptive transfer of B cells isolated from *H. polygyrus* infected mice, dramatically reduced EAE severity in uninfected recipients (86) and B cells from helminth infected MS patients suppressed T cell activation *in vitro* (78). The production of B-cell IL-10 and the induction of Treg cells were important in the reduction of inflammation. Treg cell induction was further shown to be dependent on expression of B7 co-stimulatory molecules, as B7-deficient B cells

failed to efficiently recruit Treg cells into the CNS and mediate recovery from EAE clinical disease (193). Overall, there is a strong case for the capacity of helminths to induce functional Breg cells that are protective against inflammation-driven pathology (Fig. 3).

Pathogen-driven pathways for the induction and expansion of Breg cells

Several studies have highlighted the relevance of Breg cells in down-modulating inflammation in both auto-immune and allergic disorders. In addition to the direct effects via cytokine production, Breg also function indirectly via the induction or recruitment of regulatory T cells and therefore may have promising therapeutic potential. However, the mechanism underlying the formation of regulatory B cells and their implications in existing therapies must be fully understood, before these pathways can be exploited for therapeutic purposes. As demonstrated in Figure 4, Breg cells can be induced by bacterial or parasitic infections. Therefore, the identification of the secreted or excreted pathogenic compound(s) driving Breg cell induction provides useful information for the development of therapeutic interventions. Indeed, the fact that live schistosome worms could induce IL-10 producing Breg cells from splenic B cells in an *in vitro* culture system, suggests that helminth antigens have a direct effect on B cells (81). Helminth-related TLR ligands may be a likely candidate responsible for helminth-induced Breg cell formation, given the implication of certain TLR ligands in the induction of

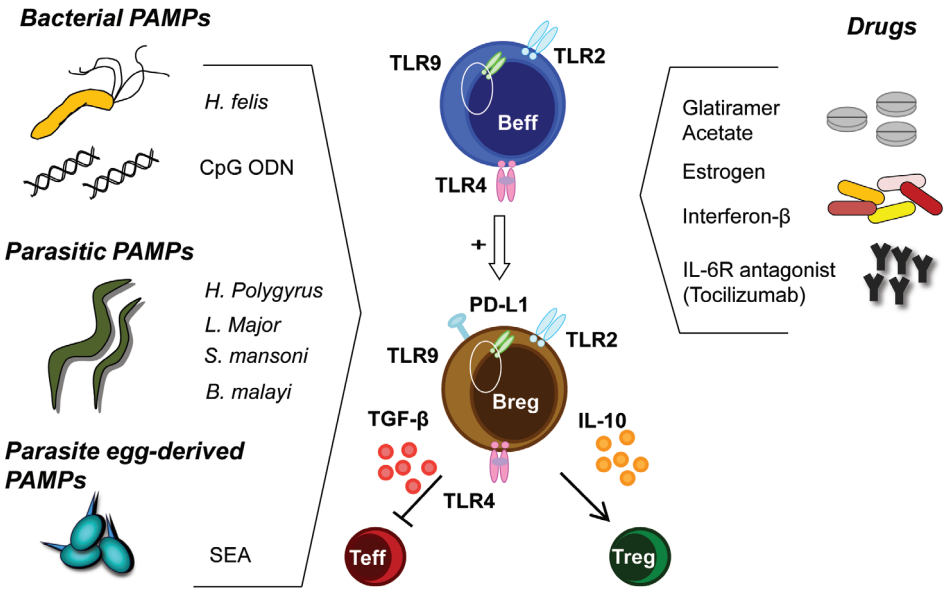

Figure 4. Pathways for the induction and expansion of Breg cells. Different secreted or excreted (non) pathogenic compounds of bacteria, parasites or their eggs can drive Breg cell induction. These compounds have been shown to bind to TLR and, thereby induce Breg cell development. As a consequence, Breg cells start to produce anti-inflammatory cytokines IL-10 and TGF-β, inhibit effector T cell proliferation and induce Treg cells. PAMPs: pathogen associated molecular patterns, TLR: Toll-like receptor, Breg: regulatory B cells, Teff/reg: effector/regulatory T cells, LNFPIII: lacto-N-fucopentaose-III, SEA: soluble egg antigens.

Breg cells in auto-immune models (Fig. 4). Notably, lacto-N-fucopentaose-III (LNFPIII), a milk-derived sugar similar to those found on soluble egg antigens (SEA) interacts with TLR-4 and stimulates splenic B cells to produce IL-10 (201). Likewise, microfilarial extracts from *Leishmania major*, and *Brugia malayi*, which both bind to TLR-4, can induce IL-10 production by B cells (202). Furthermore, lyso-phosphatidylserine, a lipid derived from *S. mansoni* worms ligated TLR-2 on human monocyte-derived DC and promoted Treg cell activity (203). Although it is unclear whether this TLR-2 ligating molecule has an effect on the formation of Breg cells, SEA stimulation of human B cells did result in TLR-2 mediated elevated IL-10 production (204). Overall, the identification of the secreted or excreted pathogenic compound(s) driving Breg cell induction provides useful information for the development of therapeutic interventions.

Scope of this thesis

In the last few decades, childhood allergy has alarmingly increased to epidemic levels not only in westernized countries, but is now also rising in middle-income countries. An explanation is suggested by the 'hygiene hypothesis', stating that this increase may be in part the result of a decreased exposure to microbial agents, leading to reduced education of the regulatory arm of the immune system and allowing the development of more inflammatory responses to essentially harmless entities, such as allergens. Indeed, epidemiological field studies and experimental murine models have pointed at an important role for helminth parasites such as schistosomes in protection against allergic diseases. During chronic schistosome infections, a profound T-cell hypo-responsiveness is found preventing excessive inflammation and tissue damage and in which the induction of a strong regulatory network and the cytokine IL-10 are central. Several immune cells can be the source of this protective IL-10, including Treg cells. However, studies with auto-immune models have suggested that also B cells can be an important source for IL-10. These IL-10-producing B cells inhibit T-cell proliferation, induce Treg cells and, therefore, were named regulatory B cells.

Regulatory B cells are an exciting new player on the regulatory side, however, most data are obtained from auto-immune models. In the context of helminth infections, the possible roles of schistosome-induced IL-10-producing B cells are unknown, what their role is in protection against allergic airway inflammation and how this may be exploited for therapeutic intervention. The work in this thesis tries to address several of these questions and aims to improve our understanding of these cells which forms a relatively young field of research and explores its future applications in controlling allergic diseases.

In **Chapter 2**, we first addressed the question whether IL-10-producing B cells, when developed during chronic schistosomiasis, can contribute to protection against allergic airway inflammation in mice. Next to splenic IL-10-producing marginal zone B cells, another Breg subset, located in the lungs,

provided protection against airway inflammation, although independently of IL-10. The characteristics and regulatory activities of these pulmonary B cells are discussed in **Chapter 3**.

Targeting Breg cells for therapeutic applications holds great promise for future treatment of autoimmune and allergic inflammatory conditions, however, conditions to promote IL-10-producing B cells are still ill defined. Pathogen-associated molecular patterns, such as Toll-like receptor ligands can stimulate a regulatory function in B cells by inducing IL-10. We have optimized *in vitro* assays to investigate which TLR ligands can stimulate B-cell IL-10 and to assess whether these IL-10-producing B cells have functional regulatory activities (**Chapter 4**).

Since schistosomes were capable of inducing IL-10-producing B cells in both mice and men (chapter 2), we next investigated the functional characteristics of human regulatory B cells in Gabonese individuals with *S. haematobium*, focusing on their influence on effector T-cell cytokine secretion and regulatory T-cell induction *in vitro* (**Chapter 5**).

Allergic disorders are less frequent in individuals with schistosomiasis. In addition, allergic responses were suppressed by schistosome-induced Breg cells in mice and increased Breg cell frequencies are found in a schistosome-infected individuals. The question remains whether in people with allergies their regulatory compartment, including Breg cells, is less well developed. Indeed, a reduced frequency of IL-10-producing peripheral blood B cells in response to the milk or bee venom antigens was found in allergic subjects compared to healthy tolerant (exposed) individuals (165;167), supporting the notion that Breg cells may be weakened in some allergic disorders. Here, we studied the frequency of IL-10-producing B cells in peripheral blood of HDM-allergic asthmatic patients compared to healthy controls and investigated their inhibitory potential of allergen-specific immune responses (**Chapter 6**). Finally, the main findings presented in this thesis are deliberated in the summarizing discussion (**Chapter 7**).

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