Regulatory B cells in allergic asthma and schistosomiasis: controlling inflammation

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About the cover:
In this thesis, both mouse and human experiments were conducted, represented by a hand carrying a mouse. The mouse is placed centrally on the cover as an awareness that without the mouse experiments we could not have studied immune regulation during helminth infection in such great detail. The speech bubble refers to the infection and is drawn in the shape of a *Schistosoma mansoni* egg. Field work was conducted in Gabon, represented by a blood vessel drawn in African style and by the yellow background, referring to the middle colour of the Gabonese flag.

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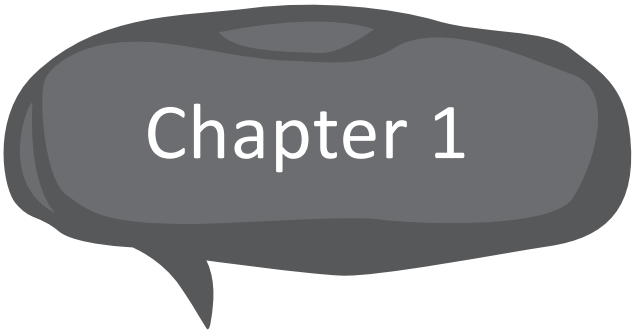

General introduction

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

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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

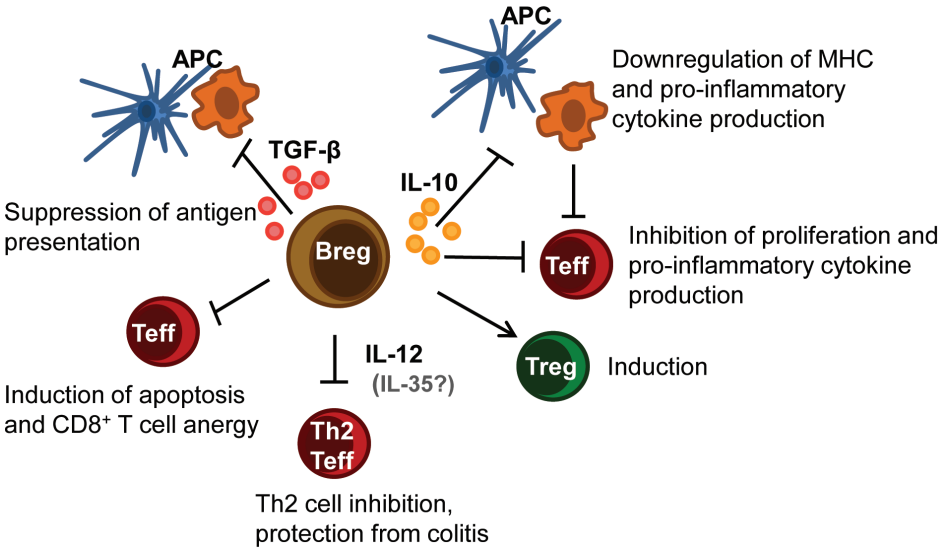

Figure 1. Suppressive functions of Breg cells mediated by the release of cytokines. Breg cells secrete immunosuppressive cytokines causing downregulation of antigen presenting cell function, inhibition of T effector cell function and induction of regulatory T cells. Breg: regulatory B cells, Teff/reg: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

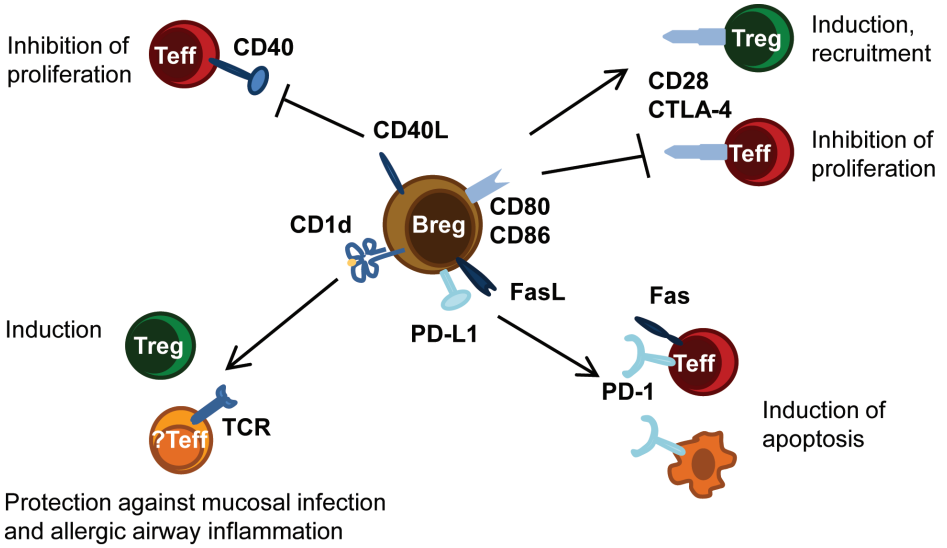

Figure 2. Suppressive functions of Breg cells mediated by cell contact-dependent mechanisms. Breg cells express several cell surface molecules that cause inhibition of T effector cell function, induction of target cell apoptosis and induction of regulatory T cells. Breg: regulatory B cells, Teff/reg: 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

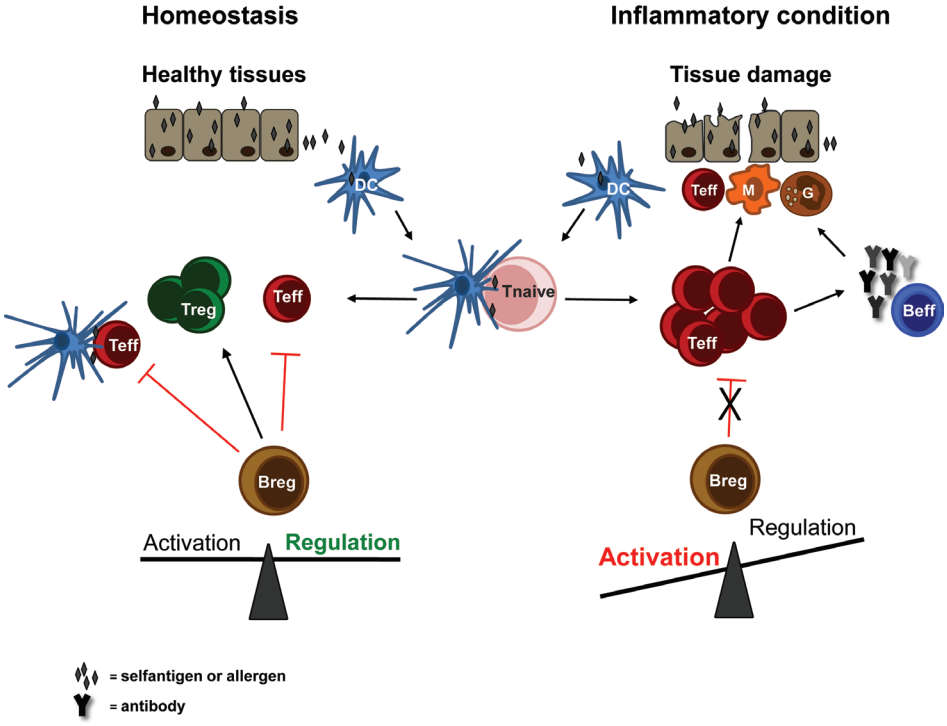

Figure 3. Regulatory B cells in homeostasis and disease. Under normal conditions regulatory B cells control T effector cell activation and proliferation in response to harmless self-antigens and allergens, and induce and activate regulatory T cells. If this Breg cell mediated control fails, effector 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

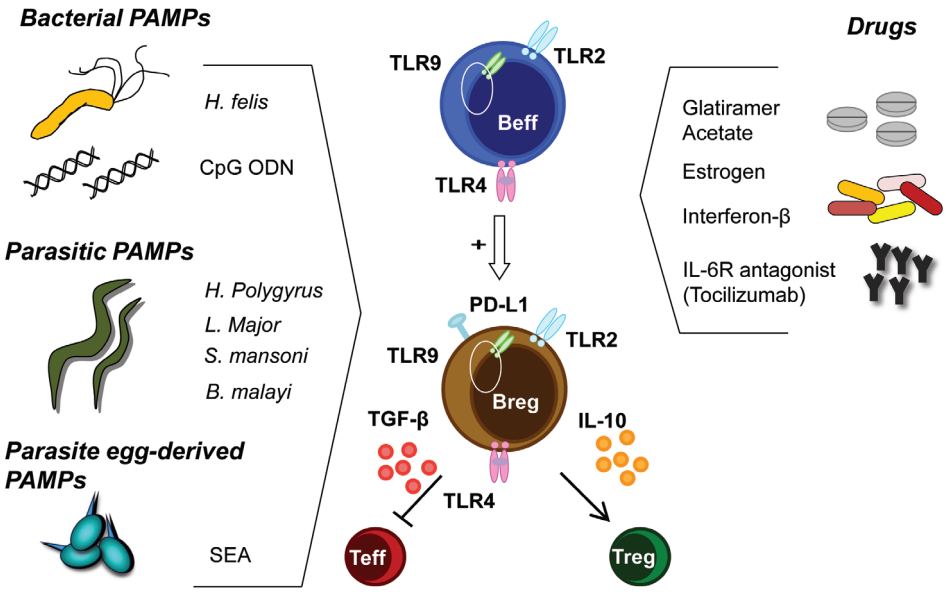

Figure 4. Pathways for the induction and expansion of Breg cells. Different secreted or excreted (non) pathogenic compounds of bacteria, parasites or their eggs can drive Breg cell induction. These compounds have been shown to bind to TLR and, thereby induce Breg cell development. As a consequence, Breg 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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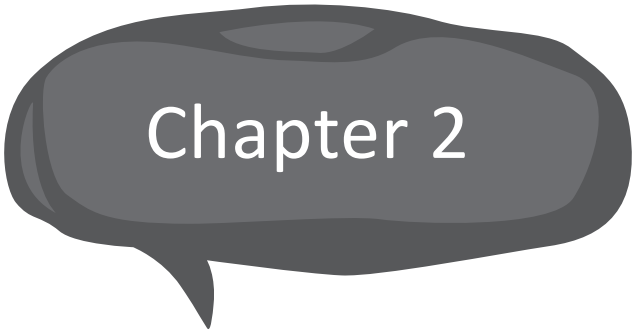

Schistosomes induce regulatory features in human and mouse CD1d^{hi} B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells

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Abstract

Chronic helminth infections, such as schistosomes, are negatively associated with allergic disorders. Here, using B cell IL-10-deficient mice, *Schistosoma mansoni*-mediated protection against experimental ovalbumin-induced allergic airway inflammation (AAI) was shown to be specifically dependent on IL-10-producing B cells. To study the organs involved, we transferred B cells from lungs, mesenteric lymph nodes or spleen of OVA-infected mice to recipient OVA-sensitized mice, and showed that both lung and splenic B cells reduced AAI, but only splenic B cells in an IL-10-dependent manner. Although splenic B cell protection was accompanied by elevated levels of pulmonary FoxP3$^+$ regulatory T cells, *in vivo* ablation of FoxP3$^+$ T cells only moderately restored AAI, indicating an important role for the direct suppressory effect of regulatory B cells. Splenic marginal zone CD1d$^+$ B cells proved to be the responsible splenic B cell subset as they produced high levels of IL-10 and induced FoxP3$^+$ T cells *in vitro*. Indeed, transfer of CD1d$^+$ MZ-depleted splenic B cells from infected mice restored AAI. Markedly, we found a similarly elevated population of CD1d^{hi} B cells in peripheral blood of *Schistosoma haematobium*-infected Gabonese children compared to uninfected children and these cells produced elevated levels of IL-10. Importantly, the number of IL-10-producing CD1d^{hi} B cells was reduced after anti-schistosome treatment. This study points out that in both mice and men schistosomes have the capacity to drive the development of IL-10-producing regulatory CD1d^{hi} B cells and furthermore, these are instrumental in reducing experimental allergic inflammation in mice.

Introduction

The prevalence and severity of allergic diseases and asthma has increased over the last five decades in industrialized countries (1). Conversely, lower prevalence of allergic diseases is seen in low income countries. Many epidemiological studies have reported an inverse association between helminth infections, which are highly prevalent in developing countries, and allergic or auto-immune disorders (2–4). In order to study the interaction between helminth infections and protection against allergic diseases, murine models of allergic airway inflammation (AAI) and helminth infection have been developed. For example, *H. polygyrus* or *T. spiralis* infections protected against house dust mite-induced and/or ovalbumin (OVA)-specific AAI (5;6). In addition, *S. mansoni* infection protected mice against OVA-induced airway hyperresponsiveness (AHR) (7); protection was optimal during the chronic stage of infection, but not the acute stage (8).

Helminth infections are characterized by potent type 2 effector responses and a strong regulatory network (9). Regulatory T (Treg) cells are well-known for their suppressive capacity, but recent studies in auto-immunity models have indicated that also B cells can be important players in immune regulation (10). These so-called regulatory B cells are known to influence the immune system by the regulatory cytokines IL-10 and TGF-β, influencing T cell proliferation, downregulating CD4^{+}, CD8^{+}, NK T cell activation and promoting FoxP3^{+} Treg cell induction (11). Interestingly, a number of studies have reported that B cells may have an active regulatory role in various parasitic infections. For example, IL-10-producing B cells in *L. major*-infected BALB/c mice are essential in suppressing type 1 responses that are necessary to clear infection (12) and *S. mansoni*-infected B cell-deficient μMT mice show more extensive hepatic granulomas (13). In addition, in a model of systemic anaphylaxis or AAI, in combination with *S. mansoni* infection with adult stage worms only, B cells appeared to be major players in addition to IL-10 and Treg cells (7;14).

In auto-immunity models several Breg subsets have been identified, including marginal zone (MZ), transitional or CD5^{+}CD1d^{hi} B cells (15). Recent studies in human auto-immune diseases have substantiated these findings by showing human Breg cells in peripheral blood characterized as CD24^{hi}CD38^{hi} (16), CD24^{hi}CD27^{+} (17) or CD1d^{hi} B cells (18).

Here, we investigated both in mice and humans whether schistosome infections can induce functional Breg cells. Indeed, we identified for the first time in peripheral blood of *S. haematobium*-infected children elevated numbers of IL-10-producing CD1d^{hi} regulatory B cells, which were decreased after treatment. The functional capacity of those schistosome-induced Breg cells was confirmed in a mouse model of allergic airway inflammation where Breg-derived IL-10 and Breg-induced Treg cells mediated suppression.

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 in accordance with the guidelines and protocols (DEC-07062, 07152, 08034, 09141) approved by the Ethics Committee for Animal Experimentation of the University of Leiden, The Netherlands. The human study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL). Written informed consent was obtained from parents or legal guardians of children participating in the study.

Animals

Six week-old female C57/Bl6 OlaHsd mice were purchased from Harlan. For the generation of chimeric IL-10-producing B cell-deficient mice, B cell-deficient μMT mice were irradiated to remove the bone marrow (BM). Subsequently, the irradiated mice were reconstituted with 80% of μMT BM and 20% IL-$10^{-/-}$ BM cells (IL-10-deficient) or with 80% of μMT BM and 20% wild-type (WT) BM as described (10). DEREG (DEpletion of REGulatory T cells) mice were kindly provided by Dr. T. Sparwasser (19).

Parasitic infection and AAI induction

Mice were infected percutaneously with 40 *S. mansoni* cercariae and the infection lasted until 14 weeks (chronic phase starts around week 12) (8). For AAI induction, mice were sensitized twice by i.p. injections of OVA (10 μg/mL, Worthington Biochemical Corp) in Imject Alum (2 mg/ml; Pierce) at 17 and 10 days before challenge. Sensitization was initiated during week 11 and 12 after the start of infection. Ten days after the last injection, mice received OVA aerosol challenges (10 mg/ml in PBS) for three consecutive days in the 14^{th} week of infection. Mice were sacrificed 24 hours after the last challenge. BAL fluids were collected and phenotyped by flow cytometry (8).

Study population

Venous blood was obtained from 20 school children living in Lambaréné (Gabon) or from a nearby village (PK15) where *Schistosoma haematobium* is endemic. *S. haematobium* infection was detected by examining 10 ml of urine passed through a 12-μm-poresize filter (Millipore) and the eggs were stained with a ninhydrin solution. Children were classified as 'infected' if at least one *S. haematobium* egg was detected in the urine or 'uninfected' if three consecutive urine samples were negative. Infections with intestinal helminths *A. lumbricoides*, *T. trichiura* and hookworm were determined by analyzing one fresh stool sample using the Kato-Katz method (20). Infection with *P. falciparum* was determined by PCR (21).

S. haematobium-infected children were treated with three doses of praziquantel (40 mg/kg) every two months. Intestinal helminth and malaria infected children received respectively a single dose of albendazole (400 mg) and an artemisinin-based combination therapy as per the local guidelines.

Mouse cell purification and cell sorting

Single cell suspensions were prepared from the spleens, mesenteric lymph nodes, and mediastinal lymph nodes by dispersion through a 70-µm cell sieve (Becton Dickinson). Perfused lungs were minced to ~1 mm pieces and digested by collagenase II/Dnase for 1 hour in 12-well plates (Greiner Bio-One). The digested lungs were sequentially dispersed through 70- and 40-µm sieves. Erythrocytes were removed from the spleen and lung single cell suspensions by lysis. B cells were purified using anti-CD19 MicroBeads (Miltenyi Biotec). Follicular B cells (CD21^{int}CD23^{hi}) and marginal zone B cells (CD21^{hi}CD23$^{int/low}$) were stained with antibodies against CD19-PE (MB19-1, eBioscience), CD21-APC (7G6, BD Pharmingen), and CD23-FITC (B3B4, eBioscience) in 15 ml tubes (Greiner Bio-One) and separated using FACSAriaII cell sorting (Becton Dickinson). The sorted subsets were routinely ~95% pure. For the depletion of MZ B cells, splenocytes were first incubated with CD19-PE (6D5, Miltenyi Biotec), followed by anti-PE multisort beads (Miltenyi Biotec) to isolate the B cells. Next, these beads were enzymatically removed and the isolated B cells were incubated with CD21-FITC (7G6, BD Pharmingen) antibody for 20 minutes. The CD21^{hi} MZ B cell fraction was depleted using anti-FITC magnetic beads (Miltenyi Biotec). For the total CD19^{+} B cells, the CD21^{hi} B cells were added back to the CD21^{neg} B cells (mock depletion). The depletion of MZ B cells was ~92% pure. CD4^{+}CD25^{-} T cells were enriched using anti-CD4 and anti-CD25 MicroBeads with a purity of 96% (Miltenyi Biotec).

Human B cell isolation and characterization

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from 20 ml of heparinized blood. B cells were isolated with anti-CD19 MicroBeads (Miltenyi Biotec) with a purity of ~95%. For immunophenotyping different B cell subsets, isolated PBMCs were fixed in 2.4% PFA and stained for CD19-PB (HIB19, eBioscience), CD1d-PE (51.1, eBioscience), CD5-APC (UCHT2, BD), CD24-PeCy7 (ML5, ITK Diagnostics), CD27-APCeFluor780 (O323, eBioscience), and CD38-FITC (HIT2, BD).

Adoptive transfer of isolated mouse B cells

Recipient mice were sensitized with two injections of OVA at day 0 and day 7. Ten days after the last injection, the OVA-sensitized animals received i.v. injection of 5 x 10^{6} pulmonary, mesenteric, splenic total or splenic CD19^{+} B cells depleted for MZ B cells from OVA sensitized-uninfected or OVA sensitized-infected mice. Blocking anti-IL-10R antibody (250 µg; kindly provided by Schering Plough Biopharma) or isotype control antibody was given i.p., one day before adoptive transfer. DEREG mice, which carry a Diphtheria toxin receptor-eGFP transgene under the control

of an additional Foxp3 promoter, were treated with two diphtheria toxin (DT, 1 µg/ml) i.p. injections: one day before and two days after the adoptive transfer of B cells in order to deplete the FoxP3$^+$ Treg cells. After two days, mice were challenged for three consecutive days and sacrificed 24 hours after the last challenge.

***In vitro* mouse B cell stimulation and co-culture with CD4$^+$CD25$^-$ T cells**

Mouse CD19$^+$ B cells and B cell subsets (1 x 10^5 cells) were cultured in medium (RPMI 1640 glutamax; Life Technologies), containing 5% FBS (Greiner Bio-One), 5 x 10^{-5} M 2-Mercaptoethanol (Sigma-Aldrich) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Life Technologies), in the presence of SEA from *S. mansoni* eggs (20 µg/mL) for five days in 96-well plates (Greiner Bio-One). Supernatants were stored for later cytokine analysis by ELISA. For *in vitro* Treg induction, B cells (1 x 10^5 cells) were first irradiated with 2600 RAD and subsequently, co-cultured with CD4$^+$CD25$^-$ T cells (1 x 10^5 cells) in the presence of medium or anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml). An isotype control anti-β-gal (10 µg/ml), or anti-IL-10 receptor (10 µg/ml) was added. After five days, cells were fixed according to the eBioscience FoxP3 fixation/permeabilization kit. Proliferation was confirmed by cell counts. Cytokines were measured in the cell culture supernatant using Luminex or ELISA (IL-1β,-4,-5,-6, -10, -12p40/70, -13, IFN-γ, TNF-α).

Human B cell stimulation and intracellular staining for IL-10

Freshly isolated B cells (1 x 10^5) were stimulated for 48 hours with 2.5 µg/ml anti-human IgG/IgM (Jackson ImmunoResearch) in the presence or absence of 10 µg/ml SEA from *S. haematobium* eggs. For ICS of IL-10, B cells were restimulated with PMA (50 ng/ml), ionomycin (2 µg/ml), and LPS (100 ng/ml; Invivogen) for 6 hours with the final 4 hours in the presence of BrefA (10 µg/ml; Sigma-Aldrich), followed by fixation with FoxP3 fixation/permeabilization kit and stained for CD1d-PE (51.1, eBioscience), CD20-APCeFluor780 (2H7, eBioscience), and IL-10-biotin (JES3-12G8, Abd Serotec) followed by second incubation with streptavidin-Qdot525 (Invitrogen).

Statistical analysis

All murine results are expressed as mean ± SEM and the different groups were tested using the Student's *t*-test (two-tailed). Differences between infection groups in humans were tested by the Mann-Whitney U test. Differences within the same group pre- and post-treatment were compared by Wilcoxon matched pairs test. Probability values less than 0.05 were considered significant. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ and # $p<0.1$.

Results

IL-10-producing B cells are important for protection against allergic airway inflammation

Elevated IL-10 characterizes chronic stages of schistosome infection and is produced by B cells starting from week 12 during infection (Fig. S1) (8). To determine whether B cells are a dominant source of IL-10 and whether this IL-10 is essential for protection against AAI during chronic schistosome infections, IL-10-deficient B cell and control wild-type (WT) chimeric mice were generated and chronically infected with *S. mansoni* followed by an allergic OVA sensitization and challenge. The IL-10-deficiency was restricted to the B cells population (confirmed by intracellular flowcytometry) as described before (10). In the uninfected allergic groups (OVA-uninfected) for both WT and IL-10$^{-/-}$ B cell chimeric mice, the bronchoalveolar lavage (BAL) fluid contained significantly more eosinophils (Fig. 1A), lymphocytes and macrophages compared to uninfected non-allergic groups (PBS-uninfected) (data not shown). Nevertheless, in OVA-infected WT mice significantly less eosinophils were found compared to OVA-uninfected WT mice (Fig. 1A), as previously described (8). In contrast, eosinophilia in the OVA-infected group of the IL-10$^{-/-}$ B cell mice was restored and was similar to the OVA-uninfected IL-10$^{-/-}$ B cell group and significantly higher compared to the OVA-infected WT group. Interestingly, in the PBS-infected group, eosinophilia was equally high, suggesting that IL-10-producing B cells were involved in controlling non-allergic inflammatory processes during natural infections as well. Furthermore, IL-5, IL-13 and/or IL-10 were equally elevated in the BAL fluid and mediastinal lymph nodes (MedLN) of OVA-infected IL-10$^{-/-}$ B cell mice compared to OVA-uninfected IL-10$^{-/-}$ B cell group, whereas these Th2 cytokines were reduced in OVA-infected WT mice (Fig. 1B, C). The IL-5 production in the BAL fluid and by T cells in the MedLN was also significantly increased in the OVA-infected IL-10$^{-/-}$ B cells mice compared to the OVA-infected WT mice as well. As expected, IL-4 remained at low levels due to the C57/Bl6 background of the chimeric mice (Fig. 1C). These results indicate that IL-10-producing B cells are critically involved in the downmodulation of eosinophilia and the Th2 response against OVA antigen leading to protection against AAI during chronic schistosomiasis.

Pulmonary and splenic B cells produce IL-10 and protect against AAI

In order to identify the dominant organ with IL-10-producing B cells during infection, we isolated B cells from organs that have previously been described to harbor regulatory B cells (spleen), drain schistosome infection sites (mesenteric lymph nodes, MLN) or are the effector site where allergic inflammation is found (lung). Both pulmonary and splenic B cells, but not mesenteric B cells, from chronically-infected mice were able to produce IL-10 upon soluble egg antigen (SEA) stimulation, with highest production by splenic B cells (Fig. 2A). To study the suppressive activity of isolated B cells from different organs in downmodulating

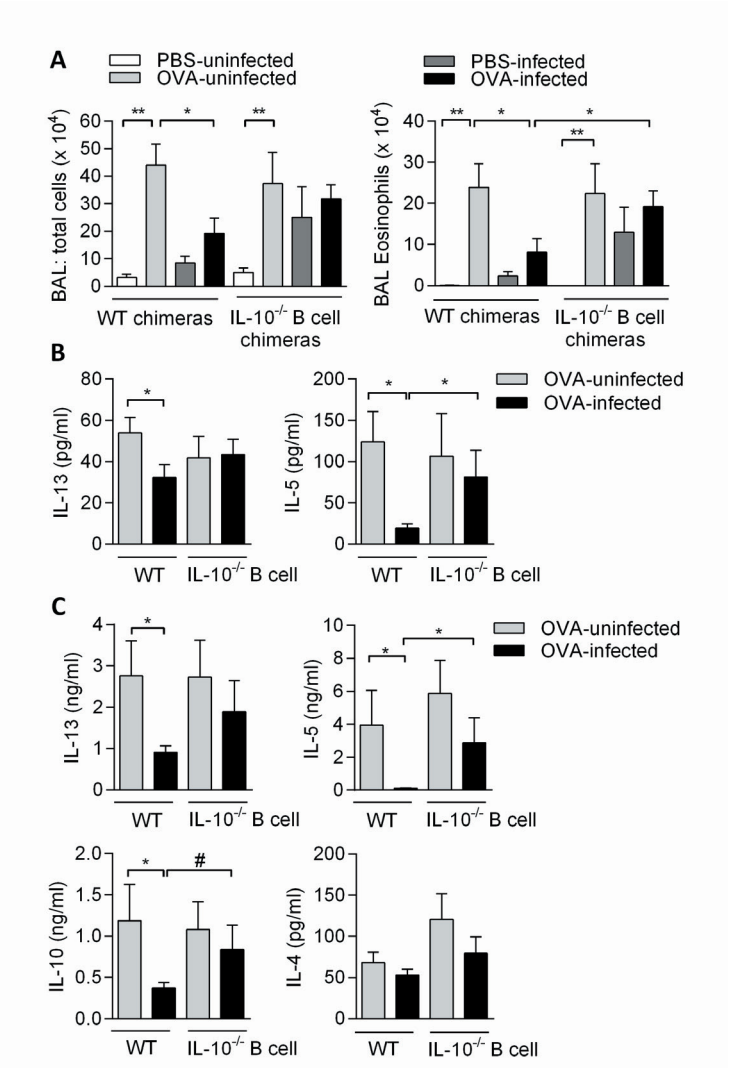


Figure 1. Role of IL-10-producing B cells on OVA-specific AAI during chronic *S. mansoni* infection. Chimeric WT and IL-10$^{-/-}$ B cell mice were infected with *S. mansoni* and sensitized and challenged for OVA at week 11 and 12 after the start of infection. BAL fluid was collected and total BAL cells (A) and eosinophils were determined. (B) In the BAL fluid, IL-5 and IL-13 were measured by ELISA. (C) MedLN cells were collected and restimulated by OVA (10 µg/mL) for four days and IL-4, IL-5, IL-10, and, IL-13 production was determined using ELISA. Results are from two independent experiments and each group consists of 6 PBS- and 6 OVA-uninfected, 8 PBS- and OVA-infected mice.

AAI, we adoptively transferred CD19^{+} B cells from OVA-infected mice into OVA-sensitized recipient mice. AAI was reduced by pulmonary or splenic but not by mesenteric B cells (Fig. 2B). Interestingly, the protective effect of the transfer of splenic B cells, but not of pulmonary B cells, was abolished by administering a

blocking IL-10 receptor antibody (Fig. 2C). Furthermore, we observed increased percentages of CD4$^+$CD25$^+$FoxP3$^+$ T cells in the lungs of recipient mice, but only after administering splenic B cells (Fig. 2D). This data indicate that pulmonary B cells can drive IL-10 and Treg cell-independent protection against eosinophilic AAI, while splenic B cells protect via an IL-10-dependent mechanism and enhance local Treg cell numbers in the lungs.

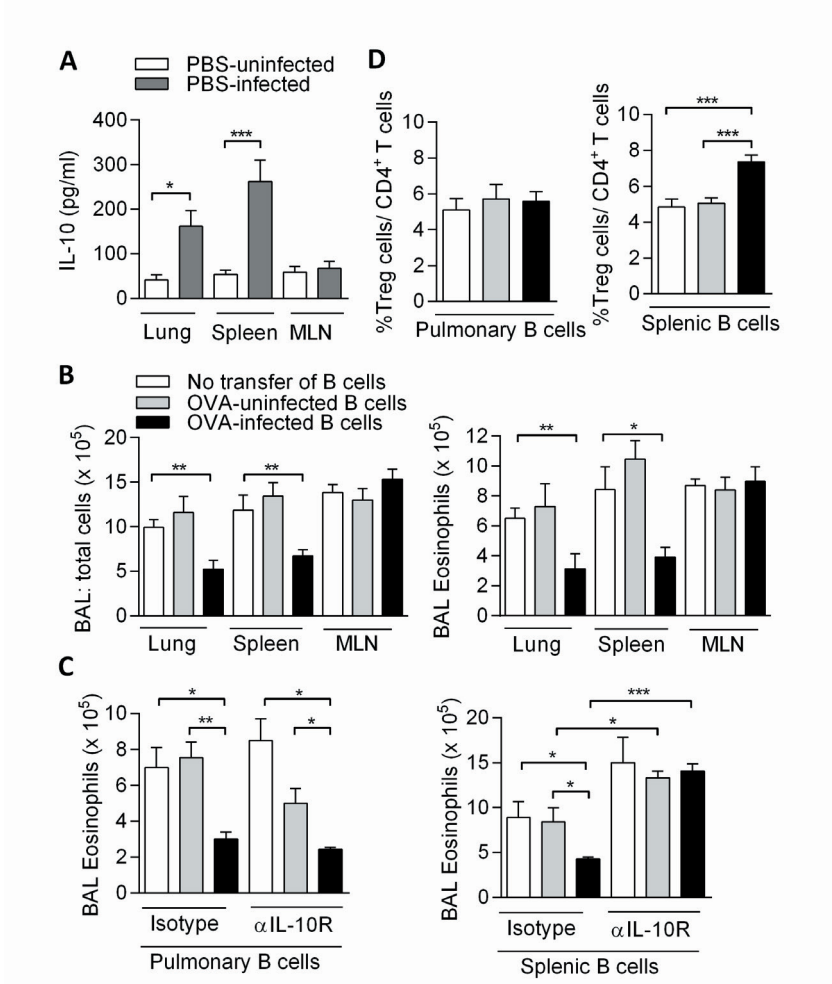


Figure 2. IL-10 production by B cells from different organs during chronic schistosomiasis and their role in protection against AAI. (A) WT mice were treated as in Fig 1A. Splenic, pulmonary or mesenteric B cells (1 x 10^5) were isolated and cultured in the presence of SEA (20 µg/ml) for five days. IL-10 production was measured using ELISA and medium value was subtracted. (B) OVA-sensitized recipient mice received 5 x 10^6 B cells from different organs. After challenge, BAL cell numbers and eosinophils were determined (C) BAL numbers and eosinophilia of mice that received 250 µg isotype control or anti-IL-10R abs per mouse one day before the adoptive transfer. (D) The percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells was determined in the lungs of recipient mice. Each graph represents three independent experiments, consisting of five mice per group.

Splenic B cells induce Treg cells, which support reduction of AAI

Regulatory B cells are known to regulate inflammation by recruitment and generation of Treg cells in auto-immune disorders (10). Therefore, we cultured irradiated splenic B cells from OVA-uninfected or OVA-infected mice with $CD4^+CD25^-$ T cells from naive mice to evaluate Treg cell development. Splenic B cells from OVA-infected mice doubled the percentage of $CD4^+CD25^+FoxP3^+$Treg cells (Fig. 3A), while pulmonary B cells did not change the level of Treg cells, as already confirmed *in vivo*. In addition, no shift towards Th1 or Th2 cytokines was found during the co-culture showing that the splenic B cells primarily influenced the development of Treg cells but not of other T cell subsets (data not shown).

Next, we studied the contribution of B cell-induced Treg cells in protection against AAI *in vivo* using OVA-sensitized FoxP3-DTR transgenic DEREG mice (19). The temporal loss of $FoxP3^+$ Treg cells during allergen challenge only

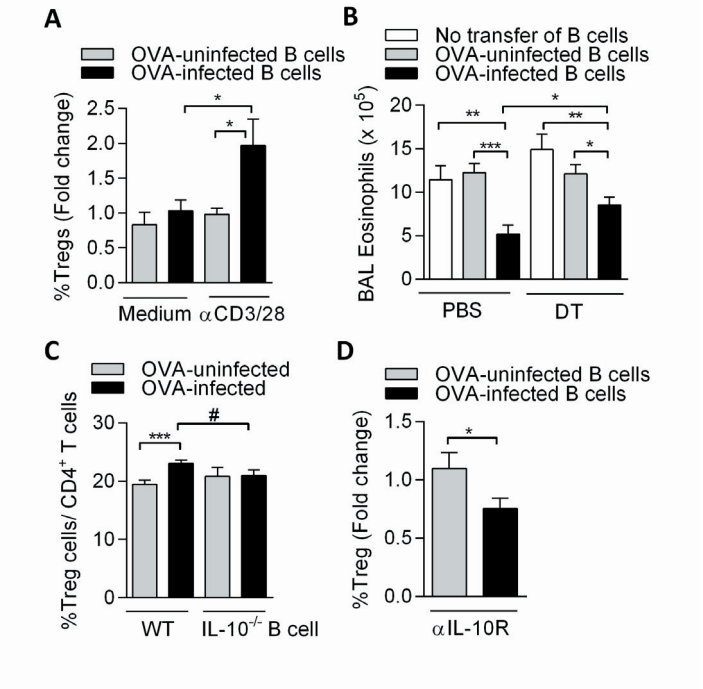


Figure 3. Treg cell induction by IL-10-producing Breg cells. (A) Irradiated splenic B cells (1 x 10^5) were cultured with $CD4^+CD25^-$ T cells (1 x 10^5) for 5 days in the presence of anti-CD3 and anti-CD28. Induction of $CD4^+CD25^+FoxP3^+$ Treg cells (in %) by PBS-uninfected B cells was set at one. Fold change in Treg cell percentage for OVA-uninfected and OVA-infected B cells was calculated. Graph expresses results from three independent experiments. (B) OVA-sensitized DEREG mice were treated as in Fig. 2B in addition to a DT or PBS injection. This graph expresses two experiments, consisting of five mice per group. (C) WT and IL-$10^{-/-}$ B cell chimeras were treated as in Fig. 1A. The MedLNs were collected and the percentage of $CD4^+CD25^+FoxP3^+$ Treg cells was determined. Figure contains two independent experiments and each group consists of 6 to 8 mice. (D) *In vitro* co-culture were performed as described in (A) in the presence of blocking anti-IL-10R or isotype control antibodies. Percentage of Treg cells induced in the presence of isotype control was arbitrarily set at 1. Fold change in Treg cell induction in the presence of anti-IL-10R was calculated. Graph represents three independent experiments.

partly restored AAI, showing that in the group with transferred B cells from OVA-infected mice, B cell-induced Treg cells are only partially involved in protection against AAI (Fig. 3B).

A causal relationship between IL-10-producing B cells and Treg cells has been suggested in antigen-induced arthritis model utilizing similar chimeric mice, where loss of IL-10-producing B cells led to significant reduction of Treg cells in draining inguinal LN (22). In OVA-infected WT mice, we found more $CD4^+CD25^+FoxP3^+$ Treg cells in the lung-draining MedLN compared to OVA-uninfected mice, which was not found in OVA-infected IL-$10^{-/-}$ B cell mice (Fig. 3C). Using the same *in vitro* co-culture as describe above, significantly less Treg cell induction by splenic B cells was found when anti-IL-10R antibodies were added compared to isotype control (Fig. 3D), underlining the role of IL-10 for Treg cell induction both *in vivo* and *in vitro*.

Schistosome-induced MZ B cells exhibit regulatory activities

We compared the two main splenic B cell subsets, the follicular (FO) B cells and the MZ B cells (which are high in CD1d, Fig. S2) for their ability to produce IL-10 during chronic infection. Sorted subsets from uninfected and infected mice were cultured in the presence of SEA, showing that the MZ B cells from infected mice produced high IL-10 levels, while FO B cells produced only low levels (Fig. 4A). Production of IL-12 and IL-1β remained low in both subsets of infected mice, while production of TNF-α and IL-6 was increased in FO cells, but remained low in MZ B cells from infected mice (Fig. S3). Based on our findings regarding Treg cell induction by total splenic B cells, we questioned whether MZ B cells, as the strongest IL-10 producers, were also responsible for Treg cell induction *in vitro*. Therefore, irradiated FO and MZ B cells were cultured with $CD4^+CD25^-$ T cells and

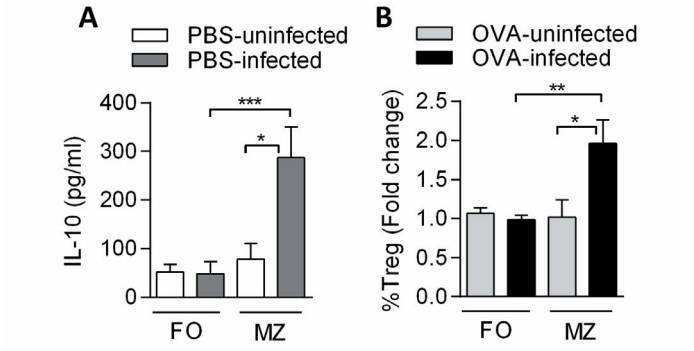


Figure 4. MZ B cells show regulatory features. (A) MZ and FO B cells from PBS-uninfected and PBS-infected were sorted using flow cytometry and cultured for 5 days in the presence of SEA for IL-10 production as in Fig. 2A. (B) Irradiated MZ B cells or FO B cells (1 x 10^5) were co-cultured with $CD4^+CD25^-$ T cells as described in Fig. 3A. Treg cell induction by MZ or FO B cells from uninfected mice was set at one. Subsequently, fold change in Treg cell induction by MZ or FO B cells from OVA-uninfected and OVA-infected mice was calculated. Each graph contains three independent experiments with five mice per group.

the highest percentage of FoxP3$^+$ T cells was observed in co-culture with MZ B cells (Fig. 4B), which was not explained by differences in proliferation as similar cell counts were found in cultures with either FO or MZ B cells (data not shown). These data indicate that MZ B cells are responsible for the regulatory features observed in total splenic B cells by producing IL-10 and by enhancement of FoxP3$^+$ T cells.

Next, the contribution of the MZ B cells *in vivo* was investigated by depletion of the CD21^{hi} cells from total splenic B cells of OVA-uninfected or OVA-infected mice by indirect magnetic labeling (Fig. 5A). The transfer of mock-treated B cells from OVA-infected mice resulted in a decrease in total BAL cell count and eosinophilia (Fig. 5B), as observed before. Importantly, the transfer of CD21$^+$-depleted B cells restored the severity of AAI and the induction of pulmonary FoxP3$^+$ Treg cells was lost (Fig. 5C), confirming the significance of MZ B cells in protection against AAI *in vivo*.

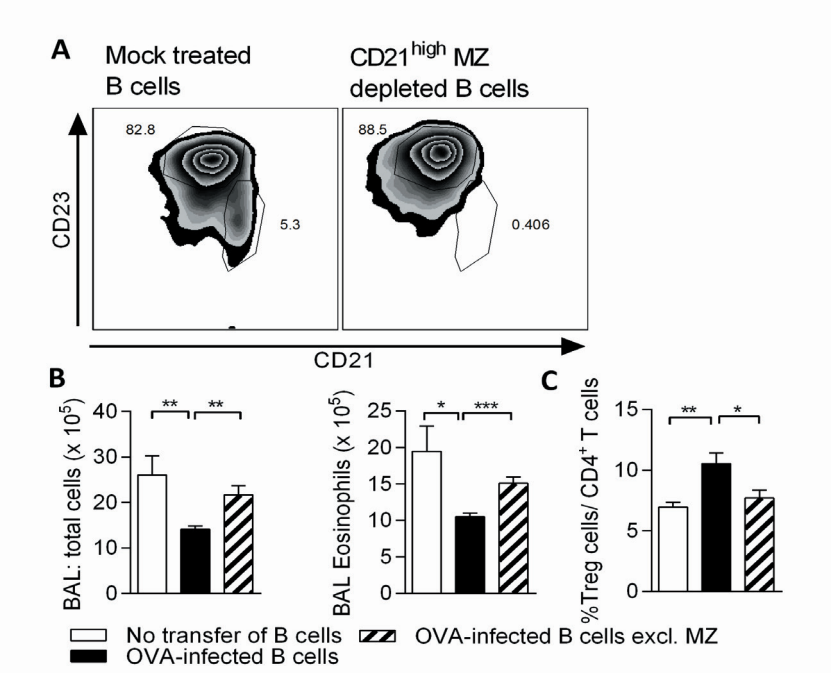


Figure 5. MZ B cells are important in the protection against AAI. (A) Mock-depleted splenic and the MZ-depleted B cells were injected in OVA-sensitized recipient mice. After challenge, total BAL cell count and the number of BAL eosinophils (B) and the percentage of CD4$^+$CD25$^+$FoxP3$^+$ T cells in the lungs (C) was measured. Figure is representative of two independent experiments, consisting of five mice per group.

Elevated levels of IL-10-producing CD1d^{hi} B cells in *S. haematobium*-infected Gabonese children

All data regarding schistosome-induced Breg cells stems from mouse models. Therefore, we asked whether in humans schistosomes can also induce IL-10-

producing regulatory B cells. PBMC were collected from 20 Gabonese children that were either *S. haematobium* positive or negative (Table 1). All samples were simultaneously analyzed for different Breg markers, including CD24^{hi}CD38^{hi}, CD24^{hi}CD27^{+} or CD1d^{hi}(CD5^{+}) (Fig. 6A). No differences were found between infected or uninfected donors for CD24^{hi}CD38^{hi} or CD24^{hi}CD27^{+} B cells (Fig. S4). However, significantly higher percentage of CD1d^{hi} B cells was found in infected children compared to uninfected children (Fig. 6B). There was also a trend for more CD1d^{hi}CD5^{+} B cells in infected children, however, the total numbers were so low (< 1 %) that the reliability of this measurement can be questioned. Importantly, after six months of treatment with praziquantel, CD1d^{hi} percentages were reduced to levels comparable to the uninfected control group (Fig. 6B). Of note, CD1d^{hi} levels in the uninfected control group were significantly increased over the same period, which may reflect seasonal changes. Alternatively, 6 out of 8 donors from the uninfected group had an increased population of plasmablasts (CD19^{+}CD24^{lo}CD38^{hi} cells; Fig. S5), suggesting a recent, unrelated infection in Lambaréné, but not in the nearby village (~15 km, PK15), which may have caused the increase in CD1d^{hi} B cells observed in the uninfected children.

Table 1. Demographic characteristics and infection status of Gabonese children.

	S.h. infected	*S.h.* uninfected
Participants pre-tx/post-tx	10/7	10/8
Mean age in years (range)	10.3 (8-14)	11.2 (8-14)
Sex male/female	5/5	4/6
Mean egg counts (range)	31.7 (1-201)	0
Co-infections:		
Plasmodium falciparum	4/10	1/10
Ascaris lumbricoides	3/9	3/8
Trichuris trichiura	3/9	6/8
Hookworm	1/9	1/8

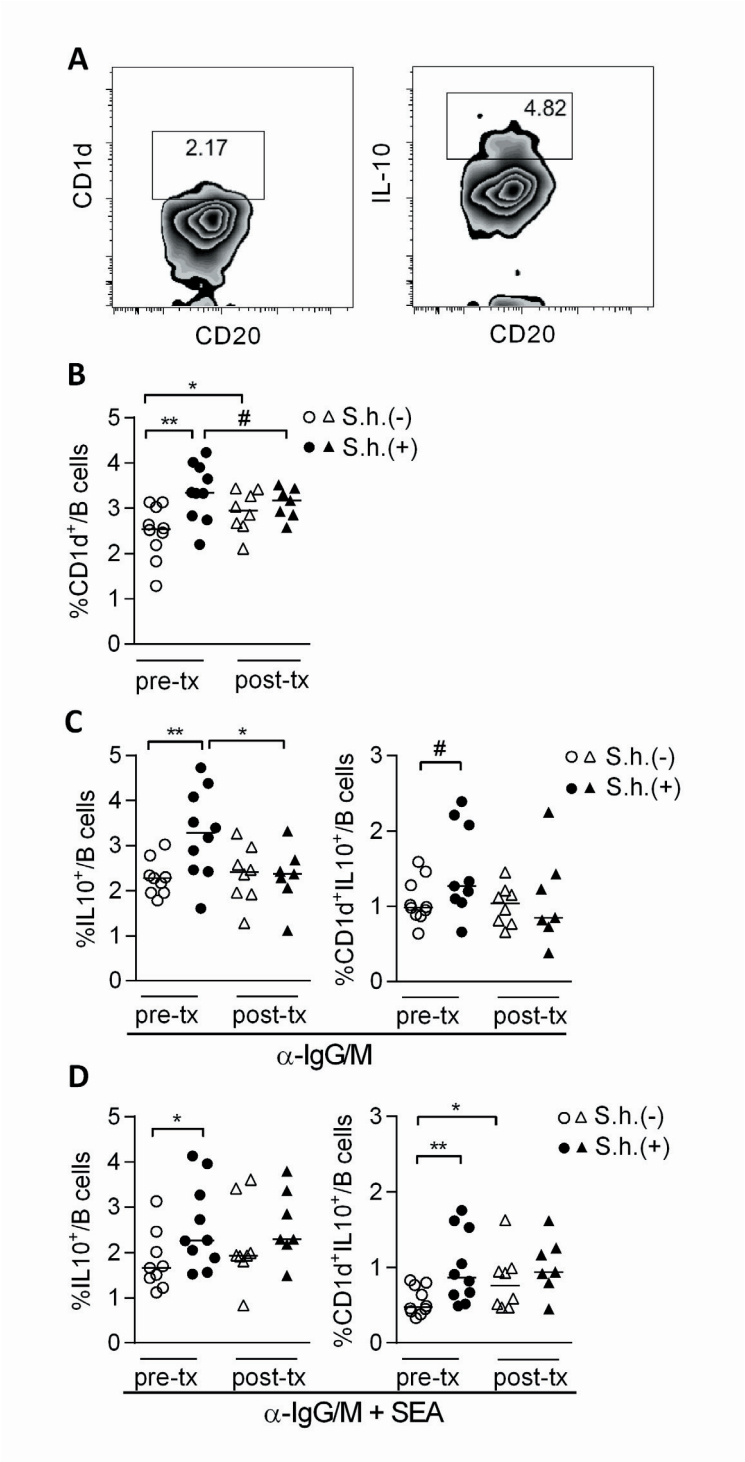
Co-infections are depicted as number of participants infected out of total number of participants tested.

To compare the IL-10 production in B cells from infected and uninfected children, total peripheral blood B cells were stimulated with anti-IgG/IgM. Subsequent intracellular analysis showed an increase in IL-10-producing B cells in the total B cell population of infected children, which was significantly reduced after treatment (Fig. 6A,C). Further gating on the CD1d^{hi} B cell subset, confirmed that in particular CD1d^{hi} B cells from infected children produce more IL-10 as compared to uninfected children (Fig. 6C). Interestingly, we observed a slightly different pattern, when the cells were stimulated with anti-IgG/IgM plus SEA (Fig. 6D). Although also in this condition, both total and CD1d^{hi} B cells from infected

children produce significantly more IL-10, this was not downregulated upon treatment. This may reflect the presence of a small, but persistent population of schistosome-specific B cells in the circulation of previously infected children that more readily produce IL-10 in response to SEA, as its cognate antigen, compared to the more aspecific stimulation with antibodies directed against IgG/IgM. Although it cannot be excluded that SEA may also act as an adjuvant, this seems to be less likely as only little IL-10 inducing activity of SEA is found in B cells from uninfected children. The fact that somewhat elevated IL-10 levels in anti-IgG/IgM plus SEA-stimulated B cells from uninfected group at post-treatment are observed compared to pre-treatment may again reflect seasonal effects or recent unrelated infections, suggested by the enhanced population of plasmablasts in this group (Fig. S5).

Altogether, these data confirm that also in humans, schistosomes can induce IL-10-producing Breg cells, of which the $CD1d^{hi}$ B subset is the most prominent. Therefore schistosomes can be recognized as powerful Breg cell inducer in both mice and humans.

Figure 6. Presence of IL-10-producing $CD1d^{hi}$ B cells during *S. haematobium (*S.h.) infection. (A) The $CD1d^{hi}$ B cells and the IL-10 production of the total B cells were gated according to the gating strategy depicted in this graph. The gating of the IL-10 production of the different Breg subsets is similar as the total B cell gating. (B) PBMC were fixed and stained for Breg markers, including CD1d, and analyzed by flow cytometry. (C) Total peripheral blood B cells were cultured with anti-IgG/IgM or (D) anti-IgG/IgM plus SEA (10 µg/ml) for two days. Intracellular IL-10 production of the total B cells and the $CD1d^{+}$ B cells was determined following PMA/Ionomycin/LPS and BrefA stimulation.


A
CD1d
2.17
CD20
IL-10
4.82
CD20
B
%CD1d+/B cells
S.h.(-)
S.h.(+)
pre-tx
post-tx
C
%IL10+/B cells
%CD1d+IL10+/B cells
pre-tx
post-tx
α-IgG/M
D
%IL10+/B cells
%CD1d+IL10+/B cells
pre-tx
post-tx
α-IgG/M + SEA

Discussion

In this study we provide evidence that during chronic schistosomiasis IL-10-producing CD1d^{hi} B cells are induced in both humans and mice. In mice, schistosome-induced IL-10-producing Breg cells were necessary for protection against AAI as shown here in chimeric IL-10$^{-/-}$ B cell mice. Splenic B cells were the most prominent source for IL-10. Interestingly, the induction of IL-10-producing splenic B cells by infectious agents has been previously documented for *L. major* (12), murine cytomegalovirus (23) and for *Schistosoma mansoni* by our group and by Amu *et al.* (7). These studies favor the concept that chronic infections, drive strong immunoregulatory processes in which IL-10-producing B cells seem to be important players (reviewed in (24)).

However, several reports have indicated that B cells can also suppress inflammation via IL-10-independent mechanisms. In a colitis model, mesenteric B cells were capable of reducing CD4^{+} T cell-dependent colon inflammation (25) and mesenteric CD23^{hi} B cells of *H. polygyrus*-infected mice were capable of inhibiting inflammation both in an EAE model and in an HDM-specific AAI model via an unknown mechanism (26). Furthermore, studies in *S. mansoni*-infected µMT mice, where the lack of B cells led to increased liver pathology have suggested the involvement of FcR-dependent mechanisms (13). Although their mode of action is still unknown, we describe here that transferred pulmonary B cells also protected against AAI independently of IL-10 and Treg cells. The putative role of antibodies or interaction with FcR has not been studied yet.

Further, we characterized the phenotype and function of the dominant IL-10-producing B cell subset, finding a regulatory function for CD1d^{hi} MZ B cells in the spleen of schistosome-infected mice. These data are in line with several other studies in auto-immunity models pointing towards MZ B cells as regulators of type 1 inflammation in SLE, ACAID or CHS (25;27). In addition, it was shown that CD1d^{hi}-expressing splenic B cells reduced inflammation in a chronic colitis model (25) and AAI during 'worm only'-schistosome infection (7). However, within the spleen, other regulatory subtypes have been suggested; a rare CD1d^{hi}CD5^{+} B cell subset, termed B10 cells, which are capable of downregulating inflammatory responses in a number of different auto-immune or contact hypersensitivity models (15) and transitional type 2 MZ precursor B cells (CD21^{hi}CD23^{hi}IgM^{hi}), described in mouse models for CIA (22;25) and SLE (28). It is not fully clear whether these B cells are complete unique subsets because there is a substantial overlap between the (co-)expression of various markers, such as CD1d, CD5, CD21, CD23 and IgM. In addition, local inflammation or chronic infection may change the expression of individual markers complicating distinctions between the different proposed cell subsets.

Here we show that helminth-induced MZ B cells not only reduced allergic inflammation via IL-10 but also via the induction/recruitment of active FoxP3^{+} Treg cells. In humans, Treg cells are known to be an important element in reducing

allergic inflammation as a mutation in the FOXP3 gene is associated with severe eczema, food allergy and high levels of IgE and eosinophilia (29). Furthermore, children with asthma show quantitative and functional impairment of CD4^{+}CD25^{+} Treg cells in the BAL fluid (30), while children that have outgrown their allergy have increased frequencies of allergen-responsive Treg cells (31;32). Therefore, having a system that embraces not only one regulatory system but in fact two seems to be a very efficient strategy to develop tolerance to environmental stimuli and prevent allergy. Indeed, in several studies Breg function has been linked to induction or recruitment of Treg cells, i.e. in a model of ACAID, colitis, EAE, SLE (24;25) and allergic inflammation (7). Importantly, in these studies including the one presented here, Breg-induced immune regulation does not fully depend on Treg cell activity, with the exception of the study by Amu *et al*. (7), where in fact an excess of regulatory CD1d^{hi} B cells was transferred to recipient mice on three consecutive days, in contrast to our study with only one B cell injection before the challenge.

Although the majority of studies on Breg cells have been conducted in mouse models there are now a few reports that confirm the existence of human Breg cells, in which both equivalents of already described 'mouse' Breg cells are identified in addition to some new subsets. Correale *et al*. have reported human IL-10-producing CD1d^{hi} B cells in helminth-infected MS patients (18). However, these patients were infected with a mixture of different helminth species. Here, we now have established a causal relation between a single species of helminth, schistosomes and increased levels of IL-10-producing CD1d^{hi} B cells, which were reduced to 'normal' levels after anti-schistosome treatment. Of note, in both schistosome-infected and uninfected children, other geohelminths were found and for ethical reasons both groups were treated with the antihelmintic albendazole in addition to treatment with praziquantel for the schistosome-infected group. Despite their initial presence, we find reduced numbers of CD1d^{hi} B cells after treatment only in the schistosome-infected group but not in the schistosome-uninfected group. These data suggest that schistosomes have a more dominant effect on Breg cell induction than other gut-associated helminths. So far, two other human Breg subsets have been described, namely CD24^{hi}CD27^{+} (17) and CD24^{hi}CD38^{hi} Breg cells in healthy individuals, of which the activity of the latter was impaired in SLE patients (16). Interestingly, we did not observe differences in these two Breg cell subsets in peripheral blood of infected versus uninfected children, indicating that schistosomes primarily induce CD1d^{hi} Breg cells. This discrepancy suggests that the various Breg cell subsets described so far may require different conditions for their development and activation.

As illustrated above, evidence from animal studies and a few human studies points towards a significant role for IL-10-producing Breg cells in modulating pathogenic hyperinflammatory responses. As such, it would be of great therapeutic interest if Breg cell activity could be specifically induced. For this, helminth infections may be of particular value, as *in vitro* exposure of splenic B cells to live schistosome worms or peritoneal injection of schistosome egg-

derived glycans or filarial glycoproteins induces IL-10-producing B cells (7;33). However, the identification of the exact helminth-derived molecules involved, is a critical step as enhanced activity of Breg cells may form a valuable new target for therapy of rhinitis and/or allergic asthma.

Acknowledgements
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Supplementary figures

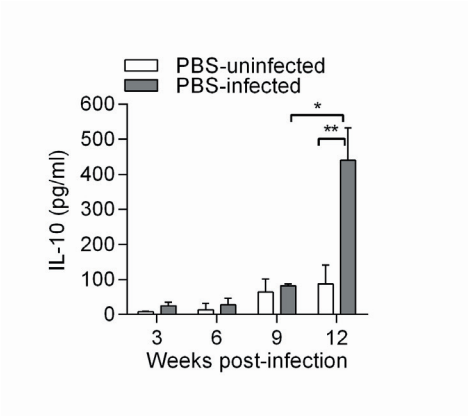


Figure S1. IL-10 production by CD19^{+} B cells during infection. Mouse CD19^{+} B cells were isolated from the spleen at different time points during *Schistosoma mansoni* infection. The B cells were cultured in the presence of SEA from *S. mansoni* eggs (20 µg/mL) for five days. Supernatants were stored for IL-10 analysis by ELISA. This experiment represents one experiment with 3-4 mice per group.

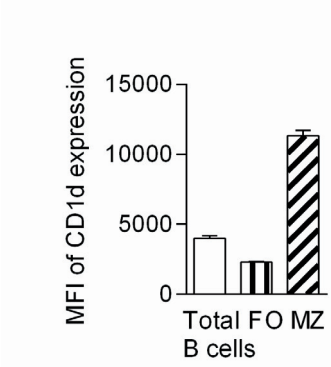


Figure S2. Geometric mean of CD1d fluorescence intensity on total B cells, FO and MZ B cells.

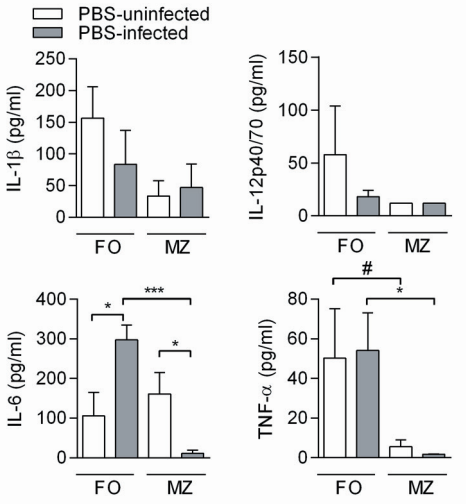


Figure S3. Production of cytokines after SEA stimulation. MZ and FO B cells from PBS-uninfected and PBS-infected were sorted using flow cytometry and cultured for five days in the presence of SEA for IL-10 production as presented in Fig. 2A. In addition, we measured IL1-β, IL-12p40/70, IL-6 and TNF-α using Luminex.

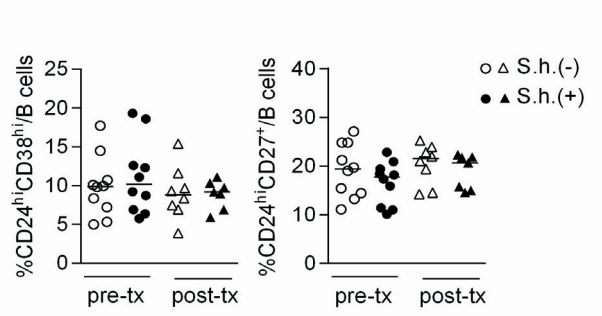


Figure S4. Percentage of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cells in peripheral blood of Gabonese children pre- and post-treatment, performed as described in legend to Fig. 6B.

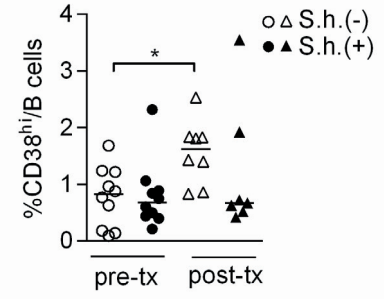
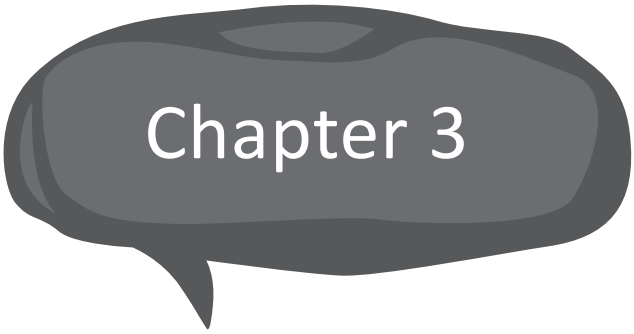


Figure S5. Percentage plasmablasts in peripheral blood of Gabonese children pre- and post-treatment, performed as described in legend to Fig. 6B.



Schistosome-induced pulmonary B cells inhibit allergic airway inflammation and display a reduced Th2-driving function

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Manuscript in preparation

Abstract

Chronic schistosome infections protect against ovalbumin (OVA)-induced allergic airway inflammation (AAI) via the induction of IL-10-producing splenic regulatory B (Breg) cells. Previous experiments have demonstrated that schistosome-induced pulmonary B cells can also reduce AAI, but they do not act via IL-10. We now have further characterized these protective pulmonary B cells phenotypically and in their inhibitory capacity.

We excluded a role for the inhibitory receptor FcγRIIB or Treg cell induction as putative AAI-protective mechanisms by schistosome-induced pulmonary B cells. However, schistosome-induced B cells showed an increased expression of CD86 and reduced cytokine response to TLR ligands compared to control B cells. To investigate the consequences for T-cell activation, we cultured OVA-pulsed schistosome-induced B cells with OVA-specific transgenic T cells and observed less Th2 cytokines and T-cell proliferation compared to control conditions. This effect was still there when sufficient co-stimulation or antigen-presentation was provided by anti-CD3/28, suggesting that schistosome infections may hamper B cells in their T(h2)-cell-stimulatory capacity and induce inhibitory molecules or receptors that can suppress Th2 cytokine production.

These data suggest that schistosome-induced pulmonary B cells have a reduced capacity to support T(h2) cytokine responses which may be achieved by the expression of inhibitory molecules and which is not dependent on their APC function.

Introduction

Chronic infections with *Schistosoma (S.) mansoni* are associated with immune hypo-responsiveness and an enhanced regulatory network (1;2). One of the regulatory cell types induced by schistosomes are regulatory B (Breg) cells and they are characterized by an enhanced production of IL-10 (3-5). They were first demonstrated in mouse models, where the absence of B-cell-derived IL-10 resulted in exacerbation of auto-immune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), lupus, or chronic colitis (6-11). Interestingly, also helminth-induced Breg cells can inhibit inflammation and were shown to protect against EAE and allergies such as systemic fatal anaphylaxis and OVA- or Derp1-induced allergic airway inflammation (AAI) (12-16). Part of the Breg cell activity was explained by their capacity to induce another regulatory cell type, i.e. Treg cells (3;14;15;17).

Murine IL-10-producing Breg cells were mostly detected within splenic B-cell subsets (18-20). However, some studies suggested that Breg cells may also reside within a mesenteric B-cell population, highly expressing the low-affinity IgE Fc-receptor CD23 during *Heligosomoides polygyrus* infection (16), or expressing the membrane-bound molecule T-cell Ig domain and mucin domain protein-1 (Tim-1), important in allograft survival (21) and controlling auto-immune (22) or allergic diseases (23). Furthermore, high CD25-expressing B cells were linked with Breg activity in humans (24;25) and in mice with inflammatory bowel disease (IBD) (26). Lastly, TGF-β-producing Breg cells controlled inflammation in inhalation tolerance (27) or diabetes models (28).

Recently, a number of alternative Breg cell suppressive mechanisms has been identified such as production of immunoglobulins with inhibitory actions. For example, an inhibitory role in OVA-induced AAI has been suggested for IgG1 ligating the inhibitory FcγRIIB, the only FcR with an immunoreceptor tyrosine-based inhibitory motif (ITIM) (29). Likewise, helminths can restrict excessive inflammatory responses during chronic infection is via the induction of polyclonal IgG molecules (30-32).

Another alternative function of Breg cells includes their capacity to suppress T-cell proliferation and cytokine production via cell-cell interactions that involve inhibitory molecules, or the induction of apoptosis. Examples of such inhibitory membrane-bound molecules are PD-1 (on T cells) and its two ligands, PD ligand 1 (PD-L1) and PD-L2 (on antigen-presenting cells (APCs)). Murine PD-1 deficiency resulted in spontaneous autoimmune diseases (33) and PD-L1 was shown to regulate Th-1 mediated immune responses, while PD-L2 was more involved in regulating mucosal and Th2 responses such as in asthma (34-36). Also during helminth infection, PD-L1 and PD-L2 were shown to be upregulated and at least important for the induction of Th2 cell exhaustion (37;38). Breg-driven apoptosis of $CD4^+$ T cells was observed during *Schistosoma* infection and

cockroach-induced asthma by Fas ligand (FasL) expressing $CD5^+$ B cells from the spleen or lungs, respectively (39;40).

B cells can also manipulate T-helper cell responses via antigen-presentation (41-43) and/or their expression of co-stimulatory signals CD80 and CD86, ligating stimulating CD28 or inhibitory CTLA-4 receptor on T cells, resulting in T cell proliferation or inhibition (44). For example, down-regulation of B-cell CD80 and CD86 expression during *Brugia pahangi* larvae infection restricted T-cell proliferation (45). In contrast, using B-cell $B7^{-/-}$mice, expression of CD86, but possibly also CD80, was essential for B-cell-mediated recovery of EAE (46), and human $CD25^{high(hi)}$ Breg cells increased CTLA-4 expression on $FoxP3^+$ Treg cells *in vitro* (24), suggesting that the interaction of some B-cell co-stimulatory molecules and CTLA-4 could be important in controlling inflammation.

We have previously shown that *S. mansoni*-infected mice are protected against OVA-induced AAI and that both splenic and pulmonary B cells from infected mice were able to transfer protection against AAI to OVA-sensitized mice (15;47). An intriguing finding in our earlier study was that splenic B cells inhibited AAI via IL-10 and the induction of Treg cells, while pulmonary B cells essentially acted in an IL-10-independent manner (15). In the current study, we aim to further explore the effector mechanism by which pulmonary B cells can protect against AAI. We demonstrate here that B cells from OVA-sensitized and -challenged mice which were infected with schistosomes do neither share the markers nor the function of splenic regulatory B cells, but have a reduced cytokine response to TLR ligands and reduced capacity to prime T cell into Th2 cells, which was independent of their antigen-presentation capacity, suggesting the involvement of other, yet unidentified, suppressive molecules.

Materials and Methods

Animals

Six week-old female C57BL/6 OlaHsd mice were purchased from Harlan. DEREG (DEpletion of REGulatory T cells) mice were kindly provided by Dr. T. Sparwasser and bred in the animal facilities of LUMC (48). FcγRIIB(CD32)-deficient mice of a C57BL/6J background were kindly provided by J. Sjef Verbeek (49). Mice were housed under SPF conditions in the animal facilities of the LUMC, Leiden, the Netherlands. All animal studies were performed in accordance with the guidelines and protocols (DEC-11166, 12182) approved by the Ethics Committee for Animal Experimentation of the University of Leiden, The Netherlands.

Parasitic infection and AAI induction

Mice were infected percutaneously with 36 *S. mansoni* cercariae and lasted until the chronic phase of infection (15 weeks) (15;47). For AAI induction, mice were sensitized twice by i.p. injections of OVA (10 μg/mL, Worthington Biochemical Corp) in Imject Alum (2 mg/ml; Pierce) at week 13 and 14. Seven days after the last injection, mice received OVA aerosol challenges (10 mg/ml in PBS) for three consecutive days. Mice were sacrificed 24 hours after the last challenge. BAL fluids were collected and phenotyped by flow cytometry (15;47).

Mouse cell purification and cell sorting

Perfused lungs were minced to ~1 mm pieces and digested by collagenase III (Worthington) and Dnase for 1 hour in 24-well plates (Greiner Bio-One). The digested lungs were sequentially dispersed through 70-μm sieves. Erythrocytes were removed from the lung single cell suspensions by lysis. Adhesive cells were removed from cell suspensions by passage over LS columns (Miltenyi Biotec). Next, B cells were purified using anti-CD19 MicroBeads (Miltenyi Biotec). B cells were stained with antibodies against CD23-PeCy7 (eBioscience) and separated using FACSAriaII cell sorting (BD). The sorted subsets were routinely ~95% pure. Untouched splenic $CD4^+$ T cells were enriched using MicroBeads (Miltenyi Biotec) and ~95% pure.

Adoptive transfer of isolated pulmonary B cells

Recipient mice were sensitized with two injections of OVA/Alum at day 0 and day 7, as described above. Ten days after the last injection, the OVA-sensitized animals received an i.v. injection of 5 x 10^6 $CD19^+$ B cells from OVA-uninfected or OVA-infected mice or PBS as a control. DEREG mice were treated with two diphtheria toxin (DT, 1 μg/ml) i.p. injections or PBS as a control: one day before and two days after the adoptive transfer of B cells in order to deplete the $FoxP3^+$ Treg cells. After two days, mice were challenged for three consecutive days and sacrificed 24 hours after the last challenge.

Phenotypic characterization

Ex vivo pulmonary B cells were characterized using: CD25-FITC, CD86-PerCP5.5 (both BD) CD40-PE (eBioscience), Tim-1-PE, B220-V510, PD-L1-PeCy7 (all Biolegend), LAP-1-PerCPeFluor710, CD23-PeCy7, CD80-APC, FasL-APC, MHC Class II-APC-Cy7, B220-APCCy7, life/dead marker Violet 450, and PD-L2 Biotin (all eBioscience) combined with streptavidin-Qdot525 (Life Technologies). For all flow cytometric measurements, FcγR-binding inhibitor (2.4G2) was added and FMOs were used for gate setting for all surface markers and cytokines.

***In vitro* B cell stimulation**

Pulmonary $CD19^+$ B cells and B cell subsets ($1x10^5$ cells) were cultured in medium (RPMI 1640 glutamax; Invitrogen Life Technologies), containing 5% heat-inactivated Fetal Bovine Serum (FBS, Greiner Bio-One), 5×10^{-5} M 2-Mercaptoethanol (Sigma-Aldrich) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Invitrogen), in the presence of LPS (100 ng/ml) or CpG1826 (5 µg/ml) for five days. Supernatants were stored for later cytokine analysis by IL-10 and IL-6 ELISA (BD).

Immunoglobulin measurements

Total and OVA-specific IgG1 and IgG2a and total IgA were measured from the first 1 ml of collected BAL fluid using ELISA kits (BD).

***In vitro* B cell stimulation and co-culture with $CD4^+$ T cells**

Pulmonary $CD19^+$ B ($1x10^6$/ml) cells were loaded with 10 µg/ml OVA17 peptide ($OVA_{323-339}$: ISQAVHAAHAEINEAGR, kindly provided by M.G.M. Camps) for 1 hour at 37 ° C, washed, and subsequently co-cultured with OT-II $CD4^+$ T cells ($1x10^5$ cells/well) at 1:1 ratio in the presence or absence of anti-CD28 (1 µg/ml). Additionally, $CD19^+$ B cells were co-cultured with $CD4^+$ T cells at 1:1, 1:2, 1:4 ratio, in the presence of medium or anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml). To assess proliferation, T cells (10×10^6/ml) were incubated with CFSE (0.5 µM) for 15 min. In some conditions, the following blocking antibodies were added to the cultures: 10 µg/ml isotype control anti-βGal and anti-TGF-β (kindly provided by L. Boon). T cells were incubated for 30 minutes at 37 °C with 10 µg/ml anti-IL-6 receptor-α/CD126 (eBioscience) or 10 µg/ml anti-IL-10 receptor ((kindly provided by L. Boon). After three days, CFSE-labelled T cell co-cultures were stained with anti-CD3-eFluor450, CD25-PE, B220-eFluor780, 7-AAD, CD4-biotin (all eBioscience) with streptavidin-Qdot525 to measure T-cell proliferation of activated T cells. For cytokine analysis, the cells were restimulated with 100 ng/ml PMA and 1 µg/ml ionomycin for six hours in the presence of 10 µg/ml Brefeldin A (all Sigma-Aldrich) for the last four hours, followed by fixation using 1.9% PFA (Sigma-Aldrich). Next, the cells were staining for IL-4-PE (BD), CD3-eFluor710, IFN-γ-FITC, IL-17-PeCy7, IL-10-APC, IL-13-eFluor450, and B220-eFluor780 (all eBioscience).

Apoptosis measurement

After 24 and 72 hours of co-culture of pulmonary B cells and $CD4^+$ T cells, T cells were stained with anti-CD3-eFluor450, 7-AAD, B220-eFluor780 (all eBioscience), CD25-FITC (BD), CD4-biotin with streptavidin-Qdot525, and AnnexinV-PE (BD) for 30 min at 4 °C in AnnexinV staining buffer (BD). Dead cells were removed from analysis on the basis of $7\text{-}AAD^+$ staining.

Statistical analysis

All results are expressed as mean ± SEM and were tested using the independent and paired Student's *t*-test (two-tailed). Probability values less than 0.05 were considered significant.

Results

Phenotypic characterization of schistosome-induced pulmonary B cells

We first set out to investigate whether a specific pulmonary B-cell subset or the expression of specific surface markers linked to Breg cell activity were selectively expanded during schistosome infection compared to uninfected mice. Pulmonary B cells did not contain typical Breg populations that have been described in the spleen, such as $CD1d^{hi}(CD5^{+})$, $CD21^{hi}CD23^{lo}$ MZ or $CD1d^{hi}CD21^{hi}CD23^{hi}IgM^{hi}$ transition type 2 MZ B cells (less than 0.5% during infection). Therefore, we analyzed several other cell-surface markers as putative markers of Breg cell activity, i.e. the membrane-bound marker latency-associated peptide (LAP), as part of a latent TGF-β complex, Tim-1, CD23 and CD25 (16;21-23;27;28;50). Interestingly, pulmonary B cells from chronically *S. mansoni*-infected, OVA-sensitized and -challenged (OVA-infected) mice expressed similar levels of LAP-1 (3%) and CD25 (0.4%) as found on B cells from uninfected OVA-sensitized and challenged (OVA-uninfected) control mice (Fig. 1A), suggesting that TGF-β- or CD25-expressing B cells are probably not involved in protection. Although the expression of Tim-1 was slightly increased, the overall expression on schistosome-induced pulmonary B cells remained rather low (MFI of 115 compared to an MFI of 79 on B cells from OVA-uninfected mice). However, the one marker that was clearly enhanced on a large subset of pulmonary B cells in OVA-infected mice was CD23 (approximately 80%) (Fig. 1B). To investigate whether this subpopulation was responsible for the protection against allergy, we sorted $CD23^{low/intermediate(int)}$ or $CD23^{hi}$ B cells from OVA-infected mice and transferred those cells to OVA-sensitized mice followed by OVA challenge. However, the experiments remained inconclusive so far as the outcome varied (data not shown). Though we did observe that $CD23^{low/int}$ B cells showed a more anti-inflammatory cytokine profile compared to $CD23^{hi}$ B cells, as they produced higher levels of IL-10 upon LPS or CpG stimulation (Fig.1C and D). It still remains to be established whether this is decisive for their capacity to inhibit AAI or not.

Role of FcγRIIB ligation and immunoglobulins in protection against AAI by pulmonary B cells

To investigate the role of immunoglobulins (Igs) in protection against AAI, we analyzed several Ig subclasses and detected a general elevated production of IgG1 and IgG2a antibodies in the BAL fluid of OVA-infected mice compared to OVA-uninfected mice, though this was not reflected in increased OVA-specific antibody responses, as described before (47) (Appendix S1A). To investigate whether the secretion of schistosome-induced IgGs by pulmonary B cells could induce protection against AAI via the FcγRIIB receptor in a similar fashion as described for OVA-specific IgGs (29;51), we transferred pulmonary B cells from OVA-uninfected and -infected mice into OVA-sensitized $FcγRIIB^{-/-}$ mice followed by OVA challenge. However, the loss of the FcγRIIB receptor did not restore AAI

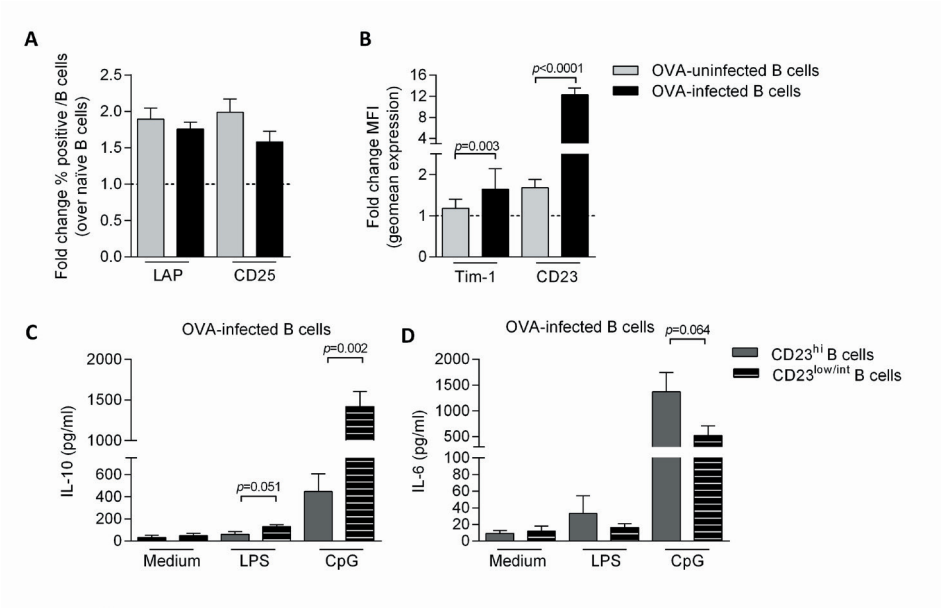

Figure 1. Characterization of Breg cell markers in schistosome-induced pulmonary B cells and inflammatory responses of CD23-sorted B cells after TLR ligation. Mice were infected with *S. mansoni* until the chronic phase (week 15). After sacrifice, the perfused lungs were minced, digested and the single cell suspension from 2-3 mice were pooled. Next, B cells were purified using anti-CD19 MicroBeads and stained for different Breg-linked markers. (A) The fold change of percentage surface LAP-1- and CD25-expressing B cells from OVA-uninfected and OVA-infected mice over control (PBS-uninfected B cells). Data are pooled from 4 independent experiments with 3-5 mice per group/experiment. (B) The fold change of geometric mean fluorescence intensity (MFI) of CD23 and Tim-1 expression over control (PBS-uninfected B cells). Data are pooled from 4 independent experiments with 3-5 mice per group. (C) OVA-infected B cells were sorted using flow cytometry based on the expression of CD23. Next, the cells were stimulated with medium, LPS (100 ng/ml) or CpG (5 µg/ml) for five days to determine (C) IL-10 and (D) IL-6 levels in the culture supernatant.

upon pulmonary B cell transfer, suggesting that despite the elevated IgG1 and IgG2a secretion in OVA-infected mice, protection against AAI was not mediated via signaling through FcγRIIB (Appendix S1B).

Schistosome-induced pulmonary B cells do not drive protection against AAI via the induction of Treg cells

One of the major effector functions of murine Breg cells centers around the induction and/or recruitment of FoxP3$^+$ Treg cells. In our previous studies, we observed that adoptive transfer of pulmonary B cells did not induce increased numbers of FoxP3$^+$ Treg cells *in vitro* nor *in vivo* (15). However, this does not exclude the possibility that, despite equal numbers, the activity of Treg cells on a per cell basis had increased. Therefore, to investigate the role of Treg cell activity, we transferred pulmonary B-cells to FoxP3-DTR transgenic DEREG mice, in which Treg cells can be temporarily depleted by DT injections and, thus allows the investigation of the contribution of Treg cell activity during pulmonary B cell-

induced protection against AAI. However, BAL eosinophil levels remained similarly reduced in both PBS- and DT-treated DEREG mice when receiving pulmonary B cells from OVA-infected compared to OVA-uninfected mice (Fig. 2). These data indicate that AAI is not restored when Treg cell activity is abolished, suggesting that schistosome-induced pulmonary B cells do not drive protection against AAI via enhanced Treg cell activity.

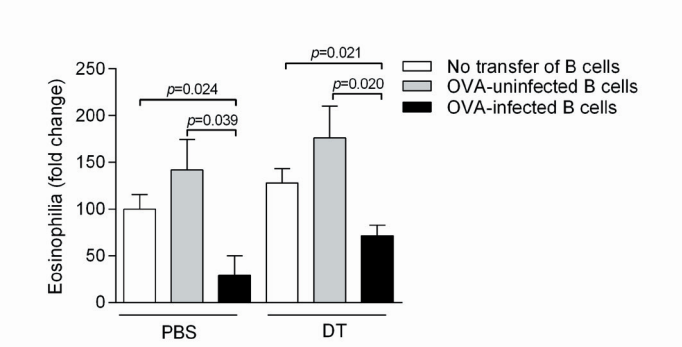

Figure 2. Role of FoxP3$^+$ T cells in pulmonary B cell-induced protection against AAI during schistosomiasis. OVA-sensitized DEREG mice, which carry a diphtheria toxin receptor-eGFP transgene under the control of an additional Foxp3 promoter, were treated with two PBS or diphtheria toxin (DT, 1 µg/ml) i.p. injections: one day before and two days after the adoptive transfer of B cells in order to deplete the FoxP3$^+$ Treg cells. After two days, mice were challenged for three consecutive days and sacrificed 24 hours after the last challenge. The number of BAL eosinophils in the PBS-injected DEREG mouse group was set at 100. Fold changes in numbers of eosinophils was calculated for the other groups. This graph represents two independent experiments.

TLR-responsiveness and antigen presentation by schistosome-induced pulmonary B cells

We next aimed to investigate the role of pulmonary B cells as APCs and stimulators of effector T-cell activation. Important signals that can influence T-cell activation, proliferation and cytokine production are provided by e.g. co-stimulatory molecules CD80 or CD86, antigen-presentation molecule MHCII, inhibitory receptors such as PD-L1, PD-L2, apoptosis-inducing FasL or various cytokines such as IL-10 and IL-6. To investigate a putative role for those (co-)stimulatory molecules and/or inhibitory receptors on schistosome-induced pulmonary B cells, we analyzed the above mentioned molecules (Fig. 3A and 3B). Pulmonary B cells from OVA-infected mice showed a significantly increased CD86 expression compared to B cells from OVA-uninfected mice (Fig. 3A). Expression levels of MHCII, CD80, PD-L1 and PD-L2 were equal between the groups (Fig. 3A), while the percentage of FasL-expressing cells was significantly reduced in OVA-infected mice compared to OVA-uninfected mice (Fig. 3B). Furthermore, we analyzed the capacity of pulmonary B cells to produce cytokines, which may support or suppress T-cell activation following stimulation by Toll-like receptor (TLR)-4 ligand LPS and TLR-9 ligand CpG1826 as being strong B-cell activators (Appendix S2). Interestingly, B cells from OVA-infected mice produced significantly less IL-10 and

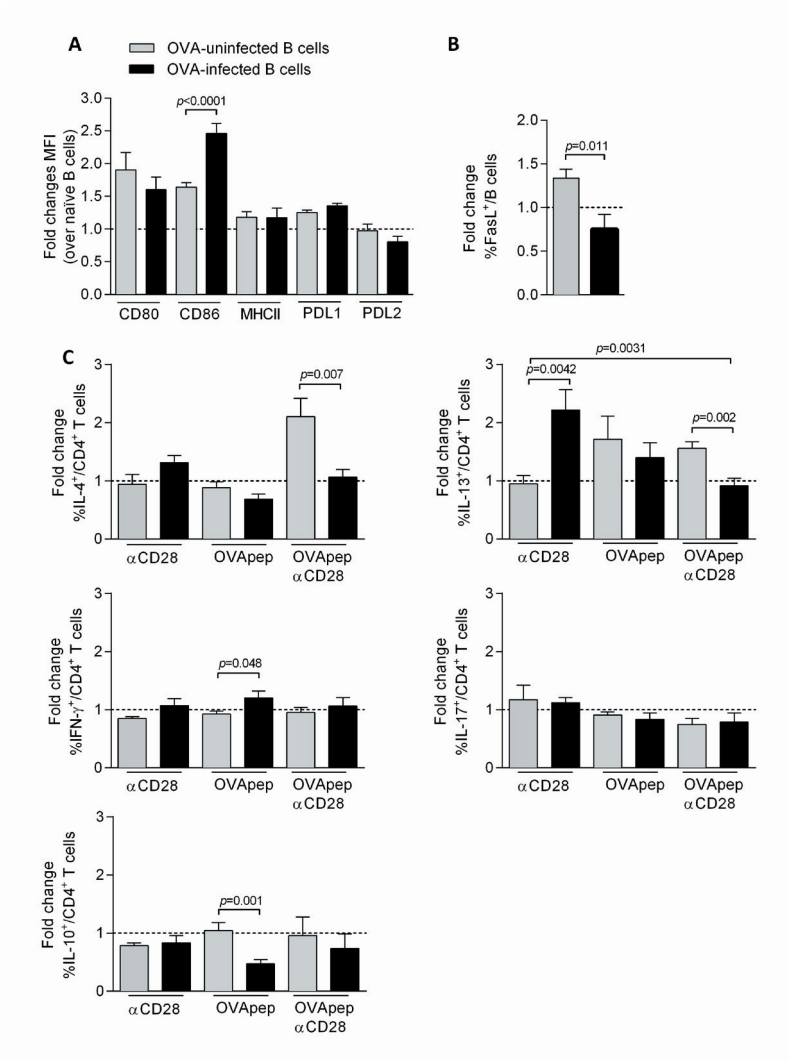

Figure 3. Schistosome-induced pulmonary B cells show elevated expression of CD86 and an impaired capacity to drive Th2 cytokines. Pulmonary B cells were isolated as described in figure 1. For both characterization and co-culture, pulmonary B cells from 2-3 mice were pooled to obtain enough cells for performing experiments. (A) Fold changes of geometric MFI expression of activation, co-stimulation, and antigen-presentation molecules on B cells from OVA-uninfected and –infected mice over control (PBS-uninfected B cells). Data are pooled from 4 independent experiments with 3-5 mice per group/experiment. (B) Fold change of percentage Fas ligand-expressing B cells in OVA-uninfected and –infected mice over control (PBS-uninfected B cells). Data are pooled from 4 independent experiments with 3-5 mice per group/experiment. (C) B cells were loaded with OVA17 peptide, washed and co-cultured 1:1 with OVA-specific CD4+ T cells in the presence or absence of anti-CD28 (1 µg/ml) for three days. As control, non-loaded B cells were cultured with T cells with anti-CD28 only. Intracellular production of Th2 cytokines IL-13, IL-4 and IL-10, Th1 cytokine IFN-γ and Th17 cytokine IL-17 cells was determined after co-culture with B cells following PMA/Ionomycin and Brefeldin A stimulation for all groups. Data are expressed as fold change over co-culture with B cells from PBS-uninfected mice. This figure represents pooled data from 3 independent experiments with 3-5 mice per group/experiment.

IL-6 in response to LPS and/or CpG compared to B cells from OVA-uninfected mice. Taken together, these data show that during chronic schistosomiasis pulmonary B cells have an increased expression of CD86, equal levels in MHCII and CD80 and lower IL-10 and IL-6 production upon TLR ligation, suggesting that pulmonary B cells may be modulated in their function as APCs.

Schistosome-induced pulmonary B cells reduce Th2 cytokine secretion *in vitro*

To assess the quality of schistosome-induced pulmonary B cells as APCs, T-cell activation was examined in a condition where antigen-presentation by B cells was essential to drive T-cell activation. To this end, we investigated OVA-specific T-cell activation of $CD4^+$ T cells from OT-II mice by OVA peptide-pulsed pulmonary B cells. After three days, OVA-presentation by B cells from OVA-uninfected or –infected mice did not significantly affect T-cell IFN-γ (3.6 vs. 3.9%), IL-17 (1.3 vs.1.6%) or IL-4 production (3.5 vs 2.1%). In contrast, IL-10 secretion (1.5 vs. 0.8%) was slightly down-modulated in co-cultures with OVA-infected B cells, while IL-13 secretion (1.3 vs 2.5%) was increased in cultures with OVA-infected B cells. We also cultured OVA-pulsed B cells and OT-II T cells in the presence of anti-CD28 to bypass differences in CD80 and CD86 and ensure optimal co-stimulation. In the presence of anti-CD28, OVA-presentation by B cells from OVA-uninfected mice increased mostly Th2 cytokines IL-4 (6.8 vs. 3.2%) and IL-13 (10.6 vs. 6.2%), while these cytokines were not induced in co-cultures with B cells from OVA-infected mice (Fig. 3C). The reduced IL-10 production found in stimulation conditions of OVA-infected B cell co-cultures was overcome in the presence of sufficient co-stimulation and may point at a hampered expression during sub-optimal stimulation. The observed reduction in Th2 cell cytokines despite, or maybe as a consequence of, the presence of optimal co-stimulation, may point at an active expression of inhibitory molecules by schistosome-induced pulmonary B cells.

Role of inhibitory molecules in the reduction of Th2 cytokines

We next aimed to investigate what suppressive factors were involved in the above described reduced capacity of schistosome-induced pulmonary B cells to induce Th2 responses. Therefore, we performed co-culture experiments of pulmonary B cells from OVA-uninfected and –infected mice with $CD4^+$ T cells from naive C57BL/6 mice in the presence of anti-CD3/28 to bypass MHCII stimulation and insufficient co-stimulation and cultured them for three days. We still observed higher Th2 cytokine production in co-cultures with B cells from OVA-uninfected mice, but also more T-cell CD25 expression and T-cell proliferation compared to co-cultures with OVA-infected B cells (Fig. 4A-C). The ability of B cells of OVA-allergic mice to induce Th2 cytokines was found to be dose-dependent and most effective at a 1:1 ratio (Fig. 4D), while the level of Th2 cytokines by OVA-infected B cells was similarly low at all indicated ratios. It thus may be argued that schistosome-induced pulmonary B cells can actively suppress Th2 cytokine production by additional factors.

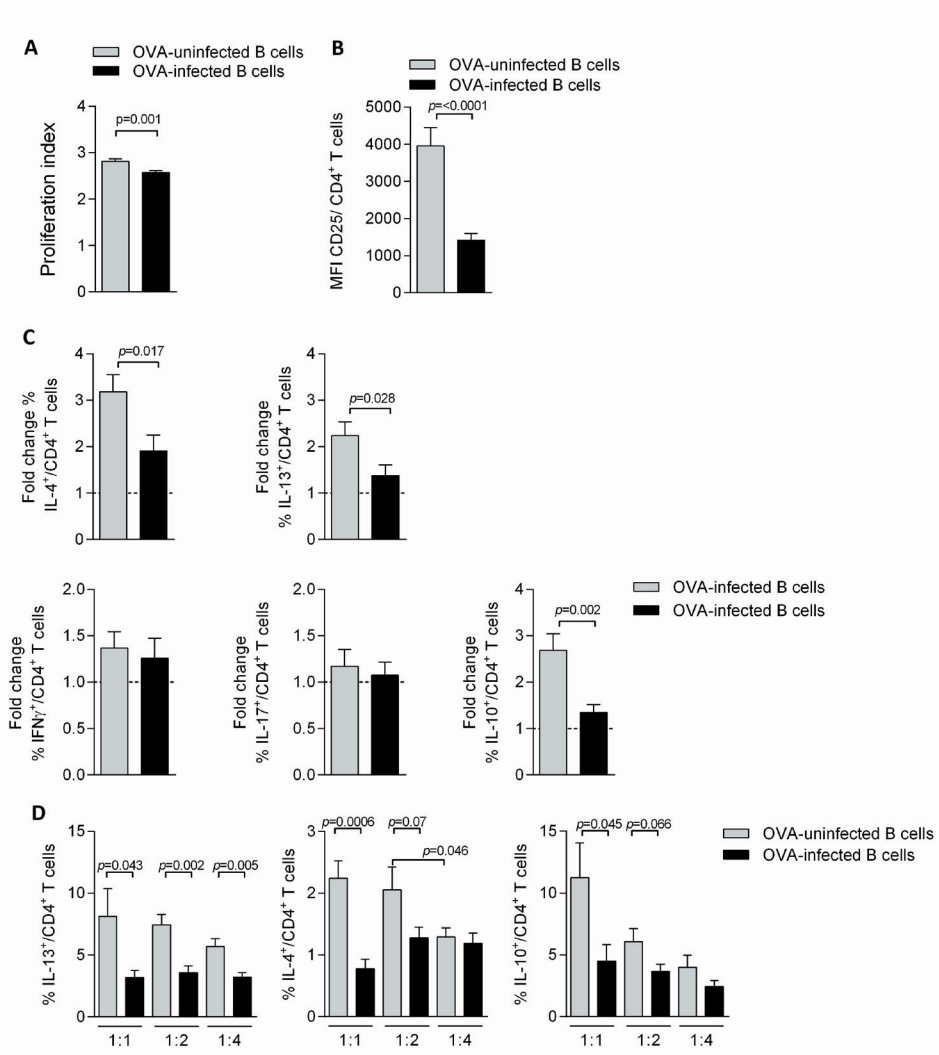

Figure 4. Role of inhibitory molecules in pulmonary B-cell mediated Th2 suppression. (A) Pulmonary B cells (1x10^5), pooled from 2-3 PBS-uninfected, OVA-uninfected, PBS-infected or OVA-infected mice, were cultured with C57BL/6 CD4$^+$ T cells (1x10^5) from naive mice for three days in the presence of anti-CD3/ CD28. (A) The proliferation of T cells using CFSE staining was measured. The graph shows cumulative data of three independent experiments. (B) The expression of CD25 on T cells after three days of co-culture. (C) Fold changes of percentage IL-4, IL-13, IL-10, IL-17 and IFN-γ-producing T cells over control (culture with PBS-uninfected B cells) was analyzed. Graphs contain 4 independent experiments. (D) 1x10^5 T cells were cultured in 1:1, 1:2 and 1:4 (B cell:T cell) ratios with B cells from OVA-uninfected and –infected mice. Bar graphs represent percentage Th2 cytokines production. The graph expresses results from two independent experiments.

We next investigated whether apoptosis, the above described differences in cytokine production, or differential expression of cell surface markers on schistosome-induced pulmonary B cells might be involved in the suppression of Th2 cytokines by $CD4^+$ T cells. First, we evaluated the induction of $CD4^+$ T-cell apoptosis by the analysis of the early apoptosis marker Annexin V after 24 and 72 hours of co-culturing. However, no differences in T-cell apoptosis between OVA-uninfected and -infected B cells were observed (data not shown). Therefore, the role of various soluble mediators was investigated by adding blocking antibodies to the IL-10 receptor (αIL-10R), the IL-6 receptor (αIL-6R) or to TGF-β (αTGFβ) into the co-cultures of pulmonary B cells and $CD4^+$ T cells (Fig. 5). We focused on the production of IL-4 and IL-13 because of the activity of these Th2 cytokines in boosting allergic responses in the airways. However, blockage of IL-6R, IL-10R or TGF-β, only slightly, though significantly, increased the IL-4, but not IL-13 production in T cells cultured with schistosome-induced pulmonary B cells, suggesting that these cytokines, if anything, only play a very minor role because IL-13 production was not affected. Since some obvious B-cell derived cytokines tested here, are not involved in controlling Th2 cytokines by schistosome-induced pulmonary B cells, other unknown mechanisms could be involved and need to be investigated.

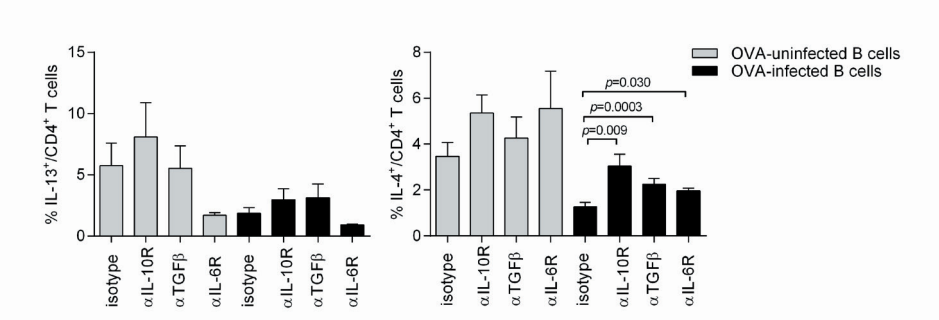

Figure 5. Role of putative inhibitory molecules in pulmonary B cell-mediated Th2 suppression. In vitro co-cultures were performed as described in figure 4 in the presence of blocking anti-IL-10R, anti-TGF-β, anti-IL-6R or isotype control antibodies. Data represents two independent experiment.

Discussion

Helminths drive strong immunoregulatory processes that limit immunopathology during chronic infection in which regulatory B cells seem to be important players. Importantly, *S. mansoni*-induced splenic and pulmonary B cells also attenuate allergic diseases such as AAI upon adoptive transfer. While splenic Breg cells mediate their suppression effect through IL-10- and Treg cell-dependent mechanisms (14;15), here we demonstrated that helminth-induced pulmonary B cells mainly have a reduced capacity to initiate Th2 cytokine responses and reduce T-cell proliferation.

By definition, Breg cells suppress inflammatory processes and induce tolerance by various mechanisms of which production of immunosuppressive IL-10 is the most widely-studied and best understood. While IL-10 has a pleiotropic suppressive effect on most hematopoietic cells, such as T cells and APCs, it also indirectly suppresses immune responses via supporting the generation and maintenance of Treg cell subsets (9;15;52). Recently, it has become evident that Breg cells utilize a number of IL-10-independent suppressive mechanisms in order to control inflammation. For example, B cells can contribute to the maintenance of tolerance via the production of TGF-β, secretory IgA, IgGs (binding to FcγRIIB (33;59)), or by induction of T-cell apoptosis (28;53).The schistosome-induced pulmonary B cells studied here did not utilize any of the Breg effector mechanisms described in the above mentioned studies. In our study, we did identify an increased population of $CD23^{hi}$ B cells in the lungs of OVA-infected mice compared to OVA-uninfected mice. Interestingly, mesenteric lymph node $CD23^{hi}$ B cells from *H. polygyrus*-infected mice were shown to suppress Derp1-induced airway inflammation independently of IL-10 (16). However, the adoptive transfer experiments of schistosome-induced $CD23^{hi}$ and/or CD23low/int pulmonary B cells into sensitized mice were inconclusive in this stage leaving it unclear whether CD23 is a marker for regulatory activity or not. Alternatively, CD23 expression may not define a specific Breg population but may be more the consequence of the local cytokine milieu (54). Indeed, strong signals to drive CD23 expression are provided by IL-4 and IgE (55;56). This is further underlined by studies showing elevated numbers of CD23-expressing B-cells during Th2 inflammation such as during helminth infections (57) or allergic asthma (58;59).

Schistosome-induced pulmonary B cells expressed enhanced levels of CD86, whereas MHC Class II and CD80 were similar. Each of these markers has been suggested to affect T-cell activation. Indeed, co-cultures of schistosome-induced pulmonary B cells presenting OVA to OVA-specific T cells resulted in reduced Th2 cytokine production compared to control conditions. However, since similar results were observed, including reduced T-cell proliferation, in co-cultures of B cells from C57BL/6 mice supplemented with anti-CD3/28 to bypass the role of B cells as APC, this may suggest the involvement of other molecules or mechanisms. An important surface molecule for the Th2 cell suppressive capacity

of schistosome-induced pulmonary B cells may be the B7 co-stimulatory molecule, CD86. Interestingly, in a TCRα KO mouse model of intestinal inflammation and EAE, CD86 has been reported to mediate suppressive effects of B cells via T cells (46;60). Furthermore, the ligation of CTLA-4 (expressed on T cells), one of the interacting receptors of CD86, was essential for T-cell hypo-responsiveness and reduced protective Th2 immunity during filarial infections (61;62) and schistosomiasis (63). However, since preliminary results showed that blocking CTLA-4 ligation did not restore Th2 cytokine production, this would exclude a potential role for CD86 in reducing Th2 cytokines, despite its enhanced expression on OVA-infected B cells.

Furthermore, studies in mice with *S. mansoni* or *Litomosoides sigmodontis* infections showed that PD-1 and interaction with its ligands (PDL-1/2) was important for T(h2)-cell hyporesponsiveness (37;64), while other studies actually suggested that Th2 hypo-responsiveness during *S. mansoni* was not related to PD-1 (65). The latter is in agreement with the data presented here, as we observed that blocking of PD-L1 or PD-L2 expression on OVA-infected B cells by blocking antibodies did not restore Th2 cytokine production or proliferation in co-cultures with T cells (data not shown).

It remains to be established what the effector mechanism is by which schistosome-induced pulmonary B cells reduce Th2 polarization and inhibit AAI. Recently, IL-35-producing B cells were described, which limited EAE by reducing the accumulation of pathogenic T cells (66;67). Here, in mice with a B-cell specific knock-out of one of the two IL-35 subunits, EBI3 or p35, B cells displayed an enhanced APC function, suggesting that IL-35 acts as a regulator of the APC function of B cells. However, IL-35 has not been studied yet during schistosomiasis and it is unclear whether this cytokine is increased and thereby may control the APC function of pulmonary B cells. Alternatively, local Treg cells, induced during helminth infection, may influence B cell function leading to reduced B-cell activation, antibody production and the APC function of B cells via e.g. TGF-β or IL-35 (68-70). Finally, secreted helminth products from *Schistosoma* may also directly attenuate the T-cell stimulatory capacity of B cells as described for DCs (71).

Here, we show that during chronic schistosomiasis the regulatory function of B cells may be affected both via the induction of a sessile splenic IL-10-producing Breg cell population in the spleen and the functional impairment of local pulmonary B cells to act as Th2-inducing APC. This suggests that in the spleen and in local tissues different B cell subsets can occur with different capacities, though eventually with the same net effect: reduction of allergic airway inflammation. In search of the most potent B cell subset, we have previously demonstrated a dominant role for IL-10-producing (splenic) B cells in the protection against AAI during a natural infection using IL-$10^{-/-}$ B cell mice (15). These data suggest that the potential of pulmonary B cells to control AAI in that particular model is not sufficient to counterbalance the loss of IL-10-producing B cells (15). However, although the functionality of these pulmonary B cells has not been investigated

in IL-10$^{-/-}$ B cell mice, it is tempting to speculate that the development of these pulmonary B cells may be dependent on local (autocrine) B-cell derived IL-10. Alternatively, the schistosome-induced pulmonary B cells could arise from a common IL-10^{+} progenitor splenic B cell that migrates to the site of inflammation. However, what argues against this is that pulmonary B cells do not resemble splenic Breg cells at all, a fact which would require a dramatic change in the B cell phenotype. Clearly, we need better markers to identify whether different AAI-suppressive splenic and pulmonary B cells arise from the same progenitors or that these pulmonary B cells are induced by the local inflammatory milieu.

Recently, various Breg cell populations have been identified in peripheral blood of humans infected with schistosomiasis or other helminths (15;72). Furthermore, in several inflammatory diseases, Breg cells were impaired in number and/or their regulatory function, i.e. in patients with SLE, RA or allergic asthma (25;73;74). Since most human studies are restricted to peripheral blood B cells, these results might not fully reflect the processes that occur in inflamed organs and B cell subsets involved may be different as suggested by the data presented here. Therefore, further studies on human B-cell biology and its activity in the inflamed organs are needed to better understand the importance and relative contribution of the various Breg cell subsets in peripheral blood and local tissues. However, what stands out is that during infection with schistosomes, potent IL-10-producing suppressive Breg cells in the spleen and impaired Th2-driving pulmonary B cells in the inflamed tissue are found which are able to suppress AAI. Identifying the mechanisms and/or molecules that influence (local) B cell function may be an interesting novel strategy to control or prevent allergic inflammatory responses at multiple sites at the same time.

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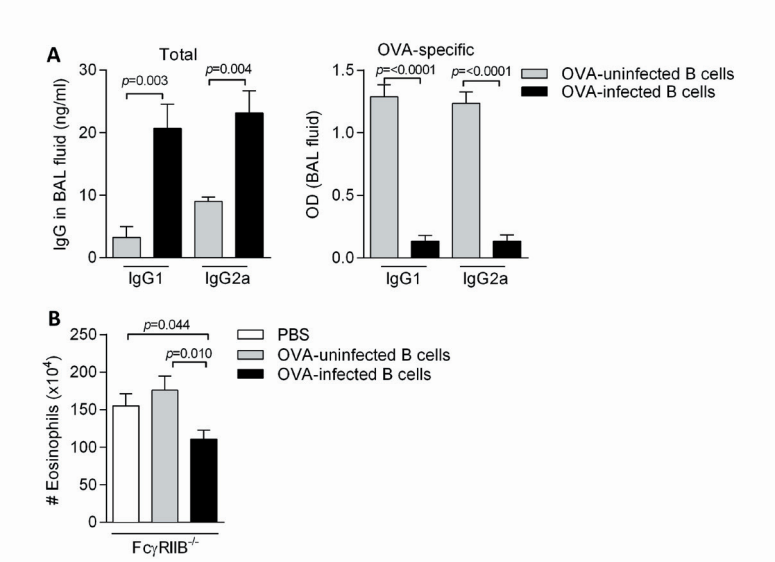
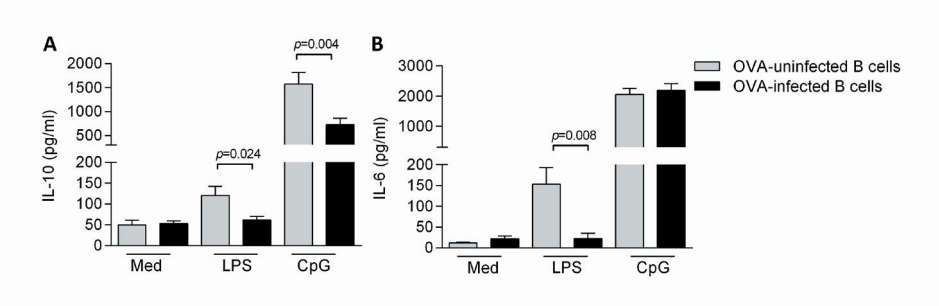
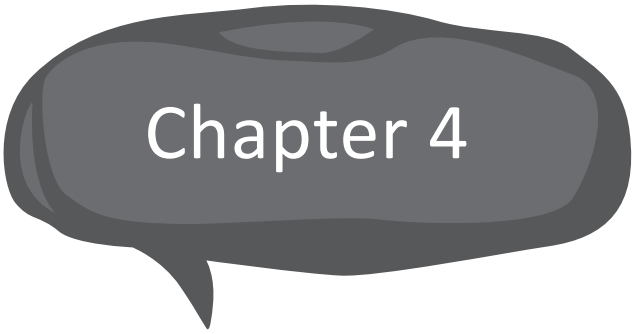
Supplementary figures



Appendix S1. Role of schistosome-induced IgG1 and IgG2a in protection against AAI. Mice were infected, OVA-sensitized and -challenged as described in figure 1. (A) Total and OVA-specific IgG1 and IgG2a were measured from the first 1 ml of collected BAL fluid using ELISA. This graph expresses one representative out of two independent experiments, consisting of five individual mice per group (B) OVA-sensitized recipient FcγRIIB-deficient or control C57BL/6 mice mice received i.v. injection of 5 x 10^6 $CD19^+$ B cells from OVA-uninfected or OVA-infected mice or PBS as control. The B cells were derived from 6-8 donor mice to obtain enough cells. This graph contains two independent experiments.



Appendix S2. OVA-infected B cells show an impaired cytokine response towards TLR ligation. OVA-uninfected and -infected B cells were stimulated with medium, LPS (100 ng/ml) or CpG (5 µg/ml) for five days to determine (A) IL-10 and (B) IL-6 responses in the supernatant.



Toll-like receptor ligation for the induction of regulatory B cells

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Abstract

Toll-like receptors (TLRs) are key components for the recognition of micro-organisms, the initiation of innate immunity and for promoting adaptive immune responses. TLR signaling in B cells, in addition to B cell receptor or CD40 ligation, plays an important role in B cell differentiation and activation. In contrast, various infectious agents and/or TLR ligands can also prime B cells to induce tolerance and downregulate inflammatory reactions; those B cells are called regulatory B (Breg) cells and are characterized by a dominant IL-10 production. Several studies have suggested that Breg cells are impaired in patients with autoimmune diseases and allergic asthma. However, the role for TLR ligands in the induction of Breg cells as a potential therapy for some of these inflammatory diseases has not yet been investigated. Here, we provide detailed instructions how to analyze and validate cytokine production in human and mouse B cells in response to various TLR ligands. Furthermore, we describe an assay to investigate the suppressive properties of TLR-induced B cells to confirm their regulatory B cell status.

1. Introduction

Toll-like receptors (TLRs) represent one of the most studied classes of pattern recognition receptors and are key components for the recognition of micro-organisms and activation of immune cells. TLR ligands have become of interest in B cell research because of their application as potent adjuvants in novel vaccine strategies. However, several reports have been published over the past years highlighting a role for TLRs in enhancing anti-inflammatory responses instead, possibly promoting the development or activation of regulatory B (Breg) cells. Several mouse studies have demonstrated a role for signaling via TLR2, TLR4, and TLR9, either or in combination with CD40 engagement, CD80/CD86 or B cell-activating factor (BAFF), to induce optimal IL-10 production by B cells with regulatory capacities (1-7). For example, during the bacterial infection by *Salmonella typhimurium*, MyD88-signaling in B cells suppressed protective immunity via IL-10, affecting natural killer cells, neutrophils, and effector T cells. Prolonged survival upon infection was displayed in mice with a B-cell specific deficiency in both TLR2 and TLR4, suggesting that the B-cell-mediated suppression resulted from direct microbial sensing by the B cells (8). Furthermore, ligation of TLR2 by ligands from *Helicobacter felis* induced IL-10-producing B cells in a MyD88-dependent manner, which suppressed helicobacter-induced pathology not through the direct production of IL-10 but via the generation of suppressive IL-10 secreting Tr1-like cells (9). Other bacterial antigens, such as the TLR4 ligand lipopolysaccharides (LPS) and the TLR9 ligand CpG oligonucleotides (ODN) were also described as strong inducers of IL-10 expression in murine B cells (10;11). Interestingly, adoptive transfer of LPS-activated B cells reduced the incidence of spontaneous type 1 diabetes for about one year in prediabetic NOD mice (12). Next to bacterial, also certain parasite-derived molecules, such as extracts from *Leishmania major* and *Brugia malayi* or milk-derived sugars comparable to the ones found in soluble egg antigens (SEA) from *Schistosoma mansoni*, induced IL-10-producing B cells most likely via TLR4 (13-15). In addition, CpG treatment of mice ameliorated spontaneous type 1 diabetes (16) and allergic conjunctivitis by TLR9 ligation and induction of IL-10 in B cells (17). These mouse studies show an important role for TLR2, TLR4 and TLR9 ligation in the development of Breg cells and highlight its potential to boost Breg cell development for treatment of various inflammatory diseases.

Human IL-10-producing Breg cells share phenotypic and functional characteristics with the murine counterpart and have been studied primarily in allergic and autoimmune diseases, where they were shown to be functionally impaired in their IL-10 response or regulatory activity (18-23). In some of the aforementioned cases, Breg cell defects also account for B cell IL-10 responses to TLR ligands. For example, CpG induced less IL-10 in B cells from patients affected by multiple sclerosis (MS) compared to healthy controls (24), which was also confirmed in autoimmune thrombocytopenia (25). Whereas TLR4 stimulation is

very potent on mouse B cells, human B cells produce rather modest IL-10 levels after exposure to LPS *in vitro* due to a relatively low TLR4 expression compared to TLR2 and TLR9 at least in healthy individuals (20;23;26-28). In several inflammatory conditions an upregulated TLR4 expression on B cells was described, suggesting a role of TLR4 activated B cells during infection (29-32). In the context of another inflammatory disease, i.e. allergic asthma, we showed that LPS-primed IL-10 from B cells of allergic asthma patients was reduced compared to that of healthy individuals and the subsequent induction of IL-10-producing T cells *in vitro* was abrogated as well (23). The impaired LPS response of B cells in allergic asthma patients may therefore play a role in the pathophysiology of asthma.

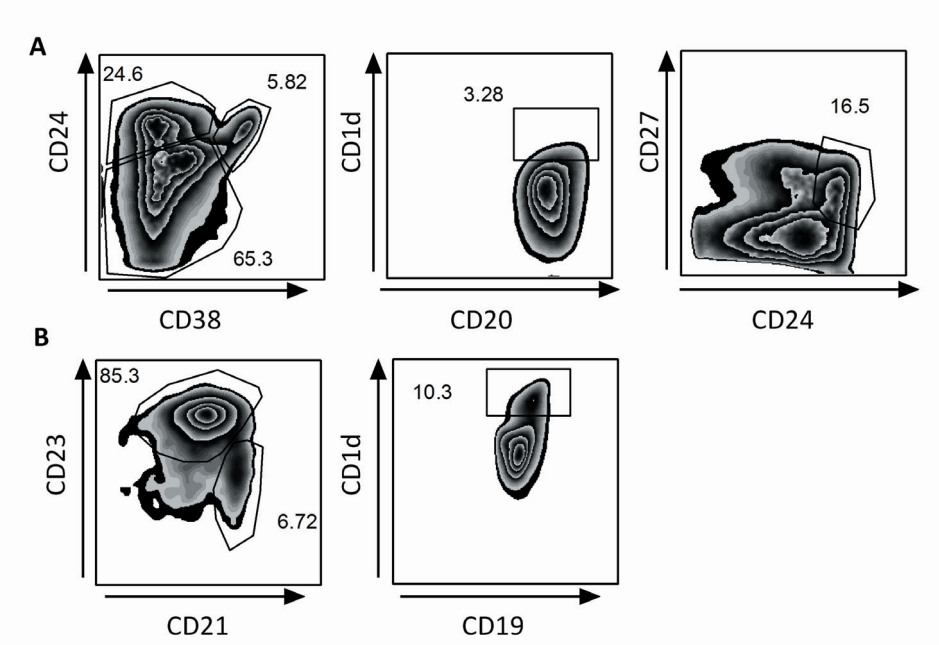


Figure 1. Main phenotypic markers of human and murine Breg cells. (A) Breg cells from human peripheral blood are $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD1d^{hi}$ (pre-gated on $CD20^{+}$ cells). (B) Murine Breg cells are $CD21^{hi}CD23^{low}$ and $CD1d^{hi}$ (pre-gated on $CD19^{+}$ splenocytes).

As outlined above, Breg cell function in response to some TLR ligands seem to be reduced in human autoimmune and allergic diseases. In contrast, recent reports suggest that the development and activity of Breg cells is increased in microbial infections, as shown in patients having chronic infections with hepatitis B virus (33), *Mycobacterium tuberculosis* (34) or the helminth *Schistosoma haematobium* (22). Although a direct role for TLR-dependent generation of Breg cells in these infections has not yet been established, TLR activation by various pathogen-derived ligands such as the TLR1/2 agonist Pam3CysK, a TLR2 ligand in SEA, and the TLR2/6 agonist Pam2CGDPKHPKSF were shown to activate IL-10 production in human B cells (20;35;36).

Various distinct and overlapping Breg subsets have been described in mice and humans of which the respective roles in response to TLRs can be investigated. For example, CD1d expressing B cells are described in both species (22), while $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD25^{hi}$ and $CD71^{hi}$ expression has been attributed to human Breg cells only (19;20;37) (as depicted in Fig. 1A) and $CD5^{+}$, $CD21^{hi}CD23^{hi}$ or $CD21^{hi}CD23^{low}$ were shown to be markers expressed by mouse Breg cells (Fig. 1B). However, as some of these markers are overlapping or these populations still contain non-IL-10-producing B cells, a unique set of markers or transcription factors that exclusively identifies Breg cells does not exist. Whether or not all B cells can develop suppressive functions following TLR activation is still under debate: Mizoguchi *et al.* proposed that only certain 'innate' B cell subsets will develop into Breg cells following TLR ligation (38), while others have suggested that all immature progenitor cells have the potential to progress into mature IL-10-producing B cells after ligation by TLRs and CD40 directly (39). Yet another hypothesis has been presented by Fillatreau and co-workers, which proposed that B cells can acquire only suppressive functions after a stepwise activation process initiated by TLR ligands and followed by BCR and CD40 reinforcement (40).

Most studies investigating the presence and function of Breg cells following TLR stimulation limit their analysis to IL-10 as the major anti-inflammatory and effector cytokine of Breg cells. However, it is often neglected that, next to IL-10, TLR ligation can simultaneously contribute to the development of inflammatory responses (10;35). For example, CpG, which is a strong IL-10 inducer, leads to strong B cell activation, proliferation, immunoglobulin production and expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (41-43). Indeed, elevated numbers of IL-6-producing B cells were observed in MS patients after *in vitro* ligation with CD40 and B-cell receptor with CpG, which was demonstrated to have a pathogenic role in the development of chronic experimental autoimmune encephalomyelitis in mice (44). Because of the recent interest to study the TLR-induced generation of Breg cells, it is crucial to use optimized assays for the phenotypic characterization of Breg cells and the analysis of both inflammatory and anti-inflammatory responses. Flow cytometry and enzyme-linked immunosorbent assay (ELISA) allow the detection of cytokines such as IL-10. However, while the qualitative IL-10 contribution of each individual Breg subset can easily be monitored by flow cytometry, ELISA provides more quantitative information, but requires a preceding cell sorting of the subsets before stimulation.

Although high IL-10 secretion is regarded as being anti-inflammatory and a marker for Breg cells, it is possible that the TLR-primed B cells did not acquire any immune suppressive activity or act regulatory in an IL-10-independent manner. Therefore, it has a strong additive value to investigate whether TLR-primed B cells show any suppressive capacity on the effector function of other cells either *in vivo* or *in vitro*. Indeed various studies have shown that Breg cells can influence several aspects of innate and adaptive immunity and either one

of these or all targets should be included in the study to the development of TLR-induced Breg cells: (1) monocyte-induced inflammatory responses are suppressed in their release of pro-inflammatory cytokines (20); (2) the Th1/ Th2 balance is manipulated by inhibition of antigen-specific T cell proliferation in both Th1 and Th2 skewed diseases; and (3) regulatory responses are potentiated by the induction and/or recruitment of IL-10-producing T cells and $FoxP3^+$ Treg cells (19;21-23).

In conclusion, various TLR ligands can be used as potent B effector or Breg cell inducers *in vitro*. However, to be able to get a better insight in the acquired regulatory capacity, a number of parameters needs to be considered, that is (1) the culture conditions (cell numbers per well, duration of stimulation, concentration of TLR ligands), (2) the method of cytokine detection (flow cytometry and ELISA, mimicking T cell interaction or not, see Fig. 2 and 3), (3) the ratio of produced anti-inflammatory versus pro-inflammatory cytokines (IL-10 versus IL-6, Fig. 2 and 3), and (4) a co-culture with other immune cells to evaluate their suppressive capacity on the effector function of other immune cells (see Fig. 4).

2. Materials

2.1 Isolation of B and T cells

1. Lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, in MilliQ, sterile.
2. MACS buffer: 0.5% BSA, 2 mM EDTA in PBS, sterile.
3. MACS Midi magnets and LS columns (Miltenyi Biotec).
4. Anti-mouse and anti-human CD19 MicroBeads (Miltenyi Biotec). (see Notes 1 and 2)
5. Anti-mouse and anti-human memory $CD4^+$ isolation MicroBeads (Miltenyi Biotec).
6. 100 µm cell strainers used for mashing the spleen in 50 ml tubes (Greiner Bio-One.
7. Ficoll/Hypaque.

2.2 Stimulation of B cells

1. Mouse: RPMI 1640 + Glutamax (Invitrogen Life Technologies) supplemented with 5% heat-inactivated FCS (Greiner Bio-One), 5×10^{-5} M 2-Mercaptoethanol, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).
2. Human: RPMI 1640 supplemented with 10% heat-inactivated FCS (Greiner Bio-One), 1 mM pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.
3. 96-well round bottom plates (Greiner Bio-One).
4. TLR ligands (stock concentrations): CpG2006 (1 mg/ml, Invitrogen), CpG1826 (1 mg/ml, Invitrogen), Ultrapure LPS (2.5 mg/ml, Invitrogen), FSL-1 (1 mg/ml, EMC microcollection), PGN (1 mg/ml, Sigma-Aldrich) and Pam3-Cys (1 mg/ml, EMC microcollection); store in aliquots at -20 °C.
5. 0.1 mg/ml Phorbol 12-myristate 13-acetate (PMA), 0.5 mg/ml ionomycin and 5 mg/ml Brefeldin A; store in aliquots at -20 °C.

2.3.1 Quantification of cytokine production by flow cytometry

1. 37% paraformaldehyde.
2. 96-well V-bottom plates (Greiner Bio-One).
3. FACS buffer: 0.5% BSA, 2 mM EDTA in PBS, non-sterile.
4. Permeabilization buffer: FACS buffer supplemented with 0.5% saponin.
5. Flow cytometer.
6. Mouse antibodies for flow cytometry: anti-mouse CD1d-PE (1B1, ebioscience), anti-mouse CD19-APC-Cy7 (1D3, BD Pharmingen), anti-mouse CD21-APC (B3B4, ebioscience), anti-mouse CD23-FITC (7G6, BD Pharmingen), anti-mouse IL-10-PE (JES5-16E3, eBioscience), and anti-mouse FcγRII/III (2.4G2, Bioceros).
7. Live-dead fixable Aqua dead cell staining kit (Invitrogen).
8. Human antibodies for flow cytometry: CD1d-PE (51.1, eBioscience), CD20-APCeFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, ITK Diagnostics),

CD27-APC (L128, BD), CD38-FITC (HIT2, BD), IL-10-biotin (JES3-12G8, Abd Serotec) with streptavidin-Qdot525 (Invitrogen), TNF-α-eFluor450 (MAB11, eBioscience) and FcγR-binding inhibitor (eBioscience) (see Note 10).

2.3.2 Quantification of cytokine production by ELISA

1. BD OptEIA mouse IL-10 and IL-6 ELISA sets (BD biosciences).
2. Human IL-10 and IL-6 ELISA sets (Sanquin).

2.4 Detection of Tr1/FoxP3 T cells by flow cytometry after co-culture

1. FoxP3 permeabilization/fixation buffer (eBioscience).
2. Human antibodies: CD3-PerCPeFluor710 (OKT-03, eBiosience), CD20-APCeFluor780 (2H7, eBioscience), CD25-PE (2A3, BD), IL-10-biotin with streptavidin-Qdot525, TNF-α-PeCy7 (MAB11, eBioscience), FoxP3-eFluor450 (PCH101, eBioscience), and FcγR-binding inhibitor (eBioscience).

Figure 2. IL-10 and IL-6 expression of human B cells in response to TLR stimulation. 3 x 10^5 isolated peripheral blood $CD19^+$ B cells from healthy donors were stimulated with the TLR ligands LPS (100 ng/ml), Pam3-Cys (5 μg/ml), FSL-1 (5 μg/ml), and PGN (5 μg/ml), or cultured in medium as a negative control. (A) The frequency of $IL\text{-}10^+$ B cells was determined after 2 days stimulation followed by 6 h culture with PMA/ionomycin (BrefeldinA added in the last 4 h) *n* =6. (B) Secreted IL-10 measured by ELISA after 2 days stimulation with TLR ligands *n* =6-12. (C) IL-10 secretion in the supernatant of B cells cultured with TLR-2 ligands for 2 days (no restimulation) or restimulated with PMA/ Ionomycin/ BrefA (PIB) for 6 h. The cytokine production before the Golgi stop resembles the flow cytometry data (Fig. 2A). In addition, the cells were cultured after TLR priming with CD40L expressing cell line (J558) to mimicking B and T cell interaction for an additional 24 h. This figure represents 3 independent experiments. (D) IL-6 secretion after 2 days of stimulation measured by ELISA *n* =6.

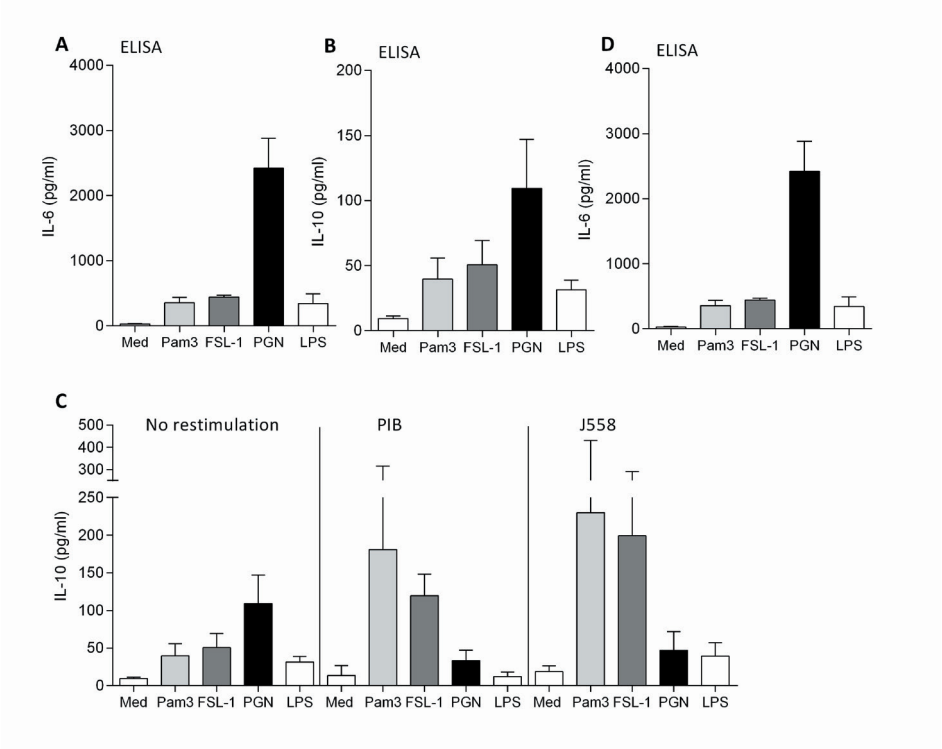
3. Methods

3.1.1 Isolation of mouse CD19$^+$ B cells

1. Single cell suspensions are prepared from mouse spleens using 100 µm cell strainers.
2. Erythrocytes are lysed from the spleen suspension using 2 minutes incubation with lysis buffer.
3. Cells are washed once with MACS buffer and are incubated with anti-mouse CD19 magnetic beads in MACS buffer and CD19$^+$ B cells are positively selected on LS columns according to the manufacturer's instructions (see Notes 1 and 2).

3.1.2 Isolation of human CD19$^+$ B and T cells

1. PBMC are isolated from blood of healthy donors (after their informed consent and in accordance with protocols approved by the local authorities) by density gradient centrifugation over Ficoll/Hypaque.
2. Cells are incubated with anti-CD19 magnetic beads (Miltenyi Biotech) in MACS buffer and positive selection of CD19$^+$ cells on LS/MS columns according to the manufacturer's instructions (see Note 3).
3. Flow through cells are collected to isolate CD4$^+$ memory T cells according to the manufacturer's instructions.



3.2 Stimulation of B cells with TLR ligands

1. MACS isolated B cells are washed twice with complete RPMI.
2. A minimum of 3.0 x 10^6 B cells/ml are needed to evaluate secreted cytokine levels in response to weak TLR ligands (see Note 4).
3. TLR ligands are diluted in complete RPMI two-fold concentrated, yielding the following final concentrations after addition of cells. Mouse: CpG1826 5 µg/mL; LPS 100 ng/mL; FSL-1 10 µg/mL; Pam3-Cys 10 µg/mL; PGN 10 µg/mL. Human: CpG2006 1 µg/ml; LPS 100 ng/ml, FSL-1 5 µg/ml; Pam3-Cys 5µg/ml; PGN 5µg/ml.
4. 100 µl of two-fold concentrated TLR ligands are added per well of a 96-well round bottom plate (Greiner Bio-One). Next 100 µl B cells (3x10^5) are added per well (total volume of 200 µl).
5. Murine cells are incubated at 37°C, 5% CO_2 and humidified atmosphere for three days (for flow cytometric IL-10 analysis, see Note 6) or five days (for ELISAs, see Notes 5 and 7). Human cells are incubated for two days (see Notes 5 and 7).

3.3.1 Quantification of cytokine expression by flow cytometry

Two different techniques can be applied for cytokine detection in TLR-activated B cells, however it is important to take into account that intracellular cytokine visualization after TLR ligation by flow cytometry needs an additional restimulation, such as PMA/ ionomycin/ BrefeldinA (PIB), which is not applied for the analysis of secreted cytokine levels in the supernatant by ELISA (see section 3.3.2). As a consequence the data of the two assays may not be fully comparable and the difference in assay processing should be carefully taken into account while drawing conclusions. This is highlighted and explained in an example where we compared different TLR2 ligands and the TLR4 ligand LPS for their capacity to induce IL-10 production in human B cells (see Fig. 2A-C, Note 8).

1. For flow cytometric detection of IL-10, cells are washed once with complete RPMI and restimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) in RPMI at 37°C, 5% CO_2 and humidified atmosphere. After two hours, Brefeldin A is added at a concentration of 10 µg/ml. In total, the cells are cultured under these conditions for six hours.
2. *Optional:* After restimulation with PIB, cells are harvested, washed with PBS and stained with Aqua dead cell staining kit (diluted 1:400 in PBS) for 15 min at room temperature.
3. The cells are fixed with 1.9% paraformaldehyde for 15 min at room temperature and stored at 4°C or -20 °C.
4. For intracellular staining, the cells are first washed once with permeabilization buffer, and then apply 50 µl/well antibody mix and incubated for 30 minutes at 4°C (see details Section 2.4).
5. After washing once with permeabilization buffer, cells are resuspended in FACS buffer and measured on a FACS Canto or similar instrument (see Note 8, Fig. 2).

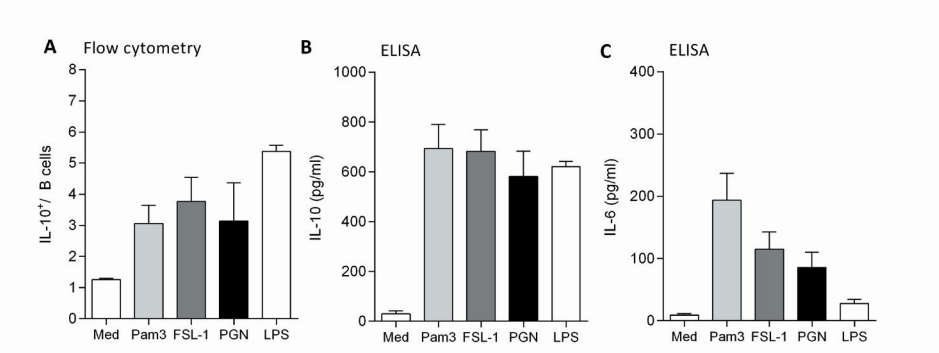


Figure 3. IL-10 and IL-6 expression of murine B cells in response to TLR stimulation. CD19$^+$ sorted splenic B cells were stimulated with the TLR ligands LPS (100 ng/ml), Pam3-Cys, FSL-1, and PGN (each 10 µg/ml), or cultured in medium as a negative control. (A) The frequency of IL-10 positive B cells was determined after 3 days stimulation followed by 6 h culture with PMA/ionomycin (BrefeldinA added in the last 4 h). Secreted IL-10 (B) and IL-6 (C) was measured by ELISA after 5 days stimulation with TLR ligands. Mean ± SEM of 3 (A) or 4 (B, C) independent experiments is given.

3.3.2 Quantification of cytokine expression by ELISA

As TLR ligation not only induces IL-10 but also other pro-inflammatory cytokines (e.g. IL-6, TNF-α), cytokine expression of various cytokines (so not only IL-10) should be taken into account to determine whether certain TLR ligands truly induce an anti-inflammatory response or not. For optimal Breg activity, a TLR ligand should display a strong IL-10 and a low IL-6 inducing capacity. For example, the TLR2 ligand PGN induced more human B cell IL-10 secretion in the supernatant compared to the other TLR2 ligands, but also the highest IL-6 production (Fig. 2B, D and Note 9). In contrast, for mouse B cells, while several TLR2 ligands induced similar levels of secreted IL-10, IL-6 levels were lowest with PGN, suggesting that in this case PGN is a more potent Breg cell inducing TLR2 ligand in mouse cells *in vitro* (Fig. 3). Of note, the TLR4 ligand LPS showed by far the highest IL-10/IL-6 ratio in murine B cells (Fig. 3 B,C).

1. Supernatants from TLR-stimulated cells are collected and stored at -20°C (see Note 9).
2. *Optional:* After TLR-priming, the cells are cultured 1:1 with CD40L expressing cell line (J558 cells, irradiated with 2600 RAD) to mimicking B and T cell interaction for an additional 24 hours. Next, the supernatant is stored at -20 °C (see Fig. 2C, Note 8).
3. *Optional:* After TLR-priming, the cells are restimulation with PMA/ionomycin/ BrefA (see section 3.3.1) and supernatant containing cytokines produced in two hours after PMA/ionomycin is stored at -20°C.
4. ELISA coating antibodies, detection antibodies and standards are used in concentrations as recommended by the manufacturer.

3.4 Detection of Tr1/FoxP3 T cell by flow cytometry after co-culture

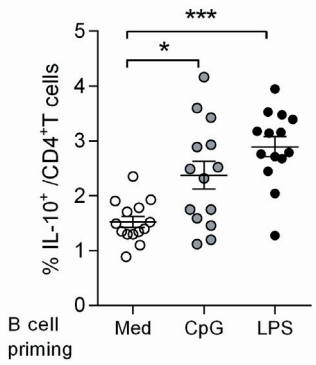


Figure 4. IL-10$^+$ T cells in co-cultures of TLR-primed B cells and T cells. B cells were stimulated for 48 h by medium, CpG (5 μg/ml) or LPS (100 ng/ml), followed by a 1:1 co-culture with autologous memory CD4$^+$ T cells for 6 days. Next, the cells were restimulated with PMA/ionomycin (BrefeldinA added in the last 4 h) for 6 h. Percentage of IL-10 producing CD4$^+$ T cells was determined by flow cytometry.

1. Even when B cells secrete elevated levels of IL-10, it is essential to elucidate whether the B cells from the test conditions really have obtained suppressive properties to confirm their regulatory B cell status (see Note 11). Most studies compared the suppressive activity of B cells from autoimmune or allergic patients with healthy controls using different culture systems *ex vivo*. In this context, we have set-up an *in vitro* co-culture assay with human B cells from healthy donors which were first primed with TLR ligands, followed by culture with autologous CD4$^+$ memory T cells to investigate their acquired function capacities (24) as described below. Figure 4 shows an example of LPS- and CpG-primed B cells both induce a clear increase in IL-10-producing T cells compared to medium-primed B cells, suggesting they have acquired an increased regulatory capacity in B cells as a consequence their TLR ligation (see Note 11, Fig. 4).
2. 1 x 10^5 human B cells are stimulated via TLR 4 (ultra-pure LPS) or TLR9 (CpG) for two days (total volume 200 μl/well) (see Section 3.2).
3. Isolated memory CD4$^+$ T cells (see Section 3.1.2) are stored at 4°C for two days.
4. After two days, supernatant is stored, and the cells are washed, and co-cultured with autologous memory CD4$^+$ T cells (ratio 1:1) in RPMI supplemented with 10% FCS for an additional six days.
5. The cells are restimulated by PMA/ionomycin and BrefA (as indicated above) and fixated with FoxP3 fixation/permeabilization buffer (eBioscience). The cells were stained for flow cytometry as indicated in Section 2.4.

4. Notes

1. Isolation of B cells using e.g Miltenyi Biotec beads reaches approximately 95-98% purity. Check for each isolation the contamination of other cells, especially dendritic cells and monocytes, as they being very responsive to TLR ligand stimulation. Depletion of other cells, prior to $CD19^+$ B cell isolation, should reduce their putative contamination.
2. The concentration of anti-mouse CD19 magnetic beads used for isolation can be reduced after empirical testing of the sorting efficiency. For C57BL/6 splenic B cells, 7 µl instead 10 µl beads per 10^7 cells can be used without reduction in cell purity.
3. PBMC and B cell isolation should be performed preferably directly after blood draw. In addition, it is recommended to use fresh B cells and not live cryopreserved PBMC or B cells, because the B cell cytokine responses are diminished after freezing and thawing.
4. Before applying these assays, the number of cells in the culture and the duration of TLR ligation should be considered. We have established that higher cell numbers of human B cells are needed to evaluate secreted cytokine levels in response to weak TLR ligands, such as LPS. A minimum of 3.0 x 10^6 B cells/ml (3.0 x 10^5/well) showed detectable levels of secreted IL-10 and IL-6 (approx. 30 pg/ml IL-10 and 612 pg/ml IL-6 above medium) in response to 100 ng/ml LPS and therefore this cell number was used in further experiments (data not shown).
5. Kinetic experiments showed that cytokine production by human B cells reached its maximum after two days of TLR exposure and longer incubations even seemed to reduce the concentration in the supernatant, which may be explained by the assumption that cytokines in the supernatant will be consumed; for example, IL-10 acts as an autocrine factor promoting expansion of IL-10-producing B cells or differentiation of B cells into plasma cells (45;46). Although in murine B cells the TLR4 expression is more abundant compared to human B cells, a minimum of 3.0 x 10^6 cells/ml stimulation of five days resulted in the most optimal cytokine detection (data not shown). Therefore, it is recommended to apply the cell numbers and time points described above to allow detecting of cytokines in response to 'weak' TLR ligands, like LPS. In case the strength of the stimulus of interest or combinations of stimuli is unknown, additional kinetic or dose range studies are recommended.
6. For flow cytometric analysis of B cell IL-10 expression, a two (human) to three (mouse) days stimulation is recommended instead of five days, because of a better cell viability.
7. Stimulation of cells should be performed at least in duplicates in order to increase the accuracy of the ELISA results.
8. Flow cytometry and ELISA may not be fully comparable because for intracellular cytokine visualization by flow cytometry, an additional restimulation, such as

PMA/ ionomycin/ BrefeldinA (PIB) is needed (see section 3.3.1). An example was represented in Figure 2, showing that applying intracellular cytokine staining, both TLR1/2 ligand Pam3CysK and TLR2/6 ligand FSL-1 induced more B cell IL-10 compared to peptidoglycan (PGN, TLR2/CD36) in human B cells (Fig. 2A), while surprisingly, PGN induced the highest IL-10 secretion as measured by ELISA (Fig. 2B). To sort out whether the second stimulation (PMA/ionomycin) changes the B cell response, TLR2 primed B cells were stimulated either for two hours with PMA/ionomycin (regular protocol for intracellular cytokine staining) or for 24 hours with a CD40L expressing cell line J558 to mimic B and T cell interaction in a more physiological manner (Fig. 2C). Both after PMA/ionomycin stimulation and after CD40 ligation, a higher IL-10 secretion was detected in FSL-1- and Pam3CysK- primed B cells in the supernatant, but not for the PGN-primed B cells. This indicates that in this example the second (putative T cell mediated) stimulation acts as an amplifying signal for B cell IL-10 production for certain TLR2 ligands (but not for all), and which is mimicked by PIB restimulation. Therefore, IL-10 production as marker for Breg cell induction by different TLR ligands should be confirmed both by flow cytometry and ELISA to be able to understand the role of certain TLR ligands for B cell activity under innate conditions and after interaction with T cells. Of note, the intracellular IL-10 levels measured by flow cytometry will be more representative for their behavior in the interaction with T cells.

9. It is important to investigate both pro- and anti-inflammatory cytokine responses when studying the role of TLR ligands as promoting adjuvant for Breg cells, and to consider species-related differences when extrapolation findings from disease models to human conditions. Thus, the Breg inducing capacity can be diverging for the same TLR ligand with different species (e.g. here: PGN for mouse and human B cells), as well as for different ligands targeting the same TLR (here: TLR2, see Fig. 2 and 3).
10. To correct for background staining of other fluorochromes in the staining mix, using FMOs (fluorescence minus one) for IL-10 (and other cytokines) is recommended. At the moment, FMOs are regarded as the best control in multicolor experiments and can also help to rule out problems in compensation or aspecific binding of the antibodies. For reference on this policy, see also Roederer *et al.* 2001 Cytometry (47), Herzenberg *et al.* 2006 Nature (48), Maecker *et al.* 2006 Cytometry (49) and TreeStar Daily Dongle 2011.
11. Next to the identification of IL-10 and other cytokine production, the functionality of the IL-10-producing B cells needs to be investigated before the term 'Breg cell' can be applied. This can be performed using memory T cells (Fig. 4) but also using different cell types as described by other groups (20;21;33) e.g. for their capacity to reduce cytokines or cell proliferation.

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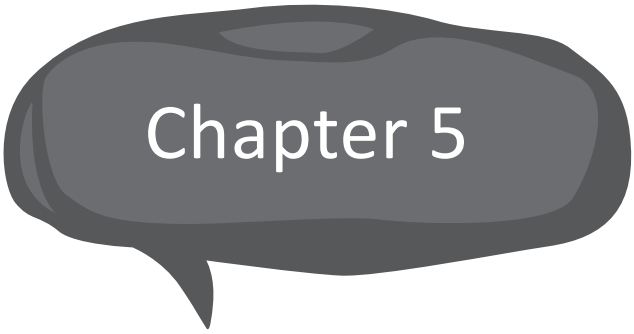

IL-10-producing CD1d^{hi} regulatory B cells from *Schistosoma haematobium*-infected individuals induce IL-10-positive T cells and suppress effector T-cell cytokines

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Abstract

Background. Chronic schistosome infections are associated with T-cell hyporesponsiveness and a strong regulatory network. Murine studies have shown that schistosome infections can induce regulatory $CD1d^{hi}$ B cells, which inhibit inflammatory responses. Here, we evaluated the influence of regulatory B cells (Bregs) on T-cell cytokines *in vitro* in human schistosomiasis.

Methods. Gabonese young adults were recruited from areas where *Schistosoma haematobium* (*S.h*) infections were high or low endemic. The study participants were categorized as infected or uninfected from a high endemic area or uninfected from a low endemic (nonendemic) area. Their B cells were studied for Breg subset markers and cocultured with allogenic anti-CD3-stimulated $CD4^+$ T cells, followed by T-cell cytokine analysis.

Results. A greater percentage of B cells from *S. haematobium*-infected donors expressed cytoplasmic interleukin(IL) 10 (IL-10) and membrane-bound latency-associated peptide/transforming growth factor β1 (LAP/TGF-β1), compared with uninfected donors. T cells produced less interferon γ (IFN-γ), IL-4 and IL-17 upon coculture with B cells from schistosome-infected individuals only, while the conversion to $CD25^{hi}FoxP3^+$ and the percentage of $IL\text{-}10^+$ T cells was enhanced. Interestingly, depletion of the prominent IL-10-producing B-cell subset, $CD1d^{hi}$ cells, resulted in less $IL\text{-}10^+$ T cells in the *S. haematobium*-infected group, while levels of $FoxP3^+$ regulatory T cells remained unaffected.

Conclusions. Schistosomes can induce functional Bregs in humans that may be instrumental in general T-cell hyporesponsiveness and may contribute to the increased regulatory milieu found in schistosomiasis.

Introduction

Helminths such as schistosomes are master regulators of host immune responses, characterized by polarized T-helper type 2 (Th2) cells (1;2) and a general T-cell hyporesponsiveness in the chronic stage of infection (3). This is facilitated by a number of potent regulatory cell types and increased levels of immunosuppressive cytokines, such as interleukin 10 (IL-10) and/or transforming growth factor β (TGF-β) (4-6). While B lymphocytes possess a variety of immune functions, more recently they were shown to be a relevant source of IL-10 and/or TGF-β (7;8). Such B cells were termed regulatory B cells (Bregs) as they were able to ameliorate various hyperinflammatory diseases (9-13). For example, IL-10-producing B cells suppressed experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) (14-17). Furthermore, TGF-β-producing B cells protected against acute allergic airway inflammation (8), and inhibited spontaneous Th1 autoimmunity in nonobese diabetic mice (18). Recently, Bregs have been considered relevant in infectious diseases, such as helminth infection. During *Schistosoma mansoni* infection, active Bregs were enriched in the splenic marginal zone, and suppressed allergic responses via elevated IL-10 production and/or FoxP3$^+$ T-regulatory (Treg) cell induction (19;20). Likewise, during chronic *Heligosomoides polygyrys* infections CD5$^-$CD23^{hi} B cells from mesenteric lymph nodes also reduced allergic airway inflammation, but they did so independently of IL-10 (21).

Although the majority of studies on Bregs were conducted in mouse models, Bregs are now also identified in humans during infectious diseases. For example, during chronic hepatitis B virus infection IL-10-producing CD24^{hi}CD38^{hi} B cells suppressed antigen-specific CD8$^+$ T-cell responses (22), while in systemic lupus erythematosis, IL-10 production by this Breg subset was impaired compared with findings in healthy controls, and as a consequence Th1 cytokine production was not reduced (23). In human immunodeficiency virus (HIV)-infected individuals, *in vitro* depletion of CD24^{hi}CD38^{hi} B cells resulted in enhanced anti-HIV CD8$^+$ T-cell responses, which was dependent on IL-10 (24). Furthermore, CD24^{hi}CD27$^+$ Bregs from healthy individuals reduced TNF-α production in monocytes *in vitro* via IL-10 (25), however this Breg subset is not yet studied in the context of infectious diseases. Lastly, during *Mycobacterium tuberculosis* infection CD1d$^+$CD5$^+$ B-cell numbers were increased and inhibited IL-17 production by T cells (26). Although active TGF-β-producing CD5$^+$ Bregs were described in healthy individuals in response to milk antigens (7), the role of human TGF-β-expressing Bregs in infectious diseases has not yet been addressed.

Enhanced numbers of IL-10-producing CD1d^{hi} B cells were first described in helminth-infected Argentine patients with multiple sclerosis (MS) and could inhibit autoreactive T-cell responses *in vitro* (27). We found a similarly elevated percentage of IL-10-producing B cells in *Schistosoma haematobium*-infected Gabonese children, which returned to baseline levels after anti-schistosome

treatment (20). However, the functional properties of these cells were not explored. In the current study, we investigated the regulatory characteristics of peripheral B cells of *S. haematobium*-infected individuals and the functional consequences for $CD4^+$ T-cell activation. We observed that $CD1d^{hi}$ B cells and $CD24^{hi}CD27^+$ B cells of *S. haematobium*-infected adults express enhanced levels of cytoplasmic IL-10 and enhanced levels of surface latency-associated peptide (LAP), part of a latent TGF-β complex, respectively. In coculture with T cells, reduced effector T-cell cytokine responses, as well as more Tregs, were associated with B cells from infected individuals, compared with B cells from uninfected controls who resided in areas where *S. haematobium* is nonendemic. Depletion of the $CD1d^{hi}$ B-cell population resulted in a loss of $IL\text{-}10^+$ T-cell induction but did not yield differences in the numbers of $FoxP3^+$ Tregs, suggesting that other $CD1d^-$ Bregs may be important for $FoxP3^+$ T-cell induction. Taken together, schistosome-induced Bregs exhibit regulatory activity that may contribute to strong regulatory responses found during chronic schistosomiasis.

Materials and Methods

Study population

Heparinized venous blood was obtained from *S. haematobium*-uninfected young adults living in Lambaréné, Gabon, a semiurban municipality where *S. haematobium* is not endemic and the prevalence of *S. haematobium* infection is low; these individuals composed the "nonendemic uninfected group". Infected donors and additional uninfected donors were recruited from the rural village Bindo, where *S. haematobium* is endemic, residents are likely to have a history of *S. haematobium* infections, and no intervention studies have been performed (28); these donor groups composed the "endemic infected group" and the "endemic uninfected group", respectively. Current users of praziquantel were excluded from study participation, as were people who had lived in either region for <1 year before enrollment.

The presence of helminth and protozoan infections was determined as previously reported (20) (Table I). Infections with *Plasmodium (P.) falciparum* and *P. malariae* were determined by polymerase chain reaction (PCR) (29) and *Loa Loa* and *Mansonella perstans* microfilariae by microscopy (30). Written informed consent was obtained from all participants, and they were treated according to local guidelines. The study was approved by the Comité d'Ethique Regional Independent de Lambaréné (CERIL N°08/10) and conducted according to the principles expressed in the Declaration of Helsinki.

Cell isolation and characterization

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation from approximately 40 ml of blood in 50 ml tubes (Greiner Bio-One). $CD19^+$ B cells, untouched $CD4^+$, and $CD4^+CD25^-$ T cells were isolated by magnetic-bead purification (purity, >96%). For $CD1d^{hi}$ depletion, B cells were first incubated with CD1d-APC (Biolegend), washed, and incubated with 20 µl/10^7 anti-APC MicroBeads (Miltenyi Biotec). The cells were then split in 2 groups, $CD1d^{hi}$-depleted B cells (86% depleted) and mock-depleted B cells, to which the eluted $CD1d^{hi}$ B cells were added back after depletion. Part of the B cells were cryopreserved in cryovials (Greiner Bio-One) and transported to Leiden University Medical Center, Leiden, The Netherlands.

In PBMCs, B cells were characterized using CD1d-PE, CD19-PacificBlue, CD27-APCeFluor780 (all eBioscience), CD24-PeCy7 (ITK), and CD38-FITC (BD). For PFA-fixed PBMCs: CD1d-PE, CD20-APCeFluor780 (eBioscience), CD24-PeCy7, CD27-Biotin (eBioscience) plus streptavidin-Qdot525 (Life Technologies), CD38-Horizon450 (BD), and LAP1-APC (Biolegend). For all flowcytometric measurements, all samples were stained in 96-well V-bottom plates (Greiner Bio-One), FcγR-binding inhibitor was added to the antibody mix, and FMOs were used for gate setting for all surface markers and cytokines.

B-cell stimulation and intracellular staining

Freshly isolated B cells ($1x10^5$) were stimulated in medium (RPMI; Life Techonologies) supplemented with 10% FCS (Greiner Bio-One), 1 mM pyruvate, 2mM Glutamate and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; all Life Technologies), in the presence of 100 ng/ml PMA, 1 µg/ml ionomycin, 10 µg/ml BrefeldinA (all Sigma-Aldrich) and 10 µg/ml ultrapure LPS (Life Technologies) (25). After 5 hours, the cells were fixated with 1.9% PFA (Sigma-Aldrich) and stained for CD1d-PE, CD20-APCeFluor780, CD24-PeCy7, CD27-APC (BD), CD38-FITC, IL-10-Biotin (Abd Serotec) plus streptavidin-Qdot525, and TNF-α-eFluor450 (eBioscience).

Functional *in vitro* assays

B cells ($1x10^5$) were cultured in 96-well U-bottom plates (Greiner Bio-One) with allogenic $CD4^+$ T cells ($1x10^{5)}$ from 1 European donor in the presence of 1 µg/ml soluble anti-CD3 (IXE M1654, Sanquin) for 3 days or, in parallel, with $CD4^+CD25^-$ T cells for 6 days (23). After 3 days, cells were restimulated with PIB, PFA-fixed and stained in 0.5% saponin buffer for IL-4-PE (BD), IL-10-Biotin plus streptavidin-Qdot525, IL-17-FITC (Biolegend), IFN-γ-HorizonV450 (BD), TNF-α-PeCy7 (eBioscience), CD3-PerCPCy5.5 (BD), and CD20-APCeFluor780. After 6 days, restimulated cells fixed with eBioscience FoxP3 fixation buffer were stained in permeabilization buffer for: CD1d-APC (Biolegend), CD3-PerCPeFluor710 (eBiosience), CD20-APCeFluor780, CD25-PE (BD), IL-10-Biotin plus streptavidin-Qdot525, TNF-α-PeCy7, FoxP3-eFluor450 (eBioscience), and Helios-FITC (Biolegend).

Statistical analysis

For non-parametric analysis Kruskal-Wallis, Mann-Whitney *U*, and Wilcoxon matched pairs tests were used. For categorical data, the Pearson Chi-squared and Fisher exact tests was used. All tests were performed used Graphad Prism. Correlations were analyzed using SPSS Statistics 20 (IBM). A *P* value <.05 was considered statistically significant.

Results

Elevated B-cell IL-10 and LAP/TGF-β1 expression in *S. haematobium*-infected subjects

Peripheral blood specimens was collected from 14 young adults in the endemic infected group and compared to specimens from 12 individuals in the endemic uninfected group and from 12 individuals from the nonendemic uninfected groups. As described in Table I, no significant differences were found between the 3 groups in the prevalence and infection intensity of other parasitic infections such as malaria, *Ascaris lumbricoides* infection, *Trichuris trichiura* infection, or hookworm infection. Furthermore, sex was equally distributed, but the age of the *S. haematobium*-infected individuals was slightly lower than that of uninfected individuals. Age was not regarded as a confounder, however, as the real age differences were very small (3-4 years). The average number of isolated PBMCs ($1.1x10^6$, $1.0x10^6$, $1.0x10^6$ cells/ml, P =.5) and the average percentage of isolated B cells (8.3%, 8.3%, 6.2%, P =.4) were comparable between the endemic infected group, endemic uninfected group, and nonendemic uninfected group, respectively.

$CD19^+$ B cells isolated from peripheral blood specimens were studied for the presence of IL-10-competent B cells by a short, 5-hour stimulation period with PMA, ionomycin, Brefeldin A and LPS (PIB+LPS) as described by Iwata *et al.* (25). TNF-α was included as an inflammatory control. The percentage of B cells expressing cytoplasmic IL-10 was the highest in the endemic infected group and lowest in the nonendemic uninfected group (Fig. 1A). TNF-α production was similar in all groups (Fig. 1A, Appendix S1A). The percentage of IL-10-producing B cells was not correlated with age, helminth burden (based on *S. haematobium* egg counts; data not shown), or *Plasmodium* parasites (Fig. 1C) (31). Of note, PIB-only stimulation also showed a greater percentage IL-10-producing B cells in the endemic infected group, compared to the other 2 groups, although in each group, levels after PIB-only stimulation were lower than those after PIB+LPS stimulation (data not shown).

We also analyzed the expression of LAP/TGF-β1. Dissociation of this complex results in bioactive TGF-β1. B cells from the endemic infected group expressed significantly more LAP, while B cells from endemic uninfected individuals showed an increased trend, compared with the nonendemic uninfected group (Fig. 1B, Appendix S1B). We did not study the coexpression of TGF-β1 and IL-10 in B cells because this investigation requires different methods (ie, B cells need to be stimulated for IL-10, but not for LAP/TGF-β dectection). These results indicate that schistosome infections or exposure induces elevated numbers of circulating IL-10-producing and/or LAP/TGF-β1-expressing B cells.

Phenotypic characterization of IL-10 and LAP/TGF-β1-expressing B cells

To determine which Breg subset was responsible for the increased IL-10 production and/or LAP/TGF-β1 expression, PBMCs were analyzed for the presence of 3 different human Breg subsets: $CD1d^{hi}$ B cells, $CD24^{hi}CD38^{hi}$ B cells and $CD24^{hi}CD27^{+}$ B cells (23;25;27). Only the $CD1d^{hi}$ subset was elevated during infection (from 1.9% to 2.5%), whereas the other 2 subsets were similar among the 3 study groups (approximately 5% $CD24^{hi}CD38^{hi}$ and 21% $CD24^{hi}CD27^{+}$) (Fig.

Table I: Characteristics of study population

	Endemic Infected (n=14)	Endemic Uninfected (n=12)	Nonendemic Uninfected (n=12)	*P*
Study area	Bindo	Bindo	Lambaréné	
Gender male/ female	7/7	6/6	6/6	
Mean age in yrs (range)	18.7 (16-23)	23 (18-28)	21.8 (18-27)	0.002#
S. haematobium	14 (100%)	0	0	
Mean egg count (range)	165 (1-798)	-	-	
Other helminths				
Any helminths	6 (43%)	4 (33%)	3 (25%)	0.631*
Ascaris (%)	4 (29%)	2 (17%)	1 (8%)	0.407*
Trichuris (%)	4 (29%)	2 (17%)	1 (8%)	0.407*
Hookworm (%)	3 (21%)	2 (17%)	1 (8%)	0.656*
Entamoeba histolytica (%)	0 (0%)	0 (0%)	1 (8%)	0.329*
Malaria (PCR)				
Any malaria	4 (29%)	7 (58%)	3 (25%)	0.172*
P. falciparum	4 (29%)	6 (50%)	3 (25%)	0.372*
P. malariae	0 (0%)	1 (8%)	1 (8%)	0.540*
Microfilaria				
Any microfilaria	3 (21%)	5 (42%)	2 (17%)	0.332*
Loa loa	3 (21%)	2 (17%)	0 (0%)	0.248*
Mansonella perstans	0 (0%)	3 (25%)	2 (17%)	0.155*

The study was initiated in January 2011, and participants were from 2 villages in Gabon.
By the Kruskal-Wallis test for age. Mann-Whitney test shows the following differences: *P* =.002 between endemic infected and -uninfected group, *P* =.006 between endemic infected and nonendemic uninfected group, and *P* =.255 between endemic uninfected and nonendemic uninfected groups.
* By the Pearson Chi-squared test. Because of sample size, the Fisher exact test was performed between the groups, and results showed no differences between the groups for all co-infections.

2A/B, Appendix S1C). Interestingly, in the endemic infected group, the subset with the greatest percentage of cells producing IL-10 was the CD1d^{hi} B cells (25 %), and only a small percentage of CD24^{hi}CD27^{+} B cells produced IL-10 (2.7 %; Fig. 2C). Within the IL-10-producing B-cell pool, approximately 10-20% of the B cells shared a CD1d^{hi} or CD24^{hi}CD27^{+} phenotype. However, only the percentage of CD1d^{hi} B cells within the IL-10-producing B-cell population significantly increased during infection, again indicating that mostly these cells are induced during schistosome infections. The percentage of the different Breg subsets was not correlated with helminth burden (data not shown).

Increased levels of LAP/TGF-β1 expression in total B cells from endemic infected individuals was mainlv attributed to the CD24^{hi}CD27^{hi}, and partly to the

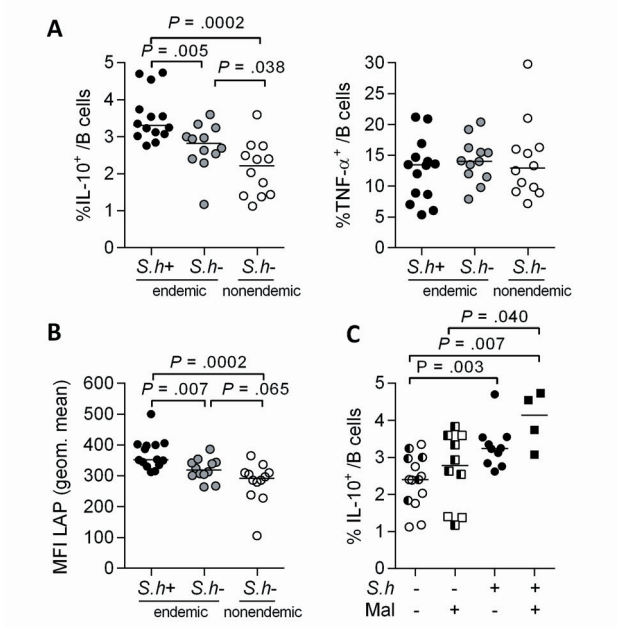

Figure 1. Intracellular interleukin 10 (IL-10) and tumor necrosis factor α (TNF-α) detection in total B cells. 1 × 10^5 blood-derived B cells from *Schistosoma haematobium*–infected individuals from a *S. haematobium*–endemic area (*S.h*+), uninfected individuals from a *S. haematobium*–endemic area (*S.h*−), and uninfected individuals from a *S. haematobium*–nonendemic area (*S.h*−) were directly stimulated for 5 hours with PMA, ionomycin, and brefeldinA (PIB) in combination with ultrapure lipopolysaccharide (LPS). (A) Intracellular IL-10 production and TNF-α production were analyzed using flow cytometry (FACS Canto II, BD Biosciences). (B) 1 × 10^6 PFA-fixed peripheral blood mononuclear cells were stained for latency-associated peptide (LAP)/ transforming growth factor β1. The geometric mean fluorescence intensity (MFI) of LAP expression on total B cells was determined. (C) Intracellular IL-10 in B cells after 5 hours of PIB + LPS stimulation. Donors were grouped according to the presence of malaria parasite infection (Mal) and/or *S. haematobium* infection. The malaria-negative *S. haematobium*–negative group contains 14 individuals (5 of whom were from Bindo), the malaria-positive *S. haematobium*–negative group contains 10 (7 of whom were from Bindo), the malaria-negative *S. haematobium*–positive group contains 10, and the malaria-positive *S. haematobium*–positive group contains 4. Donors from Bindo are depicted in Figure 1C as half-shadowed circles or squares and donor from Lambaréné as open circles or squares. Each individual is represented as a separate dot, with a line showing the median value for the group. Data were analyzed using the Mann–Whitney *U* test.

$CD24^{hi}CD38^{hi}$ B-cell population, but not to the $CD1d^{hi}$ B cells (Fig. 2D). The difference in IL-10 expression between the endemic infected group and the uninfected groups was lost when $CD1d^{hi}$ B cells were removed from the $CD24^{hi}CD27^{+}$ B-cell gate, but the difference in LAP/TGF-β1 expression was maintained (Appendix S2). Therefore, these data suggest that the $CD1d^{hi}$ B cells are the dominant subset that increases in number and capacity to produce IL-10 during *Schistosoma* infection, while increased LAP/TGF-β1 has a stronger association with $CD24^{hi}CD27^{+}$ B cells, although the frequency of this subset did not change during schistosomiasis.

Schistosome-induced B cells dampen the pro-inflammatory T-cell cytokine profile

We next performed *in vitro* $CD4^{+}$ T and B cell cocultures in the presence of anti-CD3 to investigate whether the T-cell stimulatory activity of B cells from the endemic infected group is altered. Allogenic $CD4^{+}$ T cells from a single donor were used, to prevent differences due to variation in T-cell donors. After 3 days, the cells were restimulated with PIB, and we observed a slight but significantly lower percentage of $CD4^{+}$ T cells producing IFN-γ-, IL-4- and IL-17 when stimulated in the presence of B cells from the endemic infected group, compared to nonendemic uninfected group, whereas TNF-α production was not affected (Fig. 3A-D). T cells stimulated with anti-CD3 only, in the absence of B cells, showed a variable degree of viability and were therefore excluded from the analysis. No significant differences were detected between endemic infected and endemic uninfected individuals, suggesting that past exposure to *Schistosoma* infections can yield changes in B-cell function, reflected in the slightly enhanced IL-10 and TGF-β expression (Fig. 2), which may be sufficient to control effector T-cell responses.

$CD1d^{hi}$ B cells induce IL-10-producing T cells

To investigate whether schistosome-induced B cells can prime the regulatory functions in T cells, either by enhancing IL-10 production (as in Tr1 cells) or by inducing $CD25^{hi}FoxP3^{hi}$ Tregs, $CD4^{+}CD25^{-}$ T cells were cocultured with B cells for 6 days. Interestingly, the frequency of IL-10-producing T cells was significantly higher after coculture with B cells from the endemic infected group, compared with the uninfected groups (Fig. 4A). In addition, more $FoxP3^{hi}$ Tregs were found in cocultures with B cells from the endemic infected group, compared to uninfected groups, although the variation in FoxP3 induction was greater than the variation in IL-10 induction (Fig. 4B, Appendix S3). When Helios expression was analyzed (as an additional marker for putative functional $FoxP3^{+}$ Tregs), the frequency of $Helios^{+}CD25^{hi}FoxP3^{hi}$ T cells still remained significantly higher in the endemic infected group (data not shown).

To explore the underlying mechanism, we next focused on the dominant IL-10-producing B-cell subset, $CD1d^{hi}$ B cells, to examine the effect of helminth-induced IL-10-producing B cells on regulatory functions of T cells (Fig. 4C). Because of the limited cell numbers available, we used a $CD1d^{hi}$ B cell depletion strategy instead of isolating $CD1d^{hi}$ B cells. Similar to findings for the total B-cell population

from the endemic infected group, the mock-depleted B cells induced higher levels of IL-10 and/or CD25^{hi}FoxP3^{hi} T cells compared to B cells from uninfected

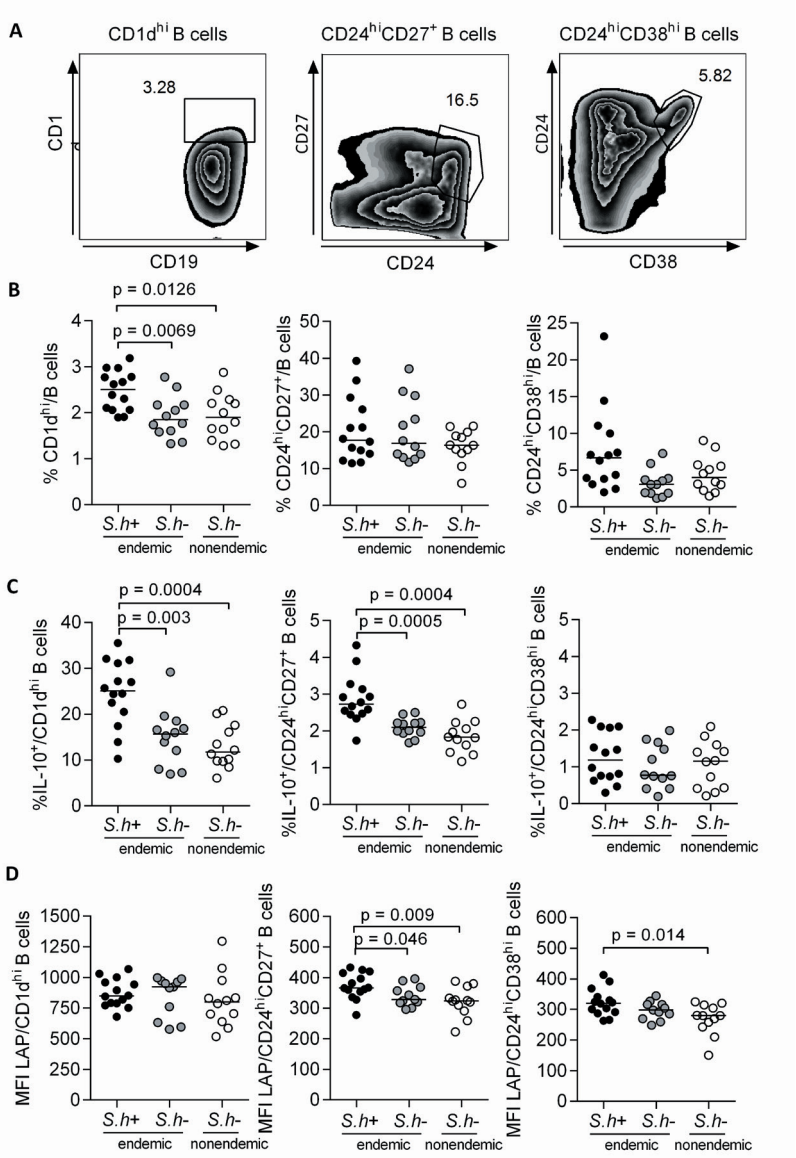

Figure 2. The frequency of different regulatory B cells in the blood specimens from *Schistosoma haematobium*-infected indviduals from a *S. haematobium*- endemic area (*S.h*+), uninfected individuals from a *S. haematobium*- endemic area (*S.h*-) and uninfected individuals from a *S. haematobium*-nonendemic area (*S.h*-). (A) *Ex vivo* PBMCs were gating for CD19^{+}, following by specific Breg subset gating as indicated in the figure by a representative gating example involving a *S. haematobium*-infected donor. (B) The percentage of CD1d^{hi}, CD24^{hi}CD27^{+}, and CD24^{hi}CD38^{hi} B cells. (C) IL-10 production of each B-cell subset after 5 hours ofstimulatuin with PIB+LPS. (D) The geometric MFI expression of LAP/TGF-β1 on the 3 Breg subsets. For all data, the Mann-Whitney *U*-test was used for analysis.

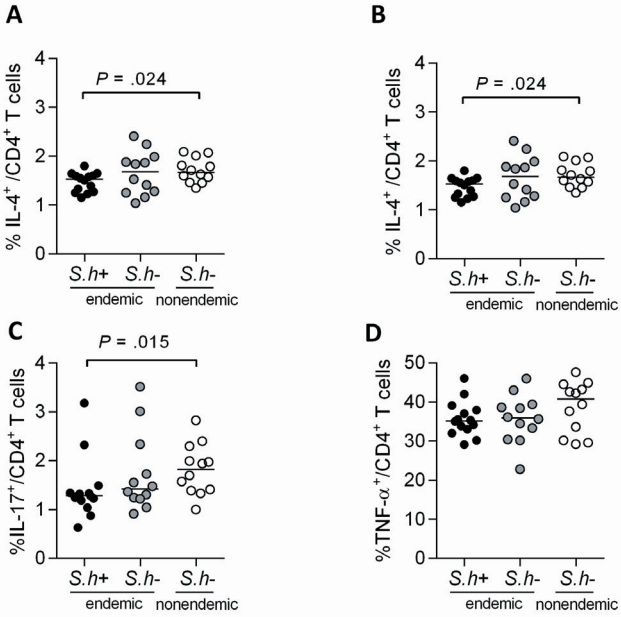

Figure 3. T-cell cytokine expression after coculture with B cells from *S. haematobium*-infected (*S.h*+) and uninfected individuals (*S.h*-) from a *S. haematobium*-endemic area, and uninfected individuals from a *S. haematobium*-nonendemic area (*S.h*-). Isolated B cells were cultured at a ratio of 1:1 with allogenic $CD4^+$ T cells (from 1 European donor) in the presence of soluble anti-CD3 (1 µg/ml) for 3 days. After coculturing, the cells were restimulated with 100 µg/ml PMA and 1 µg/ml ionomycin for 6 hours, in the presence of 10 µg/ml BrefeldinA for the last 4 hours. The cells were fixed with 1.9% PFA. Single cytokine-producing T cells were gated for pro-inflammatory cytokines IFN-γ (A), IL-4 (B), IL-17 (C) and TNF-α (D). The Mann-Whitney *U*-test was used for data analysis.

groups. After the depletion of $CD1d^{hi}$ B cells, the percentage of T cells expressing IL-10 returned to levels similar to those induced by B cells from the uninfected groups, whereas no effect on the (elevated) level of $CD25^{hi}FoxP3^{hi}$ T cells was observed (Fig. 4D, Appendix S3A-B). Indeed, B-cell IL-10 was also reduced to levels found in uninfected donors following CD1d depletion (Fig. 4D, Appendix S3C), suggesting that B-cell expression of IL-10 may play a role in the induction of IL-10-producing T cells but not the induction of $FoxP3^+$ Tregs. TGF-β is known to be important for the generation of $FoxP3^+$ Tregs (32). To investigate its role in Treg induction, cryopreserved Gabonese B cells were cocultured with freshly obtained T cells and blocking anti-TGF-β at our Dutch research institute. In contrast to the culture of fresh specimens performed at the field site, isotype-cultured B cells from infected individuals did not enhance Treg induction, suggesting that freezing and thawing the cells may have affected their function, despite a good viability after thawing (> 94 %; data not shown). Therefore, the role of TGF-β-expressing B cells in the induction of $FoxP3^+$ Tregs could not be addressed within the current study. Nevertheless, it is tempting to suggest that $CD24^{hi}CD27^+$ B cells may be involved in $FoxP3^+$ Treg induction via their enhanced LAP/TGF-β1 production (Fig. 2). Altogether, we showed in a small study set of *Schistosoma*-infected individuals that B cells not only modify effector T-cell responses by reducing their ability to produce pro-inflammatory cytokines, but also increase Treg functions by enhancing the development of $FoxP3^+$ Tregs and IL-10-producing T cells, with the latter depending on the activity of $CD1d^{hi}$ B cells.

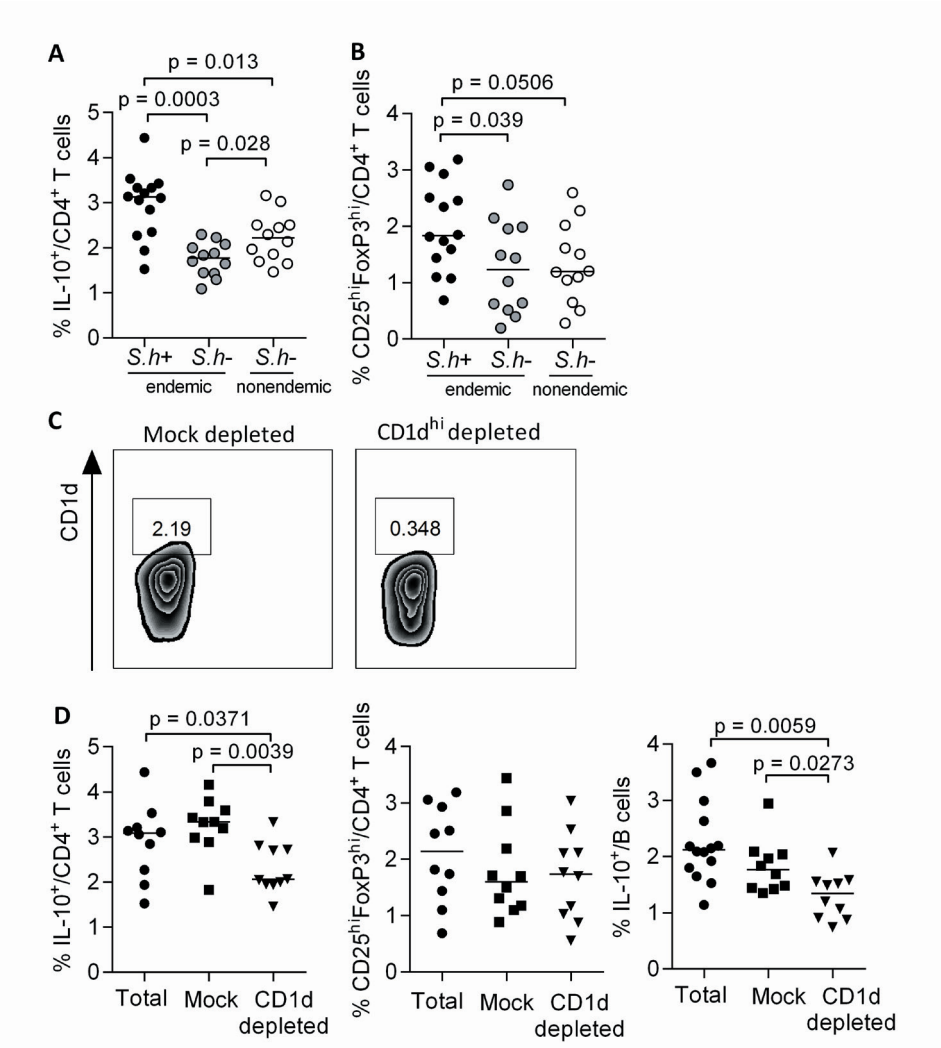

Figure 4. Induction of IL-10$^+$ T and FoxP3$^+$ regulatory (Tregs) cells by B cells. Isolated B cells were cultured at a ratio of 1:1 with allogenic CD4$^+$CD25$^-$ T cells (from 1 European donor) in the presence of soluble anti-CD3 (1 µg/ml) for 6 days. After coculturing, the cells were restimulated with 100 µg/ml PMA and 1 µg/ml ionomycin for 6 hours, in the presence of 10 µg/ml BrefeldinA for the last 4 hours. Cells were then fixed with FoxP3 fixation buffer (eBioscience). (A) T cells were gated for FoxP3$^-$ T cells and subsequently gated for IL-10$^+$(TNF-α$^-$) T cells (Tr1 cells). **(**B) The induction of CD25^{hi}FoxP3^{hi} T cells after coculture with B cells from endemic infected, endemic uninfected or nonendemic uninfected individuals. The Mann-Whitney *U*-test was used for data analysis. Median values ared denoted by horizontal lines, with dots representing individual donors. (C) A representative example of CD1d^{hi} depletion, using Miltenyi Biotec isolation beads, is shown. In each group, total untouched B cells (total), mock-depleted total B cells (mock) and CD1d^{hi}-depleted B cells were cultured with CD25$^-$CD4$^+$ T cells (See Appendix S3 for uninfected groups). (D) The induction of IL-10$^+$ T cells, FoxP3$^+$ Tregs and IL-10 production by B cells after the depletion of CD1d^{hi} B cells in *Schistosoma*-infected individuals. We were not able to perform CD1d depletion for all donors because of a limited number of B cells available. Therefore, 4 donors are missing in the *S.haematobium*-infected group (n=10). The Wilcoxon matched pairs test was used to compare data within the group.

Discussion

In this study, we demonstrated that a greater percentage of B cells from schistosome-infected individuals produce IL-10 and/or LAP/TGF-β1, compared with B cells from uninfected individuals. In coculture with $CD4^+$ T cells, these B cells reduced the production of effector T-cell cytokines and more $FoxP3^+$ and $IL\text{-}10^+$ T cells were found, compared to cultures from uninfected controls. It is suggested that IL-10-producing Bregs detected following *in vitro* stimulations may not only be derived from existing circulating IL-10-producing Bregs but may also be derived from progenitor Bregs (25;33). Indeed, it was demonstrated that these progenitor cells can develop into IL-10-competent Bregs during a 2-day *in vitro* stimulation by CD40 ligand and/or BCR ligation (34). Earlier studies exploring Breg frequencies have not investigated the presence of existing IL-10-competent Bregs, as IL-10 production was only studied following long-term *in vitro* stimulation, which does not permit discrimination between these 2 B-cell populations (20;27). Here, we specifically focused on the presence of already competent IL-10 Bregs and found that a greater percentage of peripheral blood B cells from schistosome-infected individuals could readily produce cytoplasmic IL-10, without long-term *in vitro* priming or maturation of B cells. Presumably, these processes already occurred *in vivo,* indicating that schistosome infections indeed prime for the development of readily active IL-10-producing Bregs in humans.

Several studies demonstrated that Bregs may simultaneously produce various regulatory cytokines, such as IL-10 and TGF-β. For example, LPS induced both IL-10 and TGF-β secretion in B cells, and these LPS-activated B cells suppressed the development of diabetes by promoting apoptosis of effector T cells. However, the responsible cytokine was not addressed in this study (18). Furthermore, in food-allergic mice, allergen-specific T-cell proliferation was suppressed by activated $CD5^+$ B cells secreting both TGF-β and IL-10 (35). Interestingly, similar IL-10- and TGF-β-producing $CD5^+$ B cells were found in healthy individuals upon milk antigen stimulation (7;36). Lastly, co-expression of IL-10 and TGF-β was also demonstrated in human $CD25^{hi}CD1d^{hi}CD27^{hi}$ B cells (37). However, other studies have suggested that IL-10 and TGF-β are not always expressed by the same Breg subset. For example, $CD5^+$ B cells from mice chronically exposed to ovalbumin and tolerant for allergic airway inflammation, predominantly expressed IL-10, while TGF-β was not restricted to $CD5^+$ B cells only (8). This is in agreement with the data presented here, as we also observed that IL-10 and LAP/TGF-β1 were expressed by different Breg subsets: the infected group had a greater percentage of $CD24^{hi}CD27^+$ B cells expressing LAP/TGF-β1 and had a greater percentage of $CD1d^{hi}$ B cells producing IL-10 compared with the uninfected group. This may suggest that helminth infections can promote the activity of 2 different B-cell subsets.

Enhanced Treg activity is one of the hallmarks of chronic *Schistosoma* infections (38). Because part of schistosome-induced Breg activity in mice is

centered on their capacity to induce Tregs and modulate T-cell cytokines, we focused on these aspects here. However, during a natural infection it is also possible that other cells, such as antigen-presenting cells, may also be influenced by Bregs (25;39). Here, we find a clear induction of $FoxP3^+$ Tregs and $IL\text{-}10^+$ T cells by schistosome-induced B cells, and we tried to identify the responsible Bregs. Previous studies showed that both IL-10 and TGF-β can induce $FoxP3^+$ Tregs (37;40). However in our study, cocultures with B cells depleted for $CD1d^{hi}$ B cells, the main source of IL-10, mainly reduced $IL\text{-}10^+$ T cells without affecting $FoxP3^+$ T-cell levels. This suggest that the IL-10-producing B cells mainly induced $IL\text{-}10^+$ T cells but not $FoxP3^+$ Tregs, although we cannot exclude the contribution of other (co)factors to this process. At this stage, it is unclear which schistosome-induced Breg subset is responsible for the enhanced Treg induction, although it is tempting to suggest that, given the role for TGF-β in $FoxP3^+$ Treg induction, TGF-β-producing B cells (ie, the $CD24^{hi}CD27^+$ B cells) are involved here.

We previously reported that 6 months after helminth treatment, B cells still showed increased IL-10 secretion upon *in vitro* stimulation with soluble egg antigens (SEA), suggesting the presence of a persistent pool of memory schistosome-specific B cells after clearance of infection (20). Our *S.haematobium*-uninfected donors are coming from a schistosome-endemic area and former infections are likely to have occurred, which may have promoted Breg activity in these individuals at an earlier stage. Indeed, B cells from endemic uninfected group express more IL-10 and TGF-β compared to uninfected individuals from a close-by nonendemic area. In addition, this may explain the similar levels of effector T-cell cytokines found in cocultures by B cells from schistosome-infected and uninfected individuals from the same area. Interestingly, the Treg-inducing and $IL\text{-}10^+$ T-cell-inducing capacity was not enhanced in B cells from endemic uninfected group, indicating that some of the activities or some of the Breg subsets are longer lasting after infection, while others may diminish with time.

Several mouse models showed that parasite products from either *Leishmania major* or *Brugia malayi* and the milk glycan LNFPII, containing LeX motifs similar to several schistosome glycoconjugates, can enhance B-cell-derived IL-10 via Toll-like receptor 4 ligation (41;42). Furthermore, *in vitro* stimulation of splenocytes by live schistosome worms induced IL-10-producing B cells, which dampened allergic airway inflammation upon transfer (19). Lastly, B cells from helminth-infected patients with multiple sclerosis stimulated with SEA resulted in a Toll-like receptor 2-mediated IL-10 production (43). These findings suggest that the development of Bregs, as observed in schistosome-infected individuals, may be specifically related to the presence and activity of certain helminth-derived molecules. However, we cannot exclude that Breg development may also occur as a bystander process resulting from continuous chronic infection in which the immune system desperately tries to counterbalance the inflammation induced by the continuous presence of helminths. Indeed, also other chronic infections with viral or bacterial agents show enhanced IL-10-producing B cells (22;24;26). However, what is interesting and what may point to a helminth-

specific Breg population, is that the affected Breg subsets in those studies were not characterized by CD1d^{hi}, suggesting that this population is mostly restricted to helminth infections (20;27).

Reports on patients with autoimmune (23;44;45) or allergic diseases (7;36) have shown impairments in Breg frequencies or function, suggesting a role for Bregs in the maintenance of peripheral tolerance in healthy individuals. Therapies aiming to enhance Breg numbers or activity may have potential for future treatment of inflammatory diseases. Interestingly, upregulation of Breg activity was observed in patients with bee venom allergy following successful allergen-specific immunotherapy, reaching similar levels as found in tolerant beekeepers (46). As we here observed that schistosome-induced Bregs can affect T-cell responses (ie, reduced effector T-cell cytokines and more Treg induction), applying helminth-derived molecules may form an interesting novel therapy for hyperinflammatory disorders by driving potent Bregs.

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Conflict of interest
All authors declare no conflict of interest.

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Supplementary figures

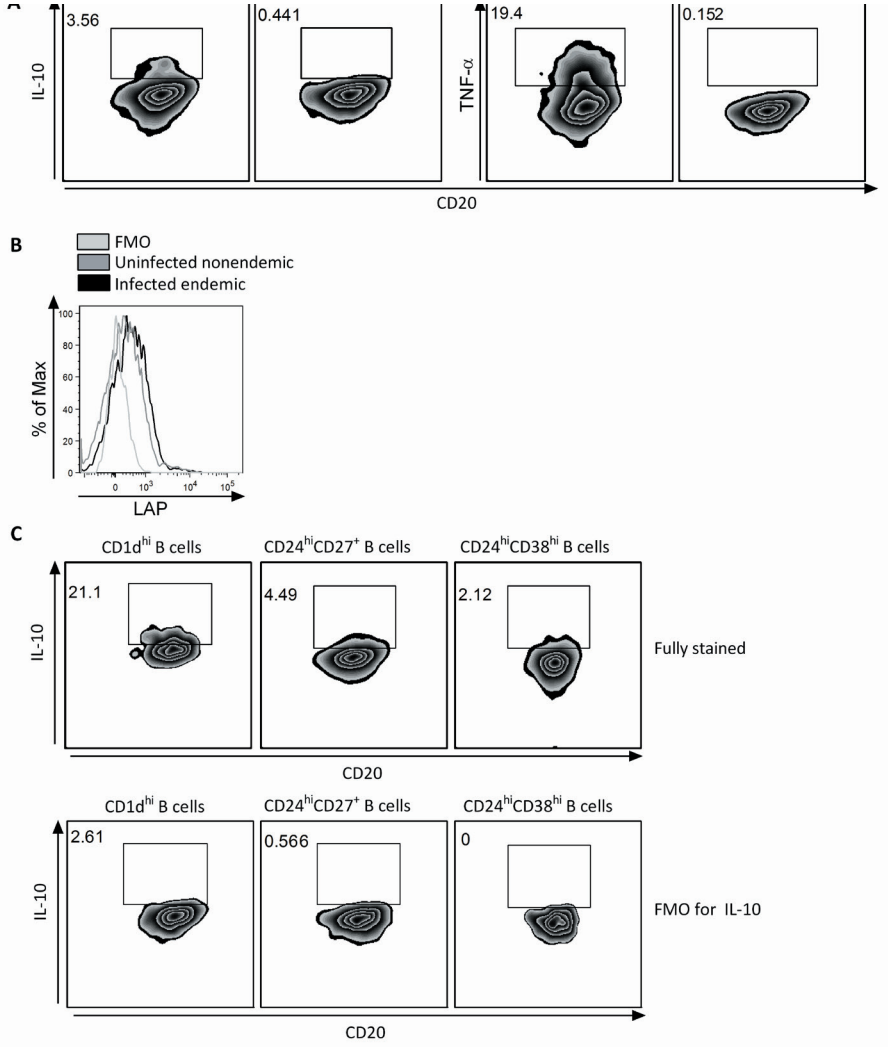


Figure S1. (A) A representative gating example of intracellular IL-10 and TNF-α production upon 5 hours PIB+LPS stimulation in total B cells from a endemic infected (*S.h*+) individual. Fluorescence minus one (FMO) shows background levels of IL-10 and TNF-α. B, A representative gating example for LAP/TGF-β1 expression on *ex vivo* total B cells from endemic infected (including FMO) and nonendemic uninfected (*S.h*-) individual. C, Intracellular IL-10 staining upon 5 hours PIB+LPS stimulation in $CD1d^{hi}$, $CD24^{hi}CD27^{+}$, $CD24^{hi}CD38^{hi}$ B cells from a endemic infected individual. Fluorescence minus one (FMO) shows background levels of IL-10 in the different Breg subsets.

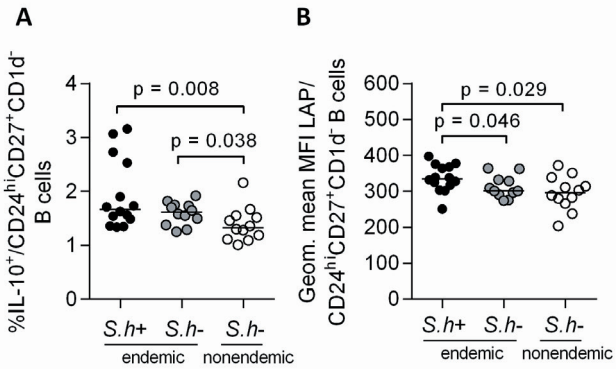


Figure S2. (A) IL-10 production and (B) LAP/ TGF-β1 membrane-bound expression of $CD24^{hi}CD27^{+}$ B cells after gating out the $CD1d^{hi}$ B cells in this population. The Mann-Whitney *U*-test was used for data analysis.

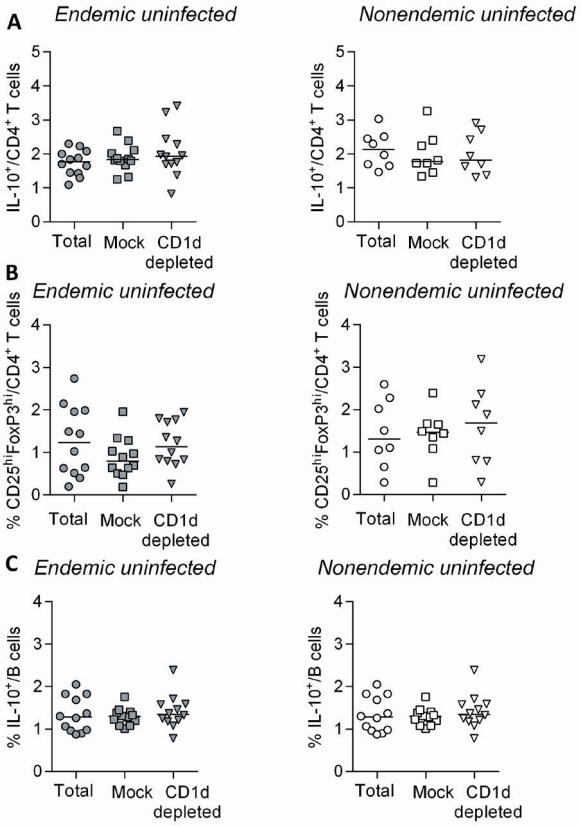
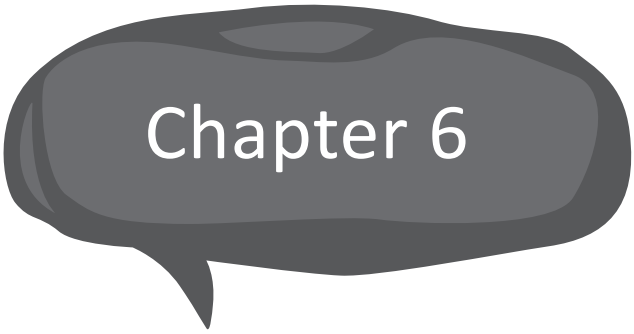


Figure S3. Induction of $IL\text{-}10^{+}$ T and $FoxP3^{+}$ Treg cells by B cells. Isolated B cells were cultured at a ratio of 1:1 with allogenic $CD25^{-}CD4^{+}$ T cells (from 1 European donor) in the presence of soluble anti-CD3 (1 µg/ml) for 6 days. After coculturing, the cells were restimulated with 100 µg/ml PMA and 1 µg/ml ionomycin for 6 hours, in the presence of 10 µg/ml BrefeldinA for the last 4 hours. The cells were then fixed with FoxP3 fixation buffer (eBioscience). (A) The induction of IL-10-producing T cells after coculture with B cells from endemic uninfected and nonendemic uninfected donors. (B) The induction of $FoxP3^{+}$ Tregs and C, IL-10 production by B cells. We were not able to perform CD1d depletion for all donors due to a limited number of B cells available. Therefore, the total individuals in the endemic uninfected group were n=12 (0 missing), and in the nonendemic uninfected group n=8 (4 missing). For all data, Wilcoxon matched pairs test was used for data analysis.



$CD24^{hi}CD27^{+}$ B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide

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Abstract

Background Regulatory B cells have been identified that strongly reduce allergic and auto-immune inflammation in experimental models by producing IL-10. Recently, several human regulatory B-cell subsets with an impaired function in auto-immunity have been described, but there is no information on regulatory B cells in allergic asthma.

Objective In this study, the frequency and function of IL-10-producing B-cell subsets in allergic asthma were investigated.

Methods Isolated peripheral blood B cells from 13 patients with allergic asthma and matched healthy controls were analyzed for the expression of different regulatory B-cell markers. Next, the B cells were activated by lipopolysaccharide (LPS), CpG or through the B-cell receptor, followed by co-culture with endogenous memory $CD4^+$ T cells and house dust mite allergen Derp1.

Results Lower number of IL-10-producing B cells were found in patients in response to LPS, however, this was not the case when B cells were activated through the B-cell receptor or by CpG. Further dissection showed that only the $CD24^{hi}CD27^+$ B-cell subset was reduced in number and IL-10 production to LPS. In response to Derp1, $CD4^+$ T cells from patients co-cultured with LPS-primed total B cells produced less IL-10 compared to similar cultures from controls. These results are in line with the finding that sorted $CD24^{hi}CD27^+$ B cells are responsible for the induction of $IL\text{-}10^+$ $CD4^+$ T cells.

Conclusions Taken together, these data indicate that $CD24^{hi}CD27^+$ B cells from allergic asthmatic patients produce less IL-10 in response to LPS leading to a weaker IL-10 induction in T cells in response to Derp1, which may play a role in allergic asthma.

Introduction

Allergic asthma (AA) is a reversible obstructive lung disease, in which cross-talk between inflammatory cells and structural lung cells is central. There is a negative association between reduced exposure to infectious agents and increased prevalence of asthma and allergy in affluent populations (1), leading to the hypothesis that childhood exposure to environmental microbes helps to prevent allergy (2). Abundant early-life microbial exposure, such as to farm animals, unpasteurized milk products and bacterial DNA and endotoxin (lipopolysaccharide, LPS), is thought to instruct a robust development of the regulatory arm of the immune system, preventing uncontrolled inflammatory responses to allergens (2-6).

In healthy individuals, immune responses to allergens are either absent or characterized by an enhanced production of IL-10. Whereas (regulatory) T cells are known for their capacity to produce IL-10 (7), IL-10 is also produced by other cell types, including B cells. Interestingly, initial studies using adoptive transfers in auto-immunity models have shown that IL-10-producing B cells have a strong potential to reduce inflammation and, therefore, are called regulatory B (Breg) cells (8-11). Importantly, recent mouse studies have demonstrated that Breg cells can reduce allergic inflammation in both anaphylactic and airway inflammation models by virtue of their IL-10 production and the induction of Treg cells (12-15).

Various Breg subsets in humans, characterized by the expression of $CD24^{hi}CD38^{hi}$ or by $CD24^{hi}CD27^{+}$, have been described and show a reduced function in certain auto-immune diseases, such as systemic lupus erythematosus (SLE) (16;17). During chronic hepatitis B virus infection, $CD24^{hi}CD38^{hi}$ B cells were described to produce IL-10 and thereby suppress antigen-specific $CD8^{+}$ T cell responses (18). In addition, an enhanced $CD1d^{hi}$ Breg subset was described in both schistosome-infected Gabonese individuals (14) and in mixed helminth-infected Brazilian multiple sclerosis (MS) patients which could inhibit auto-reactive T cell responses *in vitro* (19). Additionally, a reduced IL-10 production of B cells from MS patients in response to CD40 ligation and B-cell receptor (BCR) cross-linking was reflected mostly in the $CD27^{-}$ naive B cells (20). Taken together, different human Breg subsets may exist and for some their function is impaired in certain auto-immune diseases.

Several articles have studied the signals that control regulatory B cell number and/or activity, and have identified both BCR triggering and TLR ligation as important pathways to induce IL-10 production and functional regulatory activity. In this respect, TLR2 and 4 have been shown to promote IL-10-producing B cells in different infection models: for example, extracts from *Leishmania major* or *Brugia malayi* microfilaria use TLR4 (21), *Helicobacter felis* use TLR2 (22) and in *Salmonella typhimurium* infections MyD88-dependent signaling in B cells is crucial for IL-10 induction (23).

So far, no information is available on the presence of regulatory B cells in individuals with allergic asthma (AA) or on TLR or BCR-related pathways that can promote their development. As abundant exposure to bacterial products in the farm studies (and in particular to endotoxin) is linked to protection against allergic inflammation and bacterial products promotes IL-10-producing B cells in animal models, we focused on LPS and CpG as TLR stimuli to investigate its IL-10-inducing capacity in human B cells. Therefore, we here describe the Breg cell frequency in peripheral blood B cells from patients with AA compared to healthy controls (HC) and investigated the capacity to produce IL-10 in response to LPS, CpG or to BCR triggering and their ability to influence T-cell cytokine production.

Materials and methods

Study population

Non-smoking (or ex-smoking for more than 1 year, less than 10 pack-years) AA patients with house dust mite (HDM) allergy and control (*n*=13; age: 19-45 years; Table 1) were subjected to asthma control questionnaires (ACQ; based on (24)), 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. Nitric oxide (FeNO) 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: PC_{20} >19 mg/ml, negative SPT for all antigens and total IgE <30 IU/ml. Inclusion criteria for patients with AA: PC_{20} <9.6 mg/ml and a positive SPT for at least HDM (wheal >5 mm), total IgE ≥30 IU/ml and detectable specific IgE for at least HDM (> 0.7 kU/l). Patients with AA (seven out of 13) using inhaled corticosteroids or combination therapy were asked to cease their medication 2 weeks before blood taking. Ten healthy control subjects donated fresh blood samples twice. In addition, peripheral blood from healthy laboratory donors were used to perform some validation experiments (secretion assays, signaling and ELISA). The study was approved by the Medical Ethical Committee LUMC (P09.170) and performed according to the declaration of Helsinki. Written informed consent was given by all participants.

B- and T-cell isolation and flowcytometry

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation in 50 ml tubes (Greiner Bio-One) from heparinized blood. B cells with a purity of ~98% were isolated by anti-CD19 MicroBeads (Miltenyi Biotec). The remaining cells were collected for further isolation of memory $CD45RO^{+}CD4^{+}$ T cells using MicroBeads (Miltenyi Biotec). PBMCs or isolated B cells were stained in 96-well V-bottom plates (Greiner Bio-One) for CD1d-PE (CD1d42, BD), CD5-PercPeFluor710 (UCHT2, eBioscience), CD20-APC-eFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, Biolegend), CD27-APC (L128, BD), CD38-FITC (HIT2, BD), CD19-Pacific Blue (HIB19, Biolegend) and TLR4-biotin (HTA125, Biolegend) in combination with streptavidin-Qdot525 (Invitrogen) and FcγR-binding inhibitor (eBioscience) to define the different Breg cell subsets. CD20, which was equally expressed as CD19 on all B cell (subsets), was used to define B cells because of the MACS CD19 isolation procedure. Fluorescence minus one (FMOs) were used for proper gate setting for all markers.

In a subset of healthy control donors, cells were stained for CD24-PeCy7 (ML5, Biolegend) and CD27-APC (L128, BD Biosciences) after $CD19^{+}$ B cell isolation and sorted into $CD24^{hi}CD27^{+}$ and remaining B cells using FACSAriaIII flowcytometer (BD Biosciences).

B-cell stimulation and intracellular staining for IL-10

B cells (1 x 10^5) were stimulated with 100 ng/ml ultra-pure LPS (Invitrogen), 5 µg/ml CpG ODN2006 (Invitrogen) or 20 µg/ml anti-human IgG/IgM (Jackson-ImmunoResearch) in RPMI (Invitrogen) supplemented with 10% FCS (Bodinco). All stimulations were tested to be optimal for IL-10 production at the given concentrations (data not shown). After 48 h, supernatants were taken and B cells were restimulated with PMA (200 ng/ml), ionomycin (2 µg/ml), and LPS (100 ng/ml) for 6 h and the last 4 h in the presence of Brefeldin A (BrefA; 10 µg/ml; Sigma-Aldrich), followed by fixation with 1.9% PFA. Next, the cells were permeabilized 0.5% saponin buffer and stained for CD1d-PE (CD1d42, BD), CD5-PerCPeFluor710 (UCHT2, eBioscience), CD20-APC-eFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, Biolegend), CD27-APC (L128, BD), and CD38-FITC (HIT2, BD), IL-10-Biotin plus streptavidin-Qdot525 (JES3-12G8, Abd Serotec/Invitrogen), TNF-α-eFluor450 (MAB11, eBioscience) and FcγR-binding inhibitor (eBioscience). FMOs were used for gate setting for all surface markers and cytokine expression.

B-T cell co-culture

1 x 10^5 B cells were stimulated with medium, ultra-pure LPS, CpG or anti-human IgG/IgM for two days, followed by removal of supernatant, washed, and co-cultured with autologous memory $CD4^+$ T cells (ratio 1:1) in the presence of 1 µg/ml endotoxin-free Derp1 (LoTox Derp 1, Indoor Biotechnologies) in RPMI supplemented with 10% FCS. $CD4^+$ T cells were first pre-incubated with blocking IL-10R antibodies (2 µg/ml; R&D Systems) or IgG1 isotype control (2 µg/ml) for 30 min at 37 °C. After six days, supernatants were taken and the cells were restimulated by PMA/iono and BrefA (as indicated above) and fixated with FoxP3 fixation/permeabilization buffer (eBioscience). The cells were stained for CD3-PerCPCy5.5 (SK7, BD), CD20-Pacific Blue (2H7, Biolegend), CD25-PE (2A3, BD), IL-10-Biotin plus streptavidin-Qdot525 (JES3-12G8, Abd Serotec/Invitrogen), TNF-α-PeCy7 (MAB11, eBioscience), FoxP3-FITC (PCH101, eBioscience), and FcγR-binding inhibitor (eBioscience). FMOs were used for gate setting for all surface markers and cytokine expression.

Cytokine Secretion assay, ELISA and Luminex

From PBMC, monocytes were first depleted prior to isolation of B cells using CD14 MicroBeads (Miltenyi Biotec). After the B-cell isolation, cells were stimulated for 3 days (adapted from Milovanovic *et al*. (25)). Next, the B cells were restimulated with PMA and ionomycin for 2 h, washed, and incubated for1 hour with IL-10 catch reagent according the protocol (Miltenyi Biotec). After the incubation, cells were stained for IL-10-PE (dilution 1:25, Miltenyi Biotec), CD20-Pacific Blue (2H7, BioLegend), life death marker 7-AAD-PerCP (E00031-1632, eBioscience) and FcγR-binding inhibitor (eBioscience). Collected supernatants collected were measured by Enzyme-Linked Immunosorbent Assay (ELISA) for IL-10 (Sanquin) or by Luminex (IL-5, IFN-γ, IgG1, IgG2, IgG4, and IgE, Invitrogen/Biorad).

Western blot

For whole cell lysate, $1x10^6$ B cells from 4 healthy donors from laboratory staff were stimulated for 5, 20, 60, 180 min with medium or LPS (100 ng/ml), washed and treated with a buffer containing 8% v/w glycerol, 3% SDS, and 100mM Tris-PO4 (EBSB) for 5 min. The lysate was heated to 95 °C for 5 min and then stored at -80 °C. The protein concentration of the whole cell lysates was determined by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Whole cell lysates (10 µg/lane) were boiled in SDS sample buffer (50 mM Tris-Cl (pH 6.8), 1% 2-ME, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) for 5 min before being subjected to electrophoresis on 10% or 12.5% SDS-PAGE gels. After separation, the proteins were transferred onto nitrocellulose (Millipore). The membrane was incubated with primary antibodies against STAT3, and Phospho-STAT3 (Tyr 705) (D3A7) (all from Cell signaling Technology) and then incubated with anti-rabbit IgG HRP conjugated secondary antibodies (Promega). The bands were visualized by an enhanced chemiluminescence reagent (Thermo scientific).

Statistical analysis

For statistical analysis, Kruskal-Wallis 1-way ANOVA and the Mann-Whitney *U* tests were performed. Differences in paired conditions were analyzed by Wilcoxon matched pairs test. Correlations were analyzed using SPSS Statistics 20 (IBM, New York, USA). Probability values less than 0.05 were considered significant.

Results

Patient characteristics

To study the function and phenotype of peripheral blood B cells, 13 non-smoking patients with AA (age, 19-44 years; 7 females and 6 males) and 13 age- and sex-matched non-smoking HC were included (age, 21-45 years). The patients with AA showed clinically controlled asthma without severe exacerbations for more than 6 months (GINA guidelines). They had the following characteristics (median): an ACQ value of 4, a FEV_1-% predicted of 101, a FEV_1/FVC (as measure for obstruction) of 0.80, FeNO of 30 and a PC_{20}-methacholine of 1.4 mg/ml (Table 1), which match values for mild to moderate bronchohyperreactivity. The HC showed no signs of asthma by an ACQ outcome of 0, a FEV_1-% predicted of 112, a FEV_1/FVC of 0.87, FeNO of 13 and a PC_{20}-methacholine of >19 mg/ml.

Positive SPT reactivity with a wheal larger than 5 mm was recorded against HDM in 12 patients with AA, against grass and/or tree pollen in 10 patients and against cat and/or dog in eight patients (Table 1). The median of total serum IgE levels was 108 IU/ml and the median for specific IgE for HDM was 13 IU/ml, for grass 0.7 IU/ml, for tree pollen 0.2 IU/ml, for cat 0.4 IU/ml and for dog 0.4 IU/ml (Table 1). The HC group showed a negative SPT for HDM, cat, dog, tree and grass pollen or Aspergillus and a median total serum IgE of 7 IU/ml was recorded, which was significantly lower than the patients with AA. The blood of patients and of their matched controls was processed and analyzed simultaneously. In addition, the lymphocyte count (PBMC/ml blood) and the frequencies of B and memory $CD4^+$ T cells were similar between the groups (data not shown).

Lower numbers of $CD24^{hi}CD27^+$ B cells in patients with AA

To investigate the frequency of the different Breg cell subsets, isolated peripheral blood $CD19^+$ B cells were stained for CD1d, CD5, CD20, CD24, CD27 and CD38. Gating examples of a paired patient and healthy control are shown in Figure 1 to define the following three different subsets: $CD1d^{hi}$ B cells, $CD24^{hi}CD38^{hi}$ B cells and $CD24^{hi}CD27^+$ B cells. No differences were seen in frequency of $CD1d^{hi}$ B cells (average of 2%; Fig. 1A) or in the smaller subset $CD1d^{hi}CD5^+$ B cells (average of 0.3%; data not shown) between the groups. Of note, despite its confusing nomenclature the CD1d expression on human B cells is generally very low, but detectable as shown by figure 1. Although the percentage of transitional immature $CD24^{hi}CD38^{hi}$ Breg cells was significantly increased in patients with AA (Fig. 1B), the absolute numbers per ml blood were not similar to that described by Blair *et al.* in SLE patients (16). Interestingly, both the percentage and absolute numbers per ml blood of $CD24^{hi}CD27^+$ B cells were significantly reduced in patients with AA compared to controls (Fig. 1C), while the distribution of naïve ($CD27^-$) and memory B cells ($CD27^+$) or $CD24^+$ B cells were similar between the groups (data not shown). Of note, the frequency of $CD24^{hi}CD27^+$ B cells was the highest of the Breg cell subsets (~27 % $CD24^{hi}CD27^+$ vs. ~ 3 % $CD24^{hi}CD38^{hi}$ vs. ~2% $CD1d^{hi}$ B

Table 1. Subject characteristics

	AA (n=13)	HC (n=13)	*P* value
Sex (f)	7 (13)	7 (13)	-
Age (yrs)	26 (19-44)	24 (21-45)	0.9379
BMI	22 (19-35)	22(18-24)	0.3517
ICS/CT	6(13) + 4(13)	-	-
FeNO	30 (13-80)	13 (5-21)	0.001
ACQ	4 (0-12)	0 (0-1)	-
FEV_1-%predicted	101 (92-118)	112 (102-128)	0.0026
FEV_1/FVC	0.8 (0.64-0.91)	0.87 (0.77-0.97)	0.1637
PC_{20}-methacholine	1.4 (0.3-9.6)	>19	-
SPT (> 5mm)			
HDM	12/13	-	-
Grass and/or Tree	10/13	-	-
Cat and/or Dog	8/13	-	-
Total IgE (IU/ml)	108 (30-307)	7 (2-31)	<0.0001
Spec. IgE (IU/ml)			
HDM (n=9)	13 (0.11-50.6)	-	-
Grass (n=9)	0.7 (0.03-37.9)	-	-
Tree (n=9)	0.2 (0.02-10.3)	-	-
Cat (n=9)	0.4 (0.01-12.4)	-	-
Dog (n=9)	0.4 (0.1-2.7)	-	-

Allergic Asthma (AA); Healthy Controls (HC); female (f); years (yrs); Body mass Index (BMI); inhalation corticosteroids (ICS); combination therapy (CT); Exhaled nitric oxide (FeNO); Asthma Control Questionnaire (ACQ); 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)

cells in controls). We did not detect any correlation between the percentage of $CD24^{hi}CD27^{+}$ B cells or any of the other Breg cells and the level of total serum IgE, HDM-specific serum IgE, $PC_{20,}$ exhaled FeNO or medication usage in the asthmatic patients (data not shown).

Less intracellular IL-10 in B cell from patients with AA in response to LPS

One of the hallmarks of Breg cells is their capacity to produce IL-10. To compare the IL-10-producing capacity of B cells from AA and HC subjects, $CD19^{+}$ B cells were isolated from peripheral blood and stimulated for 2 days with medium alone, anti-IgG/IgM (general BCR trigger), LPS (TLR4 ligand) or CpG (TLR9 ligand). Subsequently, the cells were restimulated by PMA/ionomycin in the presence

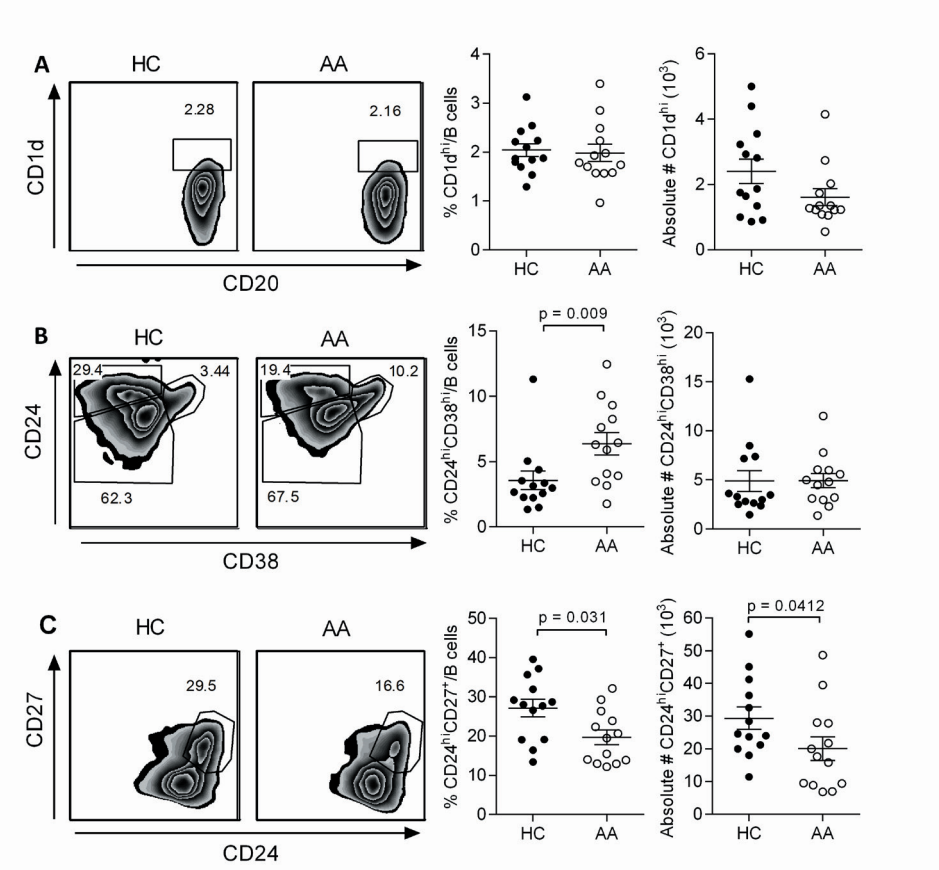

Figure 1. The frequency of different regulatory B cells in the blood from patients with AA and healthy controls. A representative gating example is depicted on the left for each Breg cell subset, showing one matched HC and AA donor pair. (A) $CD1d^{hi}$ B cells (B) $CD24^{hi}CD38^{hi}$ B cells and (C) $CD24^{hi}CD27^{+}$ B cells are expressed as percentage or as absolute numbers (#) per ml blood.

of BrefeldinA to allow intracellular cytokine detection (Fig. 2A). In medium-conditioned B cells ~2 % IL-10 producers were found, while both BCR stimulation and LPS priming significantly further enhanced intracellular IL-10 levels by almost a two-fold (Fig. 2B). CpG showed the strongest IL-10 induction, inducing approximately 10%. Strikingly, only intracellular IL-10 in LPS-primed B cells of patients with AA was significantly lower compared to those from healthy controls (Fig. 2B), while equal IL-10 levels were found for medium-conditioned, BCR- and CpG-stimulated B cells. We analyzed the mean fluorescence intensity (MFI) values of the cytokine-expressing cells, demonstrating a similar MFI for $IL\text{-}10^{+}$ cells in all conditions following background subtraction (MFI $IL\text{-}10^{+}$ approx. 5200, data not shown) and similar between HC and patients with AA. This suggests that mostly the number of responding B cells varied per condition but not so much the quantity of IL-10 produced per B cell. No correlation was found between LPS-induced IL-10 levels and serum IgE levels (and HDM-specific IgE), $PC_{20,}$ exhaled

FeNO or medication use in patients with AA. The production of the more pro-inflammatory cytokine TNF-α was also analyzed to exclude general increased cytokine levels, but no differences were found between the two groups for any of the stimuli. The IL-10-producing B cells did not co-produce TNF-α (Fig. 2C).

Secreted IL-10 levels in the supernatant of CpG-stimulated B cells confirmed the flowcytometry data and showed clearly detectable IL-10 levels, but not different between HC (210 pg/ml) and AA donors (190 pg/ml, $P = 0.47$). Unfortunately, we were unable to detect secreted IL-10 or TNF-α by ELISA in the supernatant of stimulated B cells in response to LPS or anti-IgG/M, due to either limited cell numbers (a minimum of 3 x 10^5 B cells is required; data not shown) or high background levels (caused by anti-IgG/M itself; data not shown). Therefore, we validated the LPS-induced IL-10 production by conducting an IL-10 secretion assay allowing secretion and capture of IL-10 at the B cell surface. Using this assay, secreted IL-10 was studied in B cells from healthy laboratory donors stimulated with medium, LPS, anti-IgG/M or CpG for 3 days (adapted from Milovanovic *et al.* (25). A twofold increase of B-cell IL-10 was observed for both LPS and anti-IgG/M

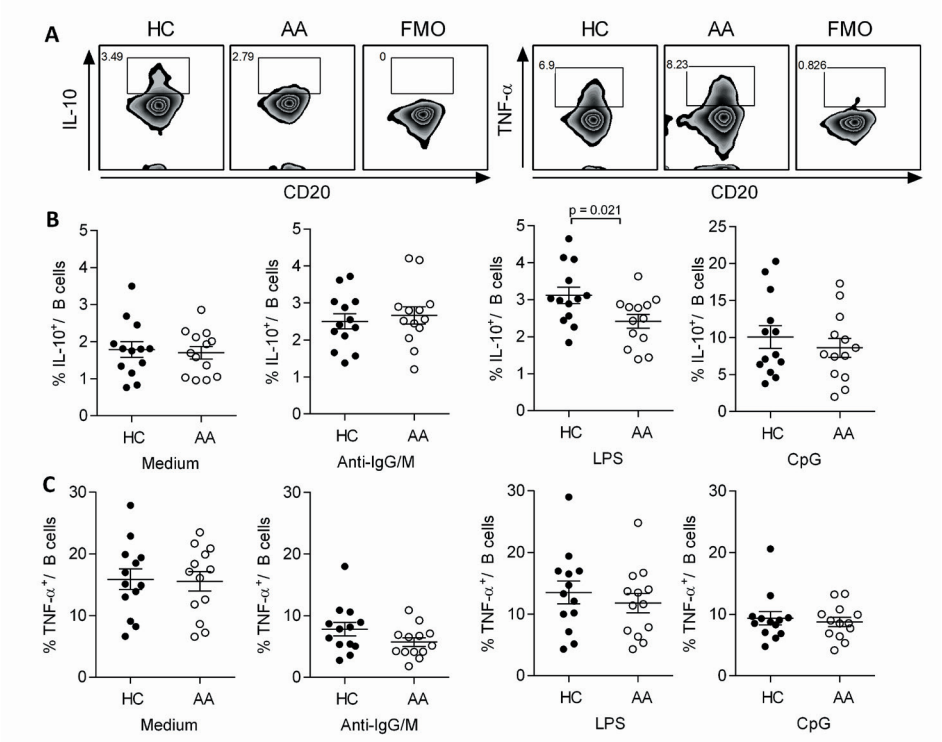

Figure 2. Intracellular IL-10 detection in total B cells. B cells were stimulated for 48 h by medium, lipopolysaccharide (LPS) (100 ng/ml), CpG (5 μg/ml) or anti-IgG/IgM (20 μg/ml) followed by restimulation with PMA/Ionomycin/LPS in the presence of BrefeldinA. (A) A representative gating example of IL-10 and TNF-α production upon LPS stimulation of a matched healthy control (HC) and allergic asthma (AA) donor. Fluorescence minus one (FMO) shows background levels of IL-10 and TNF-α. (B) Intracellular IL-10 and (C) intracellular TNF-α production were analyzed using flowcytometry.

stimulation, and an even higher increase for CpG, confirming the data obtained by intracellular cytokine staining described above (Appendix S1).

Reduced LPS responsiveness of CD24^{hi}CD27^{+} B cells in allergic asthma patients

We next questioned whether the lower LPS-induced IL-10 in B cells from patients with AA was the result of a selective impairment in one of the putative Breg subsets. Breg subset analysis showed that LPS stimulation of B cells strongly enhanced the number of IL-10^{+} cells compared to medium in all three subsets, i.e. CD1d^{hi} B cells (Fig. 3A), CD24^{hi}CD38^{hi} (Fig. 3B) and CD24^{hi}CD27^{+} B cells (Fig. 3C), although CD1d^{hi} B cells tend to have a higher spontaneous production of IL-10 (~7%) in medium conditions. When comparing patients with AA and controls, we found less intracellular IL-10 for CD24^{hi}CD27^{+} B cells in patients with AA (Fig. 3C), but similar levels in the other two Breg subsets (Fig. 3A and 3B). In the remaining CD24^{+}, CD27^{+} or CD24^{-}CD27^{-} B cells, the IL-10 production was not significantly affected in patients with AA (Appendix S2A). Despite the fact that the different Breg cell subsets have been analyzed separately in the previous studies, they do have some overlap. Interestingly, a substantial part of the CD1d^{hi} B cells (61.7% for HC and 56.1% for AA; P = 0.46) can be found within the CD24^{hi}CD27^{+} B cell subset and for both groups 17.5% of the CD24^{hi}CD38^{hi} are also CD24^{hi}CD27^{+} (P = 1.00). When CD1d^{hi} B cells were removed from the analysis of the CD24^{hi}CD27^{+} population, the decreased IL-10 production still remained in the B cells from patients with AA. Additionally, the exclusion of the CD24^{hi}CD38^{hi} B cells gave similar results (Appendix S2B). Altogether, these data suggest a diminished IL-10 response of CD24^{hi}CD27^{+} B cells from patients with AA compared to controls upon LPS stimulation.

TLR4 expression on B cells from patients with AA compared to healthy controls

To investigate whether the differences in B cell IL-10 production between the groups were a consequence from differences in TLR4 expression in B cells, we

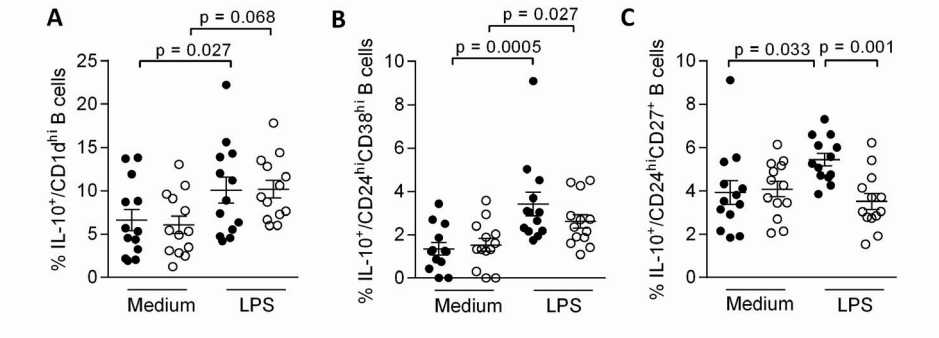

Figure 3. Intracellular IL-10 in various B cell subsets in response to lipopolysaccharide (LPS). B cells were stimulated by medium or LPS and treated as described in Fig. 2. Intracellular IL-10 in CD1d^{hi} B cells (A), in CD24^{hi}CD38^{hi} B cells (B) and in CD24^{hi}CD27^{+} B cells (C) were analyzed using flowcytometry.

evaluated the TLR4 levels by flowcytometry. Although the expression of TLR4 on B cells is rather low compared to monocytes (data not shown, MFI of 3629 (HC) and 3280 (AA), *P* = 0.10), a significant higher MFI was detected both for HC donors and patients with AA compared to total T cells (as a negative control) (Fig. 4A). Nevertheless, we did observe a trend towards a decreased TLR4 expression in AA patients, suggesting that their lower IL-10 response to LPS might be due to lower TLR4 expression, however, this was not attributed to either one of the B cell subsets (Fig. 4B). Interestingly, we detected the highest TLR4 expression on the

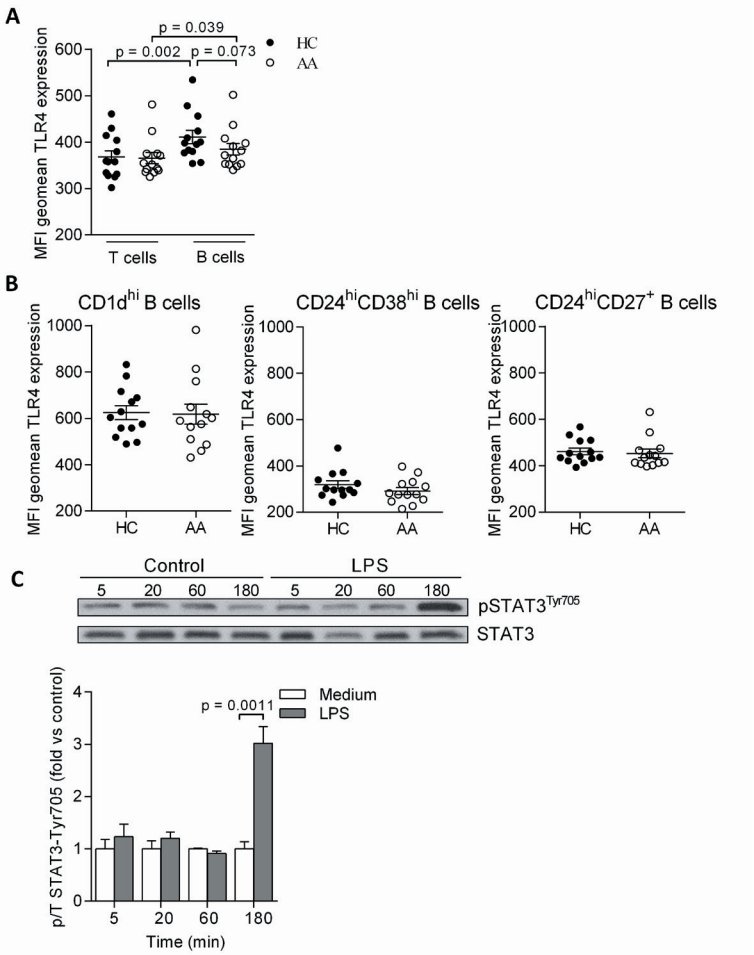

Figure 4. (A) The geometric mean fluorescence intensity of TLR4 on B cells was measured on PFA-fixed PBMCs from healthy controls (HC) and allergic asthma (AA) donors, using the TLR4 expression on total T cells as negative control. (B) TLR4 expression on the different Breg subsets. (C) 1 x 10^6 B cells from healthy laboratory donors were stimulated with medium or LPS (100 ng/ml) and incubated for 5, 20, 60 and 180 min. At the indicated time points, cells were harvested and nuclear extracts were prepared. Equal concentrations of protein were subjected to electrophoresis and blots were reacted with antibodies specific to phospho-Tyr705 of STAT3 and total STAT3, followed by development using enhanced chemiluminescence. One representative blot out of *n* = 3-4 with similar results is shown.

CD1d^{hi} B cells, following by the CD24^{hi}CD27^{+} B cells and very low expression on the CD24^{hi}CD38^{hi} B cells, which correlated well with their capacity to produce IL-10 in response to LPS (Fig. 4B).

Furthermore, to confirm LPS-induced activation of B cells, western blots were performed using whole-cell extracts from primary B cells of healthy labdonors stimulated with LPS or medium only. We focused on signal transducer and activator of transcription 3 (STAT3) signaling, as in previous studies it was shown that LPS-induced phosphorylation of STAT3 was implicated in IL-10 production in monocytes (26;27) and murine Breg cells (28). Here, we also detected strong induction of STAT3 phosphorylation after 180 min of exposure to LPS compared to medium (Fig. 4C), confirming that at least in healthy labdonors LPS induced the STAT3 signaling pathway in B cells, eventually leading to IL-10 production. Unfortunately, due to limited cell numbers we could not study the LPS-induced STAT3 phosphorylation in patients with AA.

LPS-primed B cells in patients with AA induce less IL-10-producing T cells

To study whether the lower frequency of IL-10-producing B cells from patients with AA has any functional consequences for allergen-specific T-cell cytokine responses, co-cultures were set-up with primed B cells and autologous CD4^{+} memory T cells. These cells were co-cultured for 6 days in the presence of house

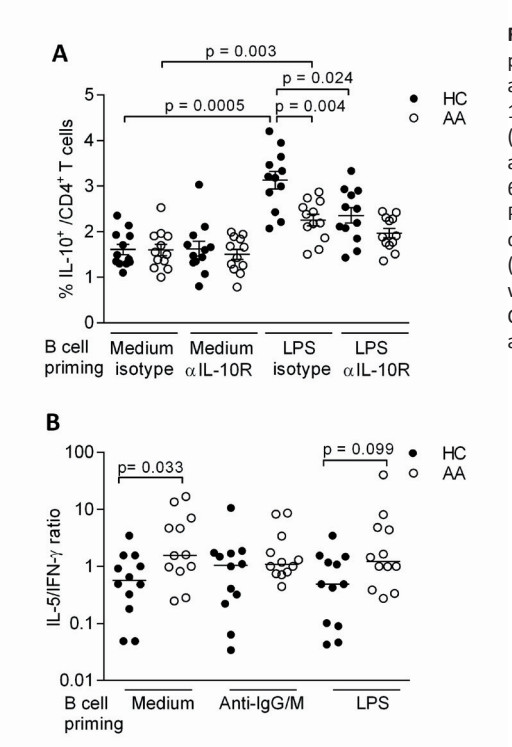

Figure 5. IL-10^{+} T cells in co-cultures of LPS-primed B cells and T cells. B cells were treated as described with Figure 2, followed by a 1:1 co-culture with autologous CD4^{+} T cells (pre-incubated with isotype or anti-IL-10R antibodies; 2 µg/ml), and DerP1 (1 µg/ml). After 6 days, cells were restimulated as in Figure 2. Percentage of IL-10-producing CD4^{+} T cells was determined by flowcytometry as represented in (A). Supernatants were taken and IL-5 and IFN-γ were measured using luminex (Invitrogen) (B). One pair was excluded as the AA patient was not allergic for HDM (n =12).

dust mite allergen DerP1 to evaluate whether primed B cells could influence the cytokine profile of allergen-specific T cells. The induction of anti-inflammatory responses such as $CD4^+CD25^{hi}FoxP3^+$ Treg cells or IL-10^+ $CD4^+CD25^{hi}FoxP3^+$ Treg cells was not different between the two groups (data not shown). Nevertheless, the intracellular IL-10 production by $CD4^+$ T cells (putative Tr1 cells) was enhanced in the presence of CpG or LPS-primed B cells. Interestingly, in patients with AA, this induction was only observed in the condition with BCR- or CpG-stimulated B cells, but not with LPS-primed B cells, suggesting a specific impairment in the case of LPS stimulation (Fig. 5A), (Appendix S3). Remarkably, despite the higher IL-10 secretion in CpG-stimulated B cells, LPS-stimulated B cells from HC induced at least equal or even more IL-10^+ T cells. The involvement of B cell derived IL-10 in the putative Tr1 cell induction was confirmed both in the CPG and the LPS condition of HC where a pre-incubation of the $CD4^+$ memory T cells with blocking IL-10R antibodies reduced T cell IL-10 production (Appendix S3). This was also observed in the condition of CpG-primed B cells of patients with AA, but not for the LPS-primed B cells (Appendix S3). Interestingly, blocking T cell IL-10 receptor in LPS-primed conditions substantially reduces the IL-10^+ T cell induction in control subjects though not fully to levels found in medium conditions, suggesting that for

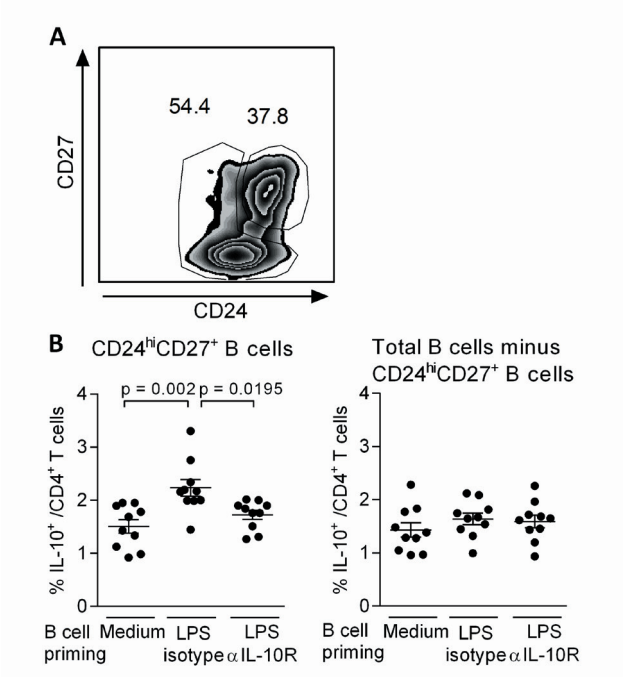

Figure 6. (A) B cells were sorted into $CD24^{hi}CD27^+$B cells and remaining cells. (B) 1 x 10^5 $CD24^{hi}CD27^+$B cells or the remaining cells were stimulated with medium or ultra-pure LPS for two days, followed by removal of supernatant, washed, and co-cultured with autologous memory $CD4^+$ T cells (ratio 1:1) in the presence of 1 µg/ml endotoxin-free Derp1. $CD4^+$ T cells were first pre-incubated with blocking IL-10R antibodies (2 µg/ml) or IgG1 isotype control (2 µg/ml) for 30 min at 37 °C. After 6 days, the cells were restimulated, fixed and staining for IL-10 using flowcytometry. The T cells were gated for $CD3^+FoxP3^-TNF\alpha^-$.

LPS-induced B cells also other factors than IL-10 may be involved in subsequent T cell IL-10 production (Fig. 5A).

Next to IL-10, intracellular cytokine production of TNF-α and IFN-γ showed similar number of these cells in both groups and IL-4 was hardly detectable after culturing with LPS-primed B cells (data not shown). However, a higher Th2/Th1 ratio (IL-5/IFN-γ) was found in the supernatant of B and T cell co-cultures from patients with AA compared to controls (Fig. 5B). Additionally, significantly more IgG1 and a trend for IgE production were detected in cultures of LPS-primed B cells and T cells from patients with AA compared to those from controls (Appendix S4), while IgG2 and IgG3 did not change and IgG4 levels were very low in both groups (data not shown) suggesting a more pro-inflammatory type 2 profile in patients with AA both with respect to cytokines and immunoglobulins.

$CD24^{hi}CD27^{+}$ B cells are essential for the induction of $IL\text{-}10^{+}$ T cells

We next assessed whether the induction of IL-10-producing T cells could be attributed to the $CD24^{hi}CD27^{+}$ B cell population (Fig. 6). To address this question, 10 HC subjects donated blood for a second time and B cells were sorted by flowcytometry into two populations, $CD24^{hi}CD27^{+}$ B cells and the remaining cells ($CD24^{low}CD27^{+}$ and $CD24^{low}CD27^{-}$ B cells) (Fig. 6A). Next, the cells were primed with medium or LPS for 2 days, followed by a co-culture with $CD4^{+}$ memory T cells, as stated above. Interestingly, the sorted $CD24^{hi}CD27^{+}$ B cells shower similar inducing capacity as the total B cells, whereas the remaining B cells could not induce $IL\text{-}10^{+}$ T cells after LPS priming (Fig. 6B). Blocking the IL-10 receptor on T cells significantly reduced the levels of IL-10 production to medium levels, confirming the exclusive role of IL-10-producing $CD24^{hi}CD27^{+}$ B cells in the induction of $IL\text{-}10^{+}$ T cells and its dependence on B-cell-derived IL-10.

Discussion

Regulatory B cells have been identified that have an impaired function in auto-immunity. Here, we demonstrated that B cells from patients with allergic asthma also have a lower capacity to produce IL-10 in response to LPS compared to B cells from controls. The $CD24^{hi}CD27^{+}$ B cell subset seems to be responsible for this effect. The expression of the pro-inflammatory cytokine TNF-α was similar in total B cells and the subsets, indicating that primarily the IL-10 response was specifically altered in B cells from patients with AA. Furthermore, this difference translates in a functional effect, namely the ability of $CD24^{hi}CD27^{+}$ B cells to induce $IL\text{-}10^{+}$ T cells and, therefore, may point at a role for a weakened Breg function in patients with AA.

We have compared the frequency of different Breg cell membrane markers on peripheral blood B cells based on reports describing functional Breg cells in humans, i.e. $CD1d^{hi}$ B cells (14;19), $CD24^{hi}CD27^{+}$ B cells (17) and $CD24^{hi}CD38^{hi}$ B cells (16). Blair *et al.* observed an increased frequency of $CD24^{hi}CD38^{hi}$ B cells in SLE patients, but with an impaired capacity to inhibit IFN-γ production in stimulated T cells. Intracellular IL-10 production in this particular B cell subset were not studied (16). Interestingly, we also observed an increase in $CD24^{hi}CD38^{hi}$ B cells in patients with AA, but with similar IL-10 frequencies between the groups. However, as we did not study the ability of isolated $CD24^{hi}CD38^{hi}$ B cells to inhibit cytokine secretion by T cells, we cannot exclude functional differences for this B cell subset in allergic asthma, despite equal intracellular IL-10 frequencies.

The regulatory capacity of $CD24^{hi}CD27^{+}$ B cells was demonstrated by Iwata *et al.* by showing the ability of this B cell subset to reduce cytokine production by monocytes (17). Interestingly, this reduction was not IL-10-dependent and these cells could not reduce cytokine production by T cells. Here, we observed a lower LPS-induced IL-10 production by $CD24^{hi}CD27^{+}$ B cells in patients with AA versus controls, which upon further co-cultures with T cells, was accompanied by a reduced IL-10 expression in T cells. Although we only studied single sorted subsets in HC subjects, but given the lower IL-10 production in $CD24^{hi}CD27^{+}$ B cells in response to LPS in patients with AA, it is tempting to speculate that this subset might be responsible for the lower induction of IL-10-producing T cells observed in the co-cultures with total B cells.

It was shown in several studies conducted with traditional European farmer families, that growing up on farms protects against the development of allergy and asthma and that both bacterial and fungal exposures may be important (4-6). Indeed, exposure to bacterial components, such as endotoxin (LPS) and muramic acid (component of peptidoglycan), were inversely related to allergic sensitization, childhood asthma or wheezing (29;30). This also accounts for microbial products found in floor dust from urban living rooms (31-33). Not only the level of microbial content but also the extent of diversity was important for the inverse relationship with allergies and asthma (33-35). Although there

is a positive correlation between the levels of bacterial DNA and the levels of endotoxin found in farm dust (36), suggesting that CpG and endotoxin will both be present to a similar extent in daily life, it is unclear whether this correlation can also be attributed to 'poor' microbe environments like urban homes. It may be questioned whether the *in vitro* stimulation conditions of B cells we have applied here are realistic to *in vivo* exposure in daily life and whether the presence of CpG may compensate for the defect in LPS-induced IL-10 production of B cells from patients with allergic asthma. Despite this assumption, it is unclear what the net effect is when CpG and LPS would act together on B cells, and whether a low concentration of CpG would still induce a similar B cell IL-10 production in both groups or that, like the weak B-cell activator LPS, it would also show a weaker response in allergic patients. Interestingly, it was showed that 'farm' bacterial DNA potentiated LPS-induced cytokine production in PBMC, while 'urban' bacterial DNA did not (36), suggesting that either the activity or the content of bacterial DNA versus endotoxin in dust is different between rich and poor microbe environments resulting in a different net effect on single cells and keeping open the option that the LPS conditions described in this paper may occur *in vivo*.

Also on the level of the innate receptors involved in the recognition of these bacterial components (CD14, TLR2 and TLR4), mRNA expression was shown to be increased on PBMC from farmer's children and in particular exposure of pregnant mothers to stables was positively correlated with increased TLR expression (37;38). Although TLR4 expression on B cells is relatively low compared to TLR2 and TLR9, B cells are clearly able to respond to LPS (39-41). Furthermore, inflammatory conditions were associated with an upregulation of TLR4 expression on B cells (41-44). In our study, we confirmed that although TLR4 expression on B cells is quite low, LPS was capable of inducing STAT3 signaling, confirming its capacity to activate human B cells of healthy controls. Because the phosphorylation of STAT3 was only significantly increased after 180 min, we cannot rule out that this effect is not a direct consequence of TLR4 signaling but alternatively the result of a secondary stimulation via other factors initially induced by LPS, but altogether still induced by LPS stimulation only. Although there was a trend in reduced TLR4 levels in patients with AA compared to HC subjects (but not in the individual Breg subsets), we cannot exclude that the lower LPS-induced IL-10 expression in B cells of patients with AA may as well be explained by differences in TLR4-induced signaling pathways, as previously suggested for monocytes from allergic patients, which showed an impaired phosphorylation of MAPK pathways upon LPS stimulation (45).

While there is clear evidence in different animal models that enhanced activity of IL-10-producing Breg cells strongly suppresses allergic inflammation (15), there is only one study available in food allergy, studying IL-10 expression in B cells in patients suffering from food allergy. IL-10-expressing B cells were downregulated in response to milk antigens in PBMC from allergic, but not from healthy individuals (46). It is also still unclear whether the IL-10-producing Breg cells in the circulation are the ones that will migrate to the target organs, such

as the lung, to act in a protective way or that maybe other local Breg cell subsets are responsible for this. In contrast, evidence for a protective role in allergy and asthma of IL-10-producing T cells or FoxP3$^+$ Treg cells is extensively reported in both animal models and human studies (7). In addition, IL-10 production plays a central role in beneficial effects of immunotherapy (47). In this study, we demonstrated a causal link between IL-10-producing B cell and IL-10$^+$ T cells, suggesting that B cell-derived IL-10 can instruct T cells for their regulatory capacity. Indeed, several animal studies have demonstrated that part of the effect of IL-10-producing B cells is mediated by induction of IL-10-producing T cells or FoxP3$^+$ Treg cells (10;15). These findings imply that targeting for regulatory B cells as a novel therapeutic approach may embrace two regulatory systems simultaneously, i.e. regulatory B cells and IL-10-producing regulatory T cells. New studies have to find out whether this may lead to a much more efficient suppression of allergen-specific immune responses in patients with AA.

In conclusion, further research should focus on the identification of pathways and molecules that enhance the number and/or activity of Breg cells, in which the CD24^{hi}CD27$^+$ B cell subset may be interesting to be targeted with respect to the treatment of allergic asthma. Those molecules will be interesting candidates to include in current immunotherapy protocols and improve their efficacy in establishing longer lasting protection against allergic asthma.

Conflict of interest

This project was supported by Netherlands Asthma Foundation (NAF grant 3.2.10.072). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflict of interest.

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Supplementary figures

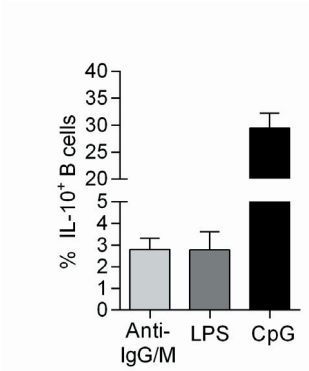


Figure S1. B cells (3x10^5) from healthy laboratory donors were stimulated for 72 h with anti-IgG/M (20 µg/ml), LPS (100 ng/ml), CpG (5 µg/ml) and medium. Medium values (~1.5%) were subtracted from the stimulated conditions for each individual. IL-10 secretion assay (Miltenyi Biotec) was performed and shows the percentage of IL-10-producing B cells. *N*=3.

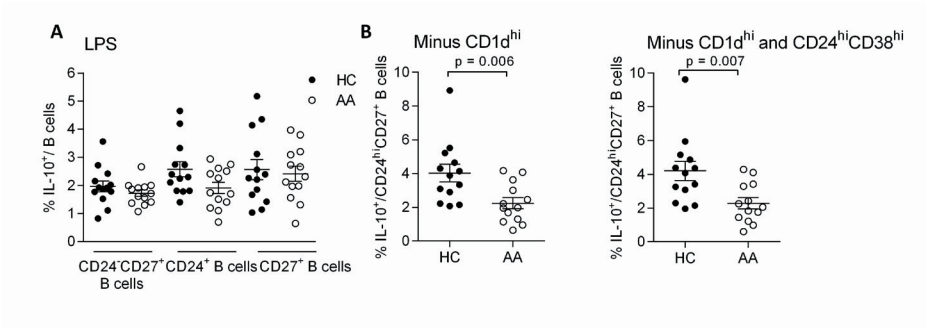


Figure S2. Intracellular IL-10 (A) in CD24^{low}CD27$^-$, CD24$^+$ or CD27$^+$ B cells. (B) The expression of IL-10 in CD24^{hi}CD27$^+$ B cells excluded for CD1d^{hi} and CD24^{hi}CD38^{hi} B cells. The B cells were treated as described in Figure 2.

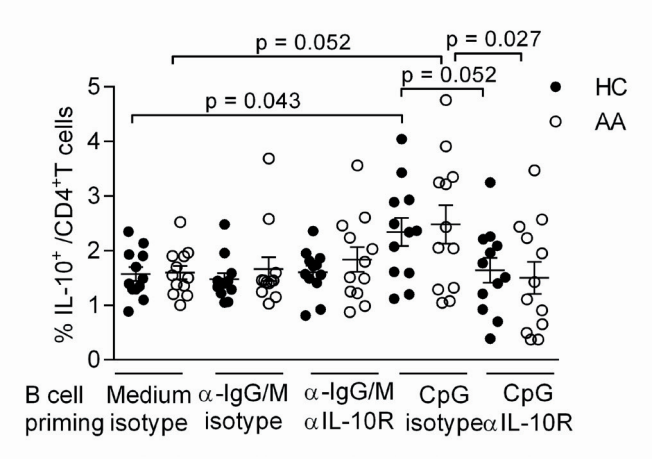


Figure S3. Co-culture of BCR- or CpG-stimulated B cells and T cells. B cells were treated as described in Figure 2, followed by a 6 day 1:1 co-culture with autologous $CD4^+$ T cells $CD4^+$ T cells (pre-incubated with isotype or anti-IL-10R antibodies; 2 μg/ml), and DerP1 (1 μg/ml). Cells were restimulated to determine intracellular cytokines by flowcytometry. Percentage in $IL\text{-}10^+$ $CD4^+$ T cells was calculated. One pair was excluded as the patient with AA was not allergic for HDM (*n* =12).

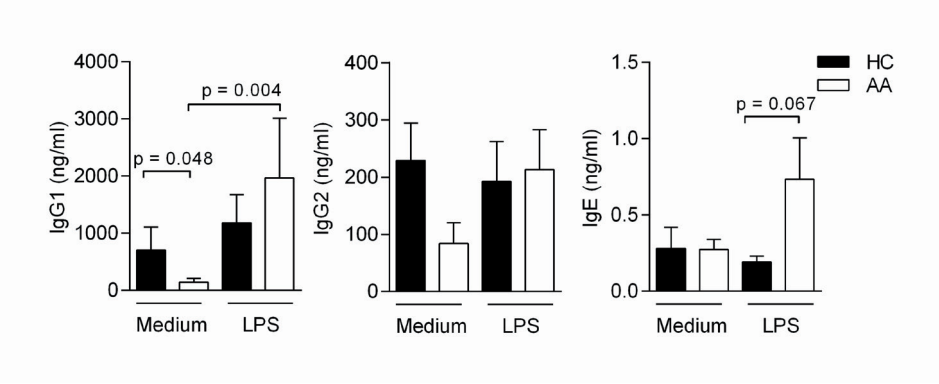
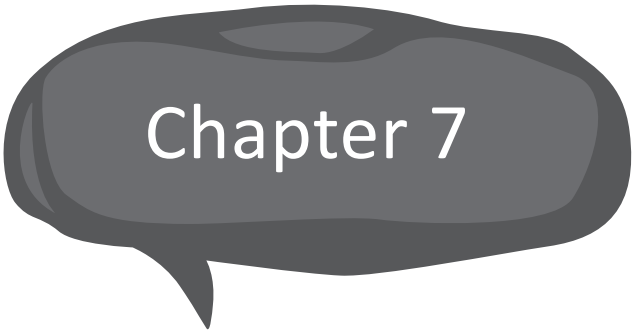


Figure S4. Immunoglobulin production of LPS-primed B cells in co-culture with T cells. Co-cultures were setup as described in Figure 4. After 6 days, supernatants were harvested and immunoglobulins (IgG1, IgG2, IgG4, and IgE) were measured by immunoglobuline isotyping assay (Biorad-luminex). Background levels of medium-primed and LPS-primed B cells are shown.



Summarizing discussion

Modified from:

Regulatory B-cell induction by helminths: Implications for allergic disease

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Regulatory B cells: suppression of auto-immune or allergic disorders

In the past decade, convincing data have demonstrated that IL-10-producing B cells, termed regulatory B (Breg) cells, are induced throughout the course of autoimmune disease in experimental models. Fillatreau and coworkers were one of the first to show that these protective B cells negatively regulated experimental autoimmune encephalomyelitis (EAE) progression through provision of IL-10 (1). Additional studies by Mizoguchi, Mauri and others confirmed the presence of active IL-10-producing Breg cells in other autoimmune models, i.e. inflammatory bowel disease (IBD) or collagen-induced arthritis (CIA) (2;3). Since then, their function has been studied in several other immune-related diseases, demonstrating that IL-10-producing Breg cells were not restricted to Th1 immune responses associated with autoimmunity, but could be induced by extrinsic factors such as helminth parasites and have the capacity to protect against Th2-skewed allergic inflammation. For example, in *Heligmosomoides* (*H.) polygyrus*-infected mice, adoptive transfer of mesenteric lymph node (MLN) B cells suppressed both DerP1-specific airway inflammation and EAE (4). Furthermore, it has been demonstrated that B cells induced by *Schistosoma (S.) mansoni* worms protected BALB/c mice against allergic reactions in both anaphylaxis and allergic airway inflammation models in an IL-10 dependent manner (5). In this model only male parasites were used for infection, yielding infections without eggs. Instead, Smits *et al.* studied Breg cell function during different stages of natural *S. mansoni* infections, and showed the existence of active regulatory mechanisms during chronic, but not acute infection. Both splenic B cells and $CD4^+$ T cells isolated from chronically, but not acutely, infected mice protected against allergic airway inflammation, revealing active roles for both B cells and $CD4^+$ T cells (6). In **Chapter 2**, we confirmed that during a natural *S. mansoni* infection the induction of IL-10-producing Breg cells was crucial for protection against AAI using chimeric IL-$10^{-/-}$ B cell mice.

Regulatory B cells induced by other pathogens

Recent reports indicated that Breg cell development is not only restricted to helminth parasites, but can also be seen during other infections, such as infection by protozoan parasites *Leishmania major* (7) and *Babesia microti* (8). During these infections, IL-10-producing B cells were critical for the development of susceptibility to infection. Recently, IL-10 expression was identified in plasma cells during a *Salmonella typhimurium* infection (9). Several viruses, such as murine cytomegalovirus, HIV, and hepatitis B, decreased virus-specific $CD8^+$ T cell responses by stimulating Breg cells (10-13). Similarly, *Plasmodium berghei*-infected mice showed elevated IL-10-producing B cells, which were important for controlling immunopathology (14). These data indicate that the functional identification of IL-10-producing B cells are not restricted to helminth infections, but may be a part of integrated network that develops to counterbalance the

inflammation induced by the presence of pathogens. What stands out, is that next to helminths, the viral and bacterial agents enhancing Breg cells are mostly inducing a chronic inflammation, suggesting that Breg cells may occur as a bystander process resulting from continuous chronic infection.

Identification of B cell subsets associated with a regulatory function

Regulatory B-cell subsets in models of autoimmunity

In the past few years, several studies focusing on the splenic compartment identified marginal zone (MZ) and transitional type 2 (T2)-MZ precursor B cells as B cells with a putative suppressive function. In addition, IL-10 production by splenic B cells has been linked to B cells expressing high levels of CD1d with or without the expression of CD5 (Table 1). In this respect, pioneering work has been performed in models of EAE (15) and oxazolone-induced contact hypersensitivity (CHS) (16) where an IL-10-dependent protective role for splenic $CD1d^{hi}CD5^{+}$ B cells was identified. This relatively rare regulatory B cell subset was termed B10 cells (16). Further characterization revealed that approximately half of the splenic $CD1d^{hi}CD5^{+}$ IL-10-producing B cells expressed high levels of CD21, similar to marginal zone (MZ) B cells ($CD21^{hi}CD23^{low}IgM^{hi}$) (16). Another major contribution to the field was made by the use of chimeric mice lacking endogenous IL-10-producing B cells in an arthritis model. These mice showed the exacerbation of the disease with a marked increase in Th1 and Th17 cells compared to WT B cell mice (17). Reconstitution of B-cell deficient mice showed that transitional type 2 (T2)-MZ precursor B cells ($CD21^{hi}CD23^{hi}IgM^{hi}$), co-expressing CD1d prevented disease and ameliorated established disease in mice with collagen-induced arthritis and lupus (17;18) (table 1). Moreover, a recent report describes another subset, peritoneal B1 cells, as a source for IL-10 and essential for suppressing late remission phase of CHS. Interestingly, they found an additional role was found for CD22, an sialic-binding Ig-like lectin, which was expressed on the IL-10-producing peritoneal B1 cells and was involved in their protective abilities (19) (Table 1).

Regulatory B cell subsets in helminth-infection models

Studies in adult stage *S. mansoni*-infected mice indicated that splenic $CD1d^{hi}$ B cells have a regulatory function as these cells provided protection against allergic airway inflammation (5). Analogous to what had been found in the models of autoimmunity (detailed above), the $CD1d^{hi}$ B cells expressed CD5, $CD21^{hi}$, $CD23^{+}$, and high levels of IgM, resembling T2-MZ precursor B cells (5) (Table 1). During natural infections with *S. mansoni*, we also found a splenic subset of IL-10-producing B cells which protected against allergic airway disease (6), however in our model the regulatory B cells were identified within the MZ B-cell compartment ($CD21^{hi}CD23^{low}$) with a majority (> 80%) co-expressing CD1d (**Chapter 2**). It is not fully clear whether these B cell subsets form completely unique subsets because there is a substantial overlap between the (co-)expression of the various markers,

such as CD1d, CD5, CD21, CD23 and IgM. In addition, local inflammation or chronic infection may change the expression of individual markers complicating distinctions between the different proposed cell subsets. As T2-MZ precursor B cells can differentiate into MZ B cells, it can be speculated that the splenic Breg cell subsets identified by several labs are in fact all similar and simply represents either precursors or mature stages of the same Breg cell subset. Furthermore, although IL-10-producing B cells are enriched in the CD1d^{hi}(CD5^{+}) and T2-MZ precursor B cells, not all B cells within these subsets produce IL-10, suggesting that this set of markers is not unique to identify IL-10-producing Breg cells. Recently, the membrane-bound marker Tim-1 (T-cell Ig domain and mucin domain protein-1) was expressed on more than 70% of the IL-10-producing B cells. Therefore, this marker is regarded as the most specific marker yet identified

Table 1. Overview of different Breg cell subsets, their phenotypic characteristics and their mode of action.

Breg cell phenotype	Additional markers	Mouse/ Human	Organ	Mechanism of Action	Disease Model/ Patients	Helminth	Ref
T2 precursor MZ	CD1d^{hi}CD5^{+}	Mouse	Spleen	IL-10, Treg cells, Th1/Th17 suppression	CIA, lupus	None	17, 18, 41
CD1d^{hi}	CD21^{hi}CD23^{hi}	Mouse	Spleen	IL-10, Treg cells	AAI (OVA)	*S. mansoni*	5, 6
CD1d^{+}CD5^{+}	CD21^{hi}	Mouse	Spleen, Peripheral LNs	IL-10, APC suppression	EAE, CHS, lupus	None	16, 36, 38
CD23^{hi}	None	Mouse	Mesenteric LNs	Unknown	AAI (Der P1) & EAE	*H. polygyrus*	4
B1-cells	CD5	Mouse	Peritoneal cavity	IL-10, CD22	CHS	None	19
CD1d^{hi}	None	Human	Peripheral Blood	IL-10, T cell suppression	MS	Mixed helminth infections	49
CD24^{hi}CD38^{hi}	CD1d^{hi}CD5^{+}	Human	Peripheral Blood	IL-10, T cell suppression	SLE		51
CD24^{+}CD27^{+}	CD1d^{hi}CD5^{+} CD38^{+}	Human	Peripheral Blood	IL-10, APC suppression	none	None	52
CD5^{+}	none	Human	Peripheral Blood	IL-10	Food allergy (milk)	None	53
IgG4^{+} B cells	IL-10^{+}CD25^{hi} CD71^{hi}CD274^{hi} CD73^{-}	Human	Peripheral Blood	IL-10	Bee venom allergy	None	44

Abbreviations: T2 is type 2; MZ is marginal zone; Treg cell is regulatory T cells; CIA is collagen-induced arthritis; AAI is allergen-induced airway inflammation; EAE is experimental auto-immune encephalomyelitis; CHS is oxazolone-induced contact hypersensitivity; MS is multiple sclerosis; SLE is systemic lupus erythematosus and refs are references.

for IL-10-producing B cells (20;21). In **Chapter 3**, we did find a small increase in Tim-1-expressing pulmonary B cells, which showed increased IL-10 production during schistosome infection. However, pulmonary B cells protected against airway inflammation in an IL-10-independent manner, suggesting that the small population of Tim-1-expressing pulmonary B cells were not dominant in reducing disease symptoms. Given that no unique marker has been identified for all Breg cells, multiple Breg cell subsets might occur depending on the activation state, inflammatory environment and the target organ involved.

Regulatory B cells located at the side of inflammation

Most studies identified regulatory B cells within the splenic compartment. However, a report describes another phenotype of parasite-induced Breg cells in the mesenteric lymph nodes (MLN) upon infection with *H. polygyrus*. These regulatory B cells expressed high levels of CD23, but no CD5 or CD1d, and suppressed allergic inflammation in an IL-10 independent manner (4). Interestingly, yet another subset with regulatory capacity, B-1a B cells, is located predominately in peritoneal and pleural cavity, but also in the spleen in very low numbers (22). Splenic CD5-expressing B-1a cells, like splenic B cells, form a relevant source for IL-10 and suppressed inflammation via the killing of $CD4^+$ T cells by FasL/Fas-dependent mechanisms in CIA and during schistosomiasis (23;24). There are multiple similarities between peritoneal and splenic B-1a cells, suggesting that they may be related to each other or possibly even be the exact same cells (25).

In the lungs, a regulatory role for pulmonary $CD5^+$ B-1a cells in a cockroach-sensitized asthma model was suggested, reducing cytokine production, pulmonary inflammation, and $CD4^+$ T cell survival via increased FasL expression (26). Interestingly, we also found, next to splenic Breg cells, a regulatory role for schistosome-induced pulmonary B cells, which could transfer protection against AAI using adoptive transfer. However, this was not conferred by any CD5-expressing pulmonary B cells (**Chapter 2 and 3**). Characterization of these pulmonary cells showed that they did not share surface markers linked to Breg cell subsets described above. These results suggest that protection against allergic inflammation is not limited to one subset of regulatory B cell, and indicates that different effector mechanisms may work in parallel in addition to IL-10-producing B cells.

Regulatory B-cell effector mechanisms

B cells as cytokine-releasing immune regulators

Breg cells can induce suppression by several effector mechanisms and by targeting different cell subsets. High IL-10 secretion is regarded as being prominent anti-inflammatory effector mechanism and a marker for Breg cells as described above. 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. TGF-β controls inflammation via suppression of Th1 and Th2 inflammatory cytokine production, maintenance of Treg cells, and inhibition the function of antigen presenting cells (APC) (27-29). Recently, IL-35-producing plasma cells were identified as a complementary arm of B cell-mediated suppression to IL-10-producing plasma cells in EAE and during *Salmonella* infection (9;30;31). Several studies demonstrated that Breg cells may simultaneously produce regulatory cytokines IL-10, TGF-β and IL-35. For example, treatment of mice with IL-35 protected mice from experimental autoimmune uveitis via the induction of Breg cells producing IL-35 as well as IL-10 (32). Furthermore, LPS-activated B cells secreted both TGF-β and IL-10 to down-regulate inflammatory immune responses during diabetes (29;33). Future studies should address whether Breg cells producing either other suppressive cytokines, apart from IL-10, or multiple regulatory cytokines are present in helminth infections and how they contribute in the regulatory network.

The production of anti-inflammatory cytokines does not automatically define B cells as Breg cells. Even when B cells secrete elevated levels of IL-10, it is essential to elucidate whether the IL-10-producing B cells truly possess suppressive capacity and whether IL-10 is essential for this process. For example, it has been demonstrated that pulmonary B cells, producing elevated levels of IL-10 during chronic schistosome infection, can reduce AAI in an IL-10-independent manner (**Chapter 3)**. Furthermore, some Toll-like receptor (TLR)-activated B cells can concomitantly secrete anti-inflammatory IL-10 and pro-inflammatory IL-6, two cytokines with opposite effects on the immune system. Importantly, B-cell derived IL-6 plays a prominent role in the pathogenesis of T cell–mediated autoimmune diseases such as EAE and MS (34). Therefore, the suppressive capacity of IL-10-producing B cells needs to be confirmed in functional *in vitro* assays investigating e.g. the effect on T cell proliferation or T cell cytokines (**Chapter 4**) or in disease models *in vivo* before the term 'Breg cell' can be applied.

Recruitment and/or induction of regulatory T cells

The concept that B cells can induce Treg cells was first introduced in a model of anterior chamber-associated immune deviation (ACAID) by Ashour and Niederkorn (35). A causal relationship between IL-10-producing B cells and Treg cells has been suggested in antigen-induced arthritis model utilizing similar chimeric mice, where loss of IL-10-producing B cells led to significant reduction of Treg cells in draining inguinal LN (17). Similar results were found in auto-immunity models, such as lupus where B10 cells reduce inflammation by the induction of Treg cells (36). Another example is shown in a model for EAE, where B cell-deficient mice displayed delayed emergence of $Foxp3^+$ and $IL\text{-}10^+$ T cells in the central nervous system, which was corrected by reconstitution with B cells and resulted in recovery from disease (37). Of note, Breg and Treg cell numbers appear to peak at different disease stages in EAE with enhanced Breg cell activity during early EAE initiation, while Treg cells were found to provide protection during late-phase

EAE (38). These findings can be extended to helminth infections, as schistosome-induced CD1d^{hi} MZ B cells promoted expansion of Foxp3^{+} Treg cells in the lung and *in vitro* cultures via IL-10 (5) (**Chapter 2**). Importantly, loss of FoxP3^{+} Treg cells via treatment with anti-CD25 antibodies during allergen challenge restored AAI (5). In contrast, our study showed that B cell-induced Treg cells are only partially involved in protection against AAI using another model to deplete Treg cells, namely FoxP3-DTR transgenic DEREG mice. Therefore, although Treg cell induction is dependent on Breg cell activity, Breg cells and Treg cells may have partly independent roles in controlling inflammation.

Suppression of T-cell responses

The capacity of B cells to suppress T cell proliferation and/or T cell cytokine production has been studied in several disease settings. Early work showed that lethal Th1 responses are expanded in schistosome-infected B-cell-deficient mice, suggesting that in WT mice Th1 cells are suppressed by schistosome-induced B cells (39;40). In addition, applying different allergy models clearly indicated that schistosome-induced B cells can also inhibit ovalbumin (OVA)-specific Th2 cytokine responses in an IL-10-dependent manner, resulting in reduced allergic symptoms (5). These findings are in line with studies in autoimmunity models, where IL-10-induced suppression of inflammation was found in EAE (15;38), lupus (41) or arthritis (18) by modulating Th cell proliferation and reducing IFN-γ, IL-2, IL-17 or TNF-α levels; in some studies this suppressive effect was potentiated via CD40 ligation (18;38;41). Furthermore, IL-10-producing B cells were described to inhibit type 2-mediated colitis in a T-cell receptor (TCR) alpha knock-out model by yet another mechanism that involves the induction of IL-12-producing B cells (42). Interestingly, IL-10-independent down-regulation of Th2 responses has also been reported by B cells from *H. polygyrus* infected mice (4), suggesting the involvement of cell-cell interaction or other soluble mediators (Table 1). Lastly, inflammation was controlled via apoptosis of CD4^{+} T cells by Fas ligand (FasL)-expressing CD5^{+} B cells from the lungs or spleen in a cockroach-based asthma model or during *Schistosoma* infection respectively (23;26). In **Chapter 3**, *S. mansoni* infection induced pulmonary B cells showed a reduced capacity to initiate Th2 cytokine responses. However, preliminary data suggested that this down-modulation is not related to enhanced FasL expression and the subsequent induction of T-cell apoptosis. Also other important signals that can influence T-cell activation and cytokine production, such as antigen-presentation molecule MHCII, inhibitory receptors PD-L1 and PD-L2, or B-cell derived cytokines IL-10, TGF-β and IL-6 did not play a major role in down-regulating Th2 cytokines (**Chapter 3**). Schistosome-induced pulmonary B cells did express enhanced levels of CD86, which has been linked to protection against EAE (37). The putative role for this molecule and/or other yet unknown inhibitory receptors/molecules of schistosome-induced pulmonary B cells in the down-modulation of Th2 responses remains to be elucidated. Altogether, these findings suggest that immune suppression by schistosome-induced B cells results in a number of different effector mechanisms

or subsets, not only involving the induction of IL-10-producing splenic Breg cells but also functionally impairs pulmonary B cells in their capacity to induce Th2 cells.

Antibody-mediated regulation

Recent studies indicate that antibodies may also be involved in the suppression of immune responses. Potential mechanisms include suppression of dendritic cell (DC) activation through the binding of IgG to FcγRIIB, as well as IgG-mediated clearance of potentially pathogenic host apoptotic cells (43). In addition, van de Veen *et al*. have suggested that human IL-10-producing B cells are designated to switch to IgG4 (44). This may be potentiated by IL-10, which is an important switch-factor for IgG4. IgG4 belongs to the group of anti-inflammatory antibody isotypes as it is not able to activate complement. IgA also belongs to this group, and recently it was reported that microbial modulation of dendritic cell function was crucial to induce allergen-specific secretory IgA in the mucosa, which suppressed the salient features of asthma (45). Interestingly, helminths are strong inducers of polyclonal IgG molecules secretion and IgG4 production in humans (39;46;47). We reported in **Chapter 3** a general elevated production of IgG1 and IgG2a antibodies in the BAL fluid of OVA-infected mice compared to OVA-uninfected mice. However, the loss of the FcγRIIB receptor did not seem to restore AAI upon pulmonary B cell transfer, suggesting that despite the elevated IgG1 and IgG2a secretion in OVA-infected mice, protection against AAI was not mediated via signalling through FcγRIIB. Additionally, we have some indications that IgA levels were not increased during *S. mansoni* infection in mice, suggesting that IgA may not contribute majorly.

DC Impairment

It is well known that IL-10 can inhibit DC antigen-processing and -presentation and expression of co-stimulatory molecules, such as CD80/CD86. This has clear consequences for their T-cell stimulatory capacity as shown by a recent report, in which purified splenic DCs from mice with EAE were cultured with MOG-specific $CD4^+$ T cells. Less T cell proliferation was seen when DCs were conditioned by $CD1d^{hi}CD5^+$ B cells compared to conditioning by $CD1d^{low}CD5^+$ B cells. This effect was IL-10-dependent (38). These findings may be extended to parasite-induced Breg cells as *L. major*-exposed B cells were shown to suppress DC cytokines in an IL-10 dependent manner *in vitro* (7). Interestingly, Everts *et al.* observed that myeloid DC function was impaired in *S. haematobium*-infected individuals (48). Although not proven, it is tempting to speculate that the increased Breg activity during schistosome infection may contribute to the altered DC function.

Human Breg cells – do they exist?

Helminth-induced $CD1d^{hi}$ B cells

The majority of the studies on regulatory B cells have been conducted in murine models, and only recently evidence for human Breg cells emerged. Four distinct human Breg cell populations, predominantly identified based on their IL-10 secretion, have mainly been studied in conditions in auto-immunity. In 2008, it was first demonstrated by Correale and co-workers that $CD1d^{+}$ B cells were present in the peripheral blood of helminth-infected patients with multiple sclerosis (MS), producing elevated IL-10 levels in response to CD40 ligation. B cells from healthy controls and infected patients with MS, but not from uninfected patients with MS, were able to suppress T-cell proliferation and IFN-γ production in an IL-10-dependent manner *in vitro* (49). Recently, we have established a causal relation between a single species of helminth, *S. mansoni*, and increased levels of IL-10-producing $CD1d^{hi}$ B cells, which were reduced to baseline levels after anti-schistosome treatment (**Chapter 2**). The functional capacities of those schistosome-induced B cells were investigated in **Chapter 5**. We observed an elevated number of $CD1d^{hi}$ B cells in the blood of *S. haematobium*-infected adults, which express enhanced levels of cytoplasmic IL-10. Interestingly, increased surface LAP(latency-associated peptide)/TGF-β1 expression was mainly attributed to the $CD24^{hi}CD27^{+}$ B cells (see below for details on this particular Breg subset). In co-culture with $CD4^{+}$ T cells, B cells from schistosome-infected individuals reduced the production of effector T-cell cytokines while more $CD25^{hi}FoxP3^{+}$ and $IL\text{-}10^{+}$ T cells were found compared to cultures with B cells from uninfected controls (**Chapter 5**). In search for the dominant Breg cell subset, we found a role for $CD1d^{hi}$ B cells, the main source of IL-10, in induction of $IL\text{-}10^{+}$ T cells, but not for $FoxP3^{+}$ T-cells. At this stage, it is unclear which schistosome-induced Breg subset is responsible for the enhanced Treg cell induction, although it is tempting to suggest that, given the role for TGF-β in $FoxP3^{+}$ Treg cell induction, TGF-β-producing B cells, i.e. the $CD24^{hi}CD27^{+}$ B cells, are involved here. This may suggest that helminth infections can promote the activity of two different B-cell subsets. Whether helminth-induced Breg cells in humans suppress allergic immune responses and participate in the maintenance of tolerance via the provision of IL-10, as indicated in murine models, is still unclear. However, as elevated IL-10 levels circulating in helminth-infected individuals was negatively associated with the outcome of skin-test reactivity to mite, it is tempting to suggest that IL-10 derived from B cells could have spill-over suppression effect on the immune responses towards allergens (50).

$CD24^{hi}CD38^{hi}$ immature B cells

In peripheral blood of healthy individuals, so-called immature $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B cells were identified by Blair and co-workers (51). Approximately 70% of these B cells also expressed CD5 and CD1d. The $CD24^{hi}CD38^{hi}$ B cell population was capable of suppressing IFN-γ and TNF-α secretion by anti-CD3-stimulated T helper cells and this suppression was dependent on IL-10 and CD80/CD86 co-stimulation. Interestingly, $CD19^{+}CD24^{hi}CD38^{hi}$ B cells isolated from SLE

patients were functionally impaired as they could not suppress autologous T helper cytokine production (51).

CD24^{hi}CD27^{+} B cells

The human equivalent of the murine competent IL-10-producing B cells was described within peripheral blood B cells, that either already produced IL-10 or first required 48 hours of priming before acquiring the ability to express IL-10 (52). B10 cells represented a small subset within the CD24^{hi}CD27^{+} B cell population, with about 60% co-expressing CD38 (52). Interestingly, both stimulated CD24^{hi}CD27^{+} and CD24^{low}CD27^{-} B cells inhibited IFN-γ and TNF-α production in T helper cells in an IL-10-independent manner. In contrast, CD24^{hi}CD27^{+} B cells inhibited TNFα production by monocytes via IL-10. In contradiction to what had been published previously (51), increased frequencies of IL-10-producing peripheral blood B cells were found in patients suffering from different autoimmune disorders (SLE, RA, Sjögren syndrome, blistering skin disease and MS compared to healthy controls upon stimulation with CD40 ligand and CpG motifs (52). However, these studies did not evaluate the functional abilities of human B10 cells from auto-immune patients compared to those from healthy controls with respect to their capacity to reduce T-cell cytokines responses nor whether other cytokines were simultaneously expressed in the B10 cells from the patients (52).

As illustrated above, evidence from a few human studies points towards a significant role for IL-10-producing Breg cells in the modulation of pathogenic responses in type 1 inflammation. Although it has become clear from a number of reports that at least the Treg cell compartment of patients with Th2-skewed diseases such as allergic asthma, rhinitis or dermatitis, is impaired in number and activity, there are not many reports on Breg cell numbers and activity in these patients as yet. One report has evaluated IL-10-producing B cells in allergic patients and controls, describing an increased frequency of IL-10-producing CD5^{+} peripheral blood B cells from healthy individuals in response to the milk antigen casein, which was not observed in PBMC cultures from cow's milk-allergic individuals (53). In **Chapter 6**, we investigated the Breg compartment in peripheral blood of patients with allergic asthma compared to healthy controls and identified a significant decrease in number of CD24^{hi}CD27^{+} B cells. This population was not only reduced in numbers, but also showed a lower capacity to produce IL-10 in response to LPS. Furthermore, this impaired B-cell IL-10 production resulted in less IL-10^{+} T cells *in vitro* cultures from patients with allergic asthma compared to similar cultures from control subjects, and, therefore, may point at a role for a weakened Breg function in the enhanced inflammation found in patients with allergic asthma.

CpG-induced CD25^{+}CD71^{+}CD73^{-} B cells

In recent report of Van de Veen and co-workers, a new subset of IL-10-producing B cells upon TLR9 ligation was identified, which were enriched in a

$CD25^+CD71^+CD73^-$ population rather than in the other described Breg subsets (44). The IL-10-producing B cells were capable of suppressing Purified protein derivative (PPD)-specific proliferation of $CD4^+$ T cells and this suppression was dependent on IL-10. Furthermore, prolonged culture of $CD25^+CD71^+CD73^-$ IL-10-producing B cells resulted in the isotype switch to anti-inflammatory IgG4 antibodies. Bee venom allergen phospholipase A_2 (PLA)-specific B cells from non-allergic beekeepers mainly expressed IgG4 and showed higher IL-10 levels compared to non-PLA-specific B cells. Interestingly, low frequencies of $IL\text{-}10^+$ PLA-specific B cells detected in patients with bee venom allergy were restored to similar levels as found in beekeepers after successful allergen-specific immunotherapy.

Are (defects in) specific Breg subsets associated with certain inflammatory diseases?

All together, these studies show that in humans different IL-10-producing Breg cells exist, however the question remains whether specific Breg cell subsets are associated to certain inflammatory milieus such as infections, auto-immunity or allergic disease. A drawback of most studies we have reviewed above is that the experiments were focused on the characteristics of one single Breg subset, while other Breg cell subsets where not taken into account. Nevertheless, we do have compared the three most studied subsets in helminth infection and asthma patients. What is interesting in this aspect, is that the affected regulatory B-cell subset in helminth infections is mostly restricted to the $CD1d^{hi}$ population, whereas defects in $CD24^{hi}CD38^{hi}$ Breg cells are mostly described for auto-immunity and we found indications for $CD24^{hi}CD27^+$ Breg cell impairment to be more linked to allergic inflammation (**Chapter 5 and 6**). However, since most human studies are restricted to peripheral blood B cells, the results published so far may not fully reflect the processes in inflamed organs and B cell subsets involved there. In Chapter 3, we have evaluated schistosome-induced Breg cell responses in the lungs of allergen-sensitized mice being very different from splenic responses, which may better reflect the type of responses found in peripheral blood. Although this was studied in mice, it clearly underlines the importance of extending this to further studies on human B-cell biology and its activity in the inflamed organs.

The concept that B cells can regulate inflammation and are important in the maintenance of peripheral or mucosal tolerance is well conceived, but before manipulation of these cells can be applied in future treatment of inflammatory diseases, further research should focus on the identification of pathways and molecules that enhance the number and/or activity of Breg cells. Identifying the mechanisms by which helminth infection and/or their molecules influence (local) B cell function may be an interesting novel strategy to control or prevent allergic inflammatory responses at multiple sites at the same time.

Signals for regulatory B cell induction

Working models

In many of the above described studies applying autoimmunity models, MyD88-dependent TLR signaling (TLR2, 4 and 9) and CD40 ligation, with or without BCR triggering, proved to be important for Breg development (Fig. 1, **Chapter 4**). Many of these findings have been paralleled in human *in vitro* studies (51;52;54). To integrate all the information on distinct signals required for Breg cell activation and development, several working models have been put forward: Mizoguchi and Bhan have suggested that more than one Breg cell subset exists, with different subsets requiring different activation signals. Innate type Breg cells are induced by TLR ligands while adaptive type Breg cells are induced by CD40 and (self) antigens that trigger BCRs (55). Instead, Fillatreau, Gray and coworkers have proposed that during autoimmunity, all activated B cells can become suppressors and that these B cell suppressive functions are acquired during a stepwise activation process initiated by TLR ligands followed by BCR and CD40 reinforcement (56). Alternatively, Tedder and coworkers have suggested a model in which immature progenitors cells progress into mature B10 cells following ligation by TLRs and CD40 (57).

Most studies investigating the presence and function of Breg cells following TLR stimulation limit their analysis to IL-10 as the major anti-inflammatory and effector cytokine of Breg cells. However, it is often neglected that, next to IL-10, TLR ligation can simultaneously contribute to the development of inflammatory responses (58;59). For example, CpG, although a strong IL-10 inducer, it also results in strong B cell activation, proliferation, immunoglobulin production and expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (60-62). Indeed, elevated numbers of IL-6-producing B cells were observed in MS patients after *in vitro* ligation with CD40 and B-cell receptor with CpG, which was demonstrated to have a pathogenic role in the development of chronic experimental autoimmune encephalomyelitis in mice (34). Because of

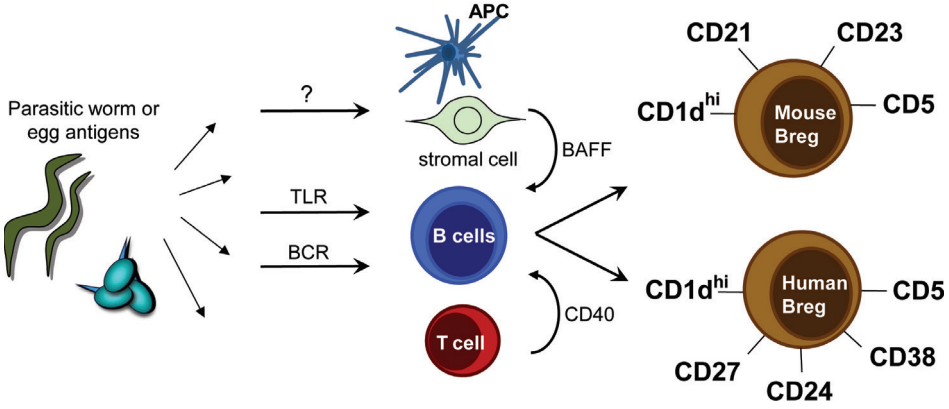

Figure 1. Signals for Breg cell development. Helminth antigens support Breg cell development either directly via TLRs and/or BCR-crosslinking plus CD40 ligation or indirectly via BAFF, produced by stromal cells or local APC. Typical mouse Breg cell markers are CD1d^{hi}, CD5, CD21 and/or CD23. Typical human Breg cell are CD1d^{hi}, CD5, CD24, CD27 and/or CD38.

the recent interest to study the TLR-induced generation of Breg cells, it is crucial to investigate both pro- and anti-inflammatory cytokine responses when studying the role of TLR ligands as promoting adjuvant for Breg cells, and to consider species-related differences when extrapolation findings from disease models to human conditions, as described in **Chapter 4**.

Signals from helminth antigens – TLR, BCR and CD40 ligation

How can this information be applied to helminth-induced Breg cell activation and development (Fig. 1)? Several reports have suggested that *S. mansoni* eggs and adult worm antigens contain TLR ligands: lacto-N-fucopentaose-III (LNFPIII), a milk-derived sugar similar to those found on soluble egg antigens (SEA) interacts with TLR4, at least on DCs, and stimulates splenic B cells to produce IL-10 (63). Furthermore, lysophosphatidylserine, a lipid derived from soluble *S. mansoni* worm antigens ligated TLR2 on human monocyte-derived DCs and promoted Treg cell activity (64). Moreover, it was demonstrated that soluble egg antigen (SEA) could modify immune responses by both human B cells and DCs via TLR2 (65). More evidence for the involvement of TLR signaling in microbial-induced Breg cell development comes from reports describing non-parasitic infections: B-cell ligation by TLR2 ligands from *Helicobacter* species suppressed *Helicobacter*-induced immunopathology by inducing Treg cells (66). MyD88-signaling in B cells suppressed protective immunity during *Salmonella typhimurium* infections via IL-10 affecting neutrophils, natural killer cells and effector T cells (9). Lastly, Amu *et al.* showed that *in vitro* exposure of splenic B cells to live schistosome worms induced functional IL-10-producing Breg cells (5), supporting the notion that direct interactions between helminth molecules and B cells may be involved in the induction of Breg cells. However, in this study the involvement of TLR signaling was not assessed. Because peritoneal injection of schistosome egg-derived glycans or filarial glycoproteins also induces IL-10-producing B cells (5;67)**,** the exact helminth-derived molecules involved in enhancing the activity of Breg cells in protection against AAI might be a shared molecule present on both worms and deposited eggs. Therefore, further studies need to be done to identify the (nature of) helminth antigens and the molecular details of their interaction with the immune system, answering the question whether different B cell subsets can be stimulated, depending on the stimuli (helminth molecules), to secrete IL-10. In addition, a possible role for CD40 ligation either alone or with BCR triggering, to mediate helminth-induced Breg cell development needs to be clarified. Importantly, as indicated in **Chapter 4**, ligation of TLRs on the surface of B cells and interaction with T cells via CD40-CD40L interaction can even generate an amplifying signal for B-cell IL-10 production. Due to the dual role of TLR ligands, namely promoting as well as restraining inflammation, it would be interesting to study whether micro-environmental changes could reprogram Breg to inflammatory B cells and induce pro-inflammatory cytokine release or vice versa. This would also provide insight in the stability or flexibility of the various cytokine producing B cell phenotypes.

Signals from helminth – soluble factors

Interestingly, endogenous apoptotic cells (68) or soluble factors, such as B cell-activating factor (BAFF) (69) may also provide signals for Breg cell activation (Fig. 1). BAFF induced IL-10-producing splenic Breg cells *in vitro* which were mainly derived from MZ B cells and had a distinct $CD1d^{hi}CD5^{+}$ phenotype. In addition, intraperitoneal injection of BAFF increased IL-10-producing B cells in the MZ areas (69). As BAFF can be produced by local dendritic cells or stromal cells, it is possible that both direct effects of helminth-derived antigens on B cells and indirect signals, such as helminth infection-induced APC production of BAFF, form an important stimulus for the development of Breg cells. As this point of view may open a totally new area of research, it is worth noting that a recent study by Phythian-Adams *et al.,* in which depletion of CD11c-expressing cells during natural schistosome infections, completely abolished infection-induced changes in the frequency of MZ B cells, but not other B cell subsets (70). Although these findings suggest that splenic $CD11c^{+}$ cells can strongly affect MZ B cells, it is still unclear whether this has any consequences for the induction of IL-10-producing (MZ) Breg cells by schistosomes.

Recently, injections with recombinant IL-33, an innate type 2 cytokine (71), protected mice from IBD induction and protection was closely associated with the induction of IL-10-producing $CD23^{-}$ B cells in the circulation (72). Interestingly, during helminth infections or exposure to allergens also enhanced IL-33 levels have been reported in the lungs (73-75). However, whether this IL-33 response is associated with the induction and/or migration of Breg cells into the mucosal tissues and may give rise to pulmonary B cells as described in **Chapter 3** remains to be clarified. Another set of recent studies focused on the role of a novel anti-inflammatory cytokine IL-35. Treatment with IL-35 protected mice from experimental autoimmune uveitis via the induction of mostly plasma B cells producing IL-35 and/or IL-10 (32). Mice in which B cells are deficient for one of the subunits of IL-35, i.e. p35 or EBI3, displayed an enhanced APC function, suggesting that IL-35 can regulate the APC function of B cells in an autocrine fashion (30). It would be interesting to study the role of IL-35-producing plasma/B cells in our asthma model and investigate whether these processes contribute to the schistosome-induced protection against AAI (**Chapter 3**).

Application of Breg cells for the treatment of allergy and asthma?

At the moment, treatment of allergic diseases is mostly centered on the alleviation of symptoms though the use of drugs such as anti-histamine, glucocorticoids and bronchodilators. These treatments are nonspecific, could induce serious side effects, and require lifelong applications. Therefore, it is warranted to search for other therapies, such as allergen-specific immunotherapy, that can target allergen-specific cells and have the possibility to induce longer lasting protective immunity. Successful immunotherapy have been correlated with increased IL-10-secreting

Tr1 and FoxP3^{+} Treg cells together with increased IgG4 and IgA responses and simultaneous decreased IgE levels (76). Recently, an increase in IL-10-producing allergen-specific B cells was demonstrated in allergic patients receiving allergen-specific immunotherapy (44). Furthermore, it has become clear from our report (**Chapter 6**) that a part of the Breg cell compartment of patients with allergic asthma is impaired in number and activity. Therefore, the restoration of impaired Breg function may hold promise as a novel therapeutic strategy. Studies on Breg cells described in this thesis, show particularly that IL-10-producing B cells are of interest because of their ability to induce/recruit Treg cells, even further amplifying regulatory responses, and their capacity to strongly reduce allergic airway inflammation, at least in mice (Fig. 2). This emphasizes that targeting for regulatory B cells may offer superior immunosuppression when compared to Treg alone. However, before therapeutic application can be evaluated in patients a number of issues first need to be addressed. Firstly, although most studies have mainly studied the potency of Breg cells in preventing onset of disease, there are only a few reports that describe the capacity of Breg cells to reverse established disease. For example, schistosome-induced CD1d^{hi} B cells could reverse asthma-like AAI in a mouse model (5), while IL-35-induced B cells could ameliorate autoimmune uveitis even when the disease was established (32). Clearly, more studies are needed evaluating immunosuppressive B cells for the treatment of established diseases.

Another open question is whether it is necessary to generate antigen- or allergen-specific Breg cells within novel treatment approaches. Applying non-specific immune suppressive B cells for treatment could lead to undesired side effects, such as general immune suppression promoting of tumor progression demonstrated in mouse models (77) and might prolong the course of infection as shown for chronic salmonella infection and HIV (78-80). Therefore, we would argue that the induction of allergen-specific Breg cells is mostly desired, if possible. Promising in this respect is a recent study describing an enhanced population of bee venom allergen-specific IL-10-producing Breg cells in patients following successful allergen-specific immunotherapy, reaching similar levels as found in tolerant beekeepers (44). Interestingly, these IL-10-producing B cells later developed into IgG4-producing B cells, a phenomenon that is also highly present in helminth-infected individuals. Therefore, it is tempting to suggest IL-10-producing B cells induced by helminths may develop into IgG4-producing B cells and thereby may contribute to protective mechanisms against allergic symptoms.

Lastly, it is unclear in this stage what is the best application form of helminth-based therapy for the treatment of respiratory diseases. Treatment options range from controlled natural infection with certain species of helminths, mixtures of helminth-derived products or even with single immunosuppressive helminth-derived molecules. However, the identification of the most potent immunosuppressive molecules is an elaborative process and may take a long time before application is possible in clinical studies. Therefore, several research

groups have taken up the approach using *Trichuris (T.) suis* eggs or *Necator (N.) americanus* larvae as a basis for helminth therapy. Treatment of *T. suis* eggs appeared to be beneficial for the clinical outcome of patients with MS or inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (81-83), suggesting that this approach may be successfull in treating these specific types of inflammatory diseases. However, since most studies have only applied a follow-up time of less than 24 weeks, more studies are needed to confirm whether this protection can sustain for a longer period of time. What is in contrast to these studies is that clinical trials with *T. suis* eggs in allergic rhinitis or *N. americanus* larvae in allergic rhinitis, asthma and coeliac disease did not show any beneficial effect with respect to clinical symptoms, medication use or skin-prick test reactivity (84-89). It remains to be established whether different types of helminths, e.g. schistosomes, would be more effective in generating protective effects in the treatment of atopic diseases (90). Furthermore, recent evidence has suggested that primarily early-life exposure is of most importance for the development of the immune system, and treatment at young age might be an decisive element in the efficacy of the treatment (91;92). Moreover, the development of immunomodulatory effects might take months or even years to be established. Currently, long-term treatment or the administration of high doses have not been assessed using helminth-based therapy, due to a greater risk of developing pathogenic effects. In order to improve the efficacy of the treatment but not run into the risk of more severe side-effects, it seems a more likely strategy to treat patients with a single parasite-derived molecule or mixture

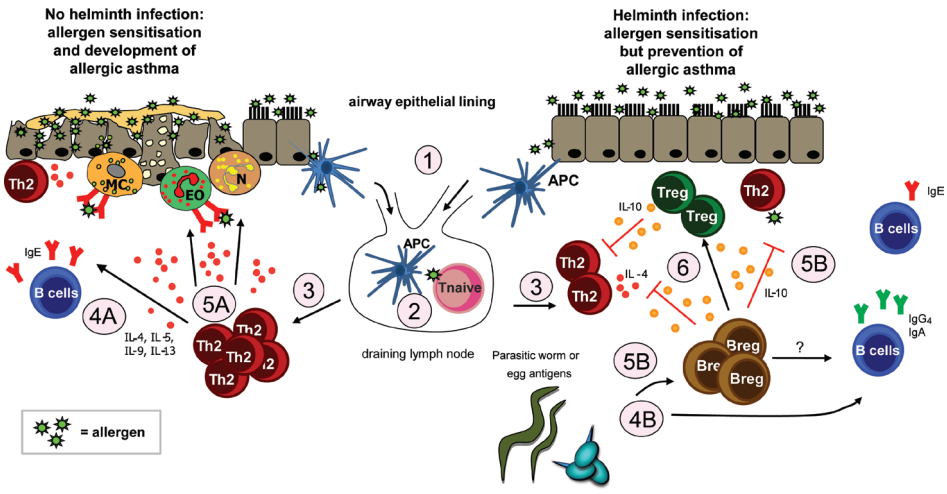

Figure 2. Breg cell-induced protection against allergic airway inflammation. In non-infected individuals, allergic sensitization leads to allergen-loaded airway DCs, driving polarized Th2 cells (1-3). These Th2 cells will subsequently drive the allergic effector cascade in the lung (4a,5a). In helminth-infected individuals, steps 1 till 3 are similar. At the same time however, helminth antigens will prime Breg cells (4b) secreting IL-10 and suppressing DCs and Th2 cells (5b). In addition, Breg cells will induce/recruit Treg cells(6), further suppressing the Th2 immune response.

of molecules that targets regulatory cells instead. In a murine model of colitis, excreted-secreted products from *Ankylostoma*, *Acanthocheilonema vitae*, and extracts from *S. mansoni* adult worms improved the clinical score or diminished local inflammation, respectively (93;94). The filarial-derived glycoprotein ES-62 has been shown to improved arthritis in mice, and reduced the production of pro-inflammatory cytokines in synovial cells from patients with rheumatoid arthritis (95;96). Furthermore, molecules derived from helminths including adult worms, eggs, and isolated protein, or extracts were described to suppress airway inflammation in a murine model of allergic asthma (97-104). Making full benefit from Breg cells actively induced during helminth infection (this thesis), the next step in this line of research would be to identify the dominant schistosome molecules or pathways driving those Breg cells and explore their beneficial effects in allergic patients.

Concluding remarks

The underlying mechanisms leading to inflammatory conditions such as autoimmune diseases and allergies are diverse and far from being fully understood. However, it has become obvious that a balance between effector and regulatory functions of different subsets of immune cells is critically important in the maintenance of a balanced steady-state condition. Under those conditions, Breg cells can be an important player to help to control effector cell activation, by releasing immunosuppressive cytokines and inducing target cell apoptosis. The broad target cell range of their cytokines allows them to inhibit pro-inflammatory functions of both innate immune cells, such as DC and macrophages as well as cells of the adaptive immune system, such as effector T cells of both the Th1 and Th2 lineage. Furthermore, they also further amplify the regulatory arm of immune responses by inducing regulatory T cells. Impaired regulatory capacity of Breg cells might play a role in the development of inflammatory diseases. Uncontrolled effector T and B cell activation can ultimately lead to inflammation and tissue damage in various target organs. Correspondingly, several treatments demonstrated to be beneficial in autoimmune and allergic diseases seem to affect the immune system at the level of B cells by amplifying their regulatory capacity. Therefore, adding Breg cells to the spectrum of regulatory cells such tolerogenic DCs and Tregs, may be of particular interest to treat a range of diseases. For this, helminth infections may be of particular value, as helminths appear to be potent inducers of Breg cells. In addition, to further characterization of Breg subtypes and their mode of action, it would be important to unravel the mechanisms underlying Breg cell induction by helminths, and to identify the helminth-derived molecules involved, as this may open novel avenues for the treatment of hyper-inflammatory diseases such as allergy and autoimmunity.

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Nederlandse samenvatting
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Nederlandse samenvatting

Afweerreacties in allergisch astma

Het afweersysteem, ook wel het immuunsysteem genoemd, heeft als doel mensen te beschermen tegen schadelijke indringers zoals bacteriën, virussen en wormen. Het afweersysteem kan worden onderverdeeld in een aspecifiek (aangeboren) en adaptief (verworven) deel. Het aspecifiek afweersysteem bestaat uit een eerstelijnsafweer van huid en slijmvliezen die een mechanische barrière vormen om het binnendringen van ziekteverwekkers tegen te gaan. Mocht een ziekteverwekker toch het lichaam binnendringen, dan stuit deze op een tweedelijnsafweer die bestaat uit witte bloedcellen. Deze afweercellen kunnen ziekteverwekkers opnemen en verteren. Hierna worden de kleine stukjes (antigeen) van de indringer gepresenteerd aan witte bloedcellen van het adaptieve immuunsysteem, zoals T cellen en B cellen. Het type afweerreactie dat geactiveerd wordt, hangt af van de binnendringende ziekteverwekker. Zo ontwikkelen T cellen zich tot T helper (Th) 1 cellen tijdens virale infecties en worden juist Th2 cellen gevormd tijdens worminfecties. De belangrijkste functie van B cellen in het afweersysteem is de productie van antistoffen, ook wel immuunglobulinen genoemd. Deze antistoffen herkennen heel specifiek een bepaald antigeen afkomstig van een ziekteverwekker en zullen vervolgens na binding aan het antigeen een reactie starten die uiteindelijk leidt tot de opruiming van deze ziekteverwekker. Aangezien dit soort afweerreacties ontstekingen bevorderen, kan het ook schade aanrichten aan de omliggende weefsels. Een goed functionerend afweersysteem zal er naar streven om de schade aan het lichaam zelf, als gevolg van heftige afweerreacties, in zekere mate af te remmen. Deze tak van het afweersysteem wordt het 'regulatoire afweersysteem' genoemd en bestaat uit verschillende regulatoire witte bloedcellen die signaalstoffen uitscheiden en zorgen voor tolerantie.

Als de verschillende componenten van het afweersysteem uit balans zijn, worden afweerreacties tegen een onschuldig antigeen of een antigeen afkomstig van het eigen lichaam niet afgeremd en kunnen ontstekingsziekten ontstaan zoals allergieën en auto-immuunziekten. Allergie is een complexe ziekte die wordt veroorzaakt door een combinatie van erfelijke aanleg die iemand heeft om allergie te ontwikkelen en omgevingsfactoren. Voorbeelden van allergische ziekten zijn eczeem, hooikoorts en astma. In dit proefschrift richten we ons op allergisch astma. Allergisch astma is een chronische longaandoening die ontstaat doordat het afweersysteem een hevige Th2-specifieke reactie ontketent tegen ingeademde onschuldige stoffen zoals huisstofmijtdeeltjes, kattenhuidschilfers of boom- en graspollen, terwijl gezonde mensen daar niet of nauwelijks op reageren. Deze onschuldige stoffen worden allergenen genoemd. Tijdens een Th2 afweerreactie komen mediatoren, zoals histamine vrij die zorgen dat de gladde spiercellen van de luchtwegen samentrekken en de bloedvaten zich verwijden. Verder zorgt een verhoogde slijmproductie en activatie van andere

ontstekingscellen voor irritatie van het weefsel en vernauwing van de luchtwegen. Als gevolg treedt er weefselschade op waardoor de luchtwegwand stugger wordt en de spierlaag verdikt. Dit leidt uiteindelijk tot symptomen zoals benauwdheid, kortademigheid, hoesten en piepende ademhaling in astmapatiënten. Tot op heden is astma niet te genezen, wel te onderdrukken met luchtwegverwijdende en ontstekingsremmende medicatie.

Parasitaire wormen beschermen tegen allergie

In de afgelopen decennia, vooral vanaf begin jaren '70, zijn allergisch ziekten bij kinderen sterk toegenomen in de geïndustrialiseerde landen. Deze bevinding kon niet verklaard worden vanuit een verandering in erfelijke aanleg, maar leidden tot de zogenaamde 'hygiënehypothese' die een verband trok tussen het elimineren van infecties en infectiebronnen door veranderingen in levensstijl en een verbeterde hygiëne, en de forse toename van allergieën in de geïndustrialiseerde landen. Epidemiologisch onderzoek toonde aan dat in ontwikkelingsgebieden, waar veel chronisch worminfecties voorkomen, allergisch aandoeningen veel zeldzamer waren en de prevalentie in de afgelopen decennia nauwelijks gestegen was. Behandeling gericht op de verwijdering van deze worminfecties resulteerde juist in een toename van prevalentie van allergieën. Verder bleek dat allergie en astma ook minder voorkwam bij kinderen die tijdens hun jeugd opgegroeid waren in een omgeving die rijk was aan micro-organismen, zoals bacteriën en schimmels. Voorbeelden zijn kinderen die opgroeiden op een boerderij, met meerdere broers en zussen, of die regelmatig in een kinderdagverblijf verbleven. Dit leidde tot de veronderstelling dat juist de micro-organismen waarmee wij tezamen zijn geëvolueerd zoals huid- en darmbacteriën en indringers die chronisch in het lichaam verblijven zoals parasitaire wormen, invloed hebben op een evenwichtige uitrijping van ons afweersysteem. Door de eliminatie of veranderde samenstelling van deze micro-organismen in onze omgeving wordt verondersteld dat het regulatoire afweersysteem minder hard hoeft te werken waardoor de balans doorslaat naar een ontstekingsbevorderende afweersysteem. Als gevolg hiervan kunnen overdreven ontstekingsreacties ontstaan tegen onschuldige stoffen. Uit verschillende onderzoeken is inderdaad gebleken dat bepaalde witte bloedcellen van het regulatoire afweersysteem, waaronder de regulatoire T (Treg) cellen, verminderd zijn in aantallen in mensen met allergisch aandoeningen en auto-immuunziekten.

Regulatoire netwerk tijdens worminfecties

Uit verscheidene muismodellen en humaan onderzoek is aangetoond dat een langdurige infectie met parasitaire wormen, zoals met de platworm van het geslacht *Schistosoma,* leidt tot een versterking van het regulatoire immuunsysteem. Men veronderstelt dat deze opgewekte onderdrukking van het afweersysteem gunstig is voor de worm omdat het de wormen beschermt tegen uitroeiing, maar ook gunstig voor de geïnfecteerde gastheer omdat die dan beschermd wordt tegen al te heftige ontstekingsreacties met mogelijk orgaanschade als gevolg. Als bijeffect

zou naast de worm-specifieke afweer, ook tegelijkertijd reacties tegen andere moleculen, zoals allergenen, kunnen worden onderdrukt. Een belangrijke en centrale speler in het door wormen opgewekte regulatoire afweersysteem bleek het ontstekingsremmende signaalmolecuul interleukine-10 (IL-10) te zijn dat onder anderen geproduceerd wordt door T cellen. Tevens werden tijdens infectie meer Treg cellen aangemaakt die met behulp van signaalstoffen, maar ook via directe interactie met andere afweercellen, de sterkte van een opkomende immuunrespons verder konden matigen.

Muismodellen van auto-immuunziekten toonden aan dat naast T of Treg cellen, ook B cellen een belangrijke bron van IL-10 konden zijn. B cellen staan eigenlijk meer bekend om hun versterkende functie binnen afweerreacties, maar onderzoek heeft aangetoond dat B cellen wel degelijk ook een regulerende en onderdrukkende functie kunnen hebben. Zij doen dit door de productie van veel IL-10 en deze cellen worden dan ook regulatoire B (Breg) cellen genoemd. Al snel werd duidelijk dat Breg cellen naast de productie van IL-10 ook op andere manieren deze rol kon uitoefenen, bijvoorbeeld door de productie van ontstekingsremmende signaalmolecuul TGF-β en/of het remmen van celdeling en signaalstofproductie van Th cellen. Verder kunnen Breg cellen het regulatoire netwerk versterken door het opwekken van meer Treg cellen. In muizen zijn verscheidene Breg celpopulaties aangetoond die gekenmerkt worden door hoge expressie van moleculen op het oppervlakte zoals CD1d, CD5 en/of CD23 en die zich voornamelijk in de milt, buikholte en in de lymfeklieren bevinden. Onlangs zijn er in het bloed van mensen ook verschillende IL-10 producerende Breg celpopulaties geconstateerd. Tevens is aangetoond dat bepaalde Breg celpopulaties in patiënten met auto-immune ziekten verminderd zijn in aantallen en/of activiteit in vergelijking met Breg cellen van gezonde mensen. In analogie met hun rol in bescherming tegen auto-immuniteit, is er wellicht ook een functie voor Breg cellen weggelegd in de bescherming tegen allergische astma tijdens chronische worminfecties.

Onderzoek beschreven in dit proefschrift

Het doel van het onderzoek beschreven in dit proefschrift is om beter inzicht te krijgen hoe chronische worminfecties bescherming kunnen bieden tegen allergisch astma en wat de rol van regulatoire B cellen hierin is. In **Hoofdstuk 2** hebben wij gebruik gemaakt van een gecombineerd muizenmodel voor astma en de parasitaire worm *Schistosoma (S.) mansoni*. Experimenten toonden aan dat ook in de longen van muizen een verminderde allergische ontstekingsreactie plaats vond tijdens een chronische worminfectie. Met behulp van muizen die geen IL-10 producerende B cellen konden aanmaken, bleek dat de aanwezigheid van IL-10 producerende B cellen essentieel was voor bescherming tegen allergie tijdens infectie. Wanneer B cellen van worm-geïnfecteerde dieren afkomstig van de milt of de longen werden overgebracht naar allergische ontvangstmuizen, resulteerde dit in bescherming tegen allergisch astma. Vervolgens werd het mechanisme waarmee deze B cellen bescherming boden onderzocht. B cellen uit

de milt beschermden tegen allergisch astma via IL-10 productie en de aanmaak van Treg cellen in de longen. Daarentegen, experimenten met beschermende B cellen uit de longen toonden aan dat ze ontsteking onderdrukten onafhankelijk van IL-10 of Treg cellen. Verder onderzoek liet zien dat de verantwoordelijke Breg celpopulatie in de milt gekenmerkt werd door de expressie van moleculen $CD21^{+}CD23^{-}CD1d^{++}$, dat sterk overeenkomt met de marginale zone B celpopulatie. Om een beter beeld te krijgen of *Schistosoma* infecties in mensen ook Breg cellen aanmaken, hebben we een veldstudie uitgevoerd in Gabon (centraal Afrika), waar *S. haematobium* infecties endemisch voorkomt. In het bloed van schoolkinderen met worminfecties werden verhoogde aantallen IL-10 producerende B cellen met een hoge CD1d expressie gevonden in vergelijking met niet-geïnfecteerde kinderen. Na een behandeling tegen worminfecties daalden het aantal IL-10 producerende B cellen weer naar aantallen die ook in niet-geïnfecteerde kinderen waren aangetoond. Hiermee hebben we een directe relatie tussen *Schistosoma* en Breg celaanmaak aangetoond zowel in de muis als in de mens.

In **Hoofdstuk 3** hebben wij de beschermende functie van de B cellen uit de longen verder onderzocht. De B cellen uit de longen bleken geen kenmerken of functies te delen die tot nu toe aan Breg cellen waren toegekend. In celkweken met T cellen bleek dat B cellen uit geïnfecteerde dieren een verminderde capaciteit hadden om T celdeling en Th2 cellen aan te maken in vergelijking met B cellen uit allergische controle dieren. Dit suggereert dat B cellen via dit mechanisme zou kunnen bijdragen aan de bescherming tegen allergisch ontsteking tijdens een worminfectie. Het is nog onduidelijk via welk mechanisme de B cellen uit de longen dit bewerkstelligen en hiervoor is nog verder onderzoek nodig.

Met oog op een eventuele therapeutische toepassing van Breg cellen in de bescherming of genezing van allergische aandoeningen, is het belangrijk om te identificeren onder invloed van welke prikkels de Breg cellen worden geactiveerd of aangemaakt. Wormmoleculen of andere moleculaire structuren van micro-organismen kunnen herkend worden door receptoren, waarvan de “Toll-like” receptoren (TLR) een van de meest bekende receptoren zijn. Naast de ontstekingsreactie die opgewekt wordt na TLR herkenning, wordt de laatste jaren ook onderzoek gedaan naar de ontstekingsremmende reacties van TLR. Uit onderzoek blijkt dat activatie van B cellen via bepaalde TLR receptoren leidt tot het aanmaken of activeren van Breg cellen. Daarom hebben wij in **Hoofdstuk 4** in detail beschreven hoe B cellen afkomstig van mens of muis in reactie op verschillende TLR receptoren geanalyseerd en gevalideerd kunnen worden in kweekmodellen. Het is belangrijk om verschillende kweekmodellen te gebruiken om te bepalen of de gestimuleerde B cellen wel ontstekingsremmende signaalstoffen maken en of de cellen zich wel gedragen als functionele Breg cellen.

Met behulp van de informatie uit de voorgaande hoofdstukken wilden we bestuderen of het verhoogde aantal IL-10 producerende B cellen in mensen met *S. haematobium* infecties ook een functionele invloed had op T cellen (**Hoofdstuk 5**). In Gabon werd het bloed van jonge volwassenen met en zonder infectie onderzocht op de aanwezigheid van Breg cellen. Ook in deze studie

bleken geïnfecteerde personen meer B cellen te hebben die IL-10, maar ook TGF-β tot expressie brachten in vergelijking met B cellen van niet-geïnfecteerde personen. De celpopulatie $CD1d^{++}$ B cellen bleek verantwoordelijk te zijn voor meer IL-10 expressie, terwijl een andere celpopulatie, de $CD24^{++}CD27^{+}$ B cellen, meer TGF-β op hun oppervlakte lieten zien tijdens infectie. Vervolgens hebben we onderzocht wat het effect was van deze verschillende Breg celpopulaties op de functie van T cellen. B cellen van geïnfecteerde donoren remden het uitscheiden van signaalstoffen door T cellen in vergelijking met B cellen van niet-geïnfecteerde donoren. Bovendien bleken de B cellen meer Treg cellen en IL-10 producerende T cellen te kunnen opwekken. Wanneer wij de $CD1d^{++}$ populatie uit de totale B cel pool verwijderden, werd het percentage IL-10 producerende T cellen weer vergelijkbaar met die van mensen zonder infectie terwijl nog steeds verhoogde aantallen Treg cellen werden opgewekt. Dit betekent dat $CD1d^{++}$ Breg cellen belangrijk zijn voor het versterken van het regulatoire afweersysteem door het vermeerderen van het aantal IL-10 producerende T cellen, terwijl een andere, nog onbekende Breg celpopulatie meer Treg cellen weet op te wekken.

Ten slotte is bestudeerd of de frequentie van regulatoire B cellen en de aanmaak van IL-10 afwijkt in patiënten met allergisch astma in vergelijking met gezonde individuen (**Hoofdstuk 6**). Wij vonden dat juist de $CD24^{++}CD27^{+}$ B cellen verminderd waren in aantal en in hun capaciteit om IL-10 te maken na blootstelling aan TLR-4 ligand LPS. Vervolgens bleek de vermindering in IL-10 producerende B cellen te leiden tot een functioneel defect in geheugen T cellen in de aanwezigheid van huisstofmijt allergenen: de T cellen maakten namelijk minder IL-10. Deze data impliceren dat een verminderde productie van IL-10 door B cellen, vooral de $CD24^{++}CD27^{+}$ populatie, kan leiden tot een verzwakte IL-10 productie in T cellen. Een verzwakte Breg celfunctie zou daardoor een mogelijk bijdrage kunnen leveren aan een verzwakte onderdrukking van overdreven allergische reacties zoals die in allergisch astma patiënten gevonden worden.

Conclusie

Het onderzoek beschreven in dit proefschrift heeft geleid tot een beter begrip hoe wormen het afweersysteem beïnvloeden en hoe zij via een verhoogde aanmaak van Breg cellen bescherming kunnen bieden tegen de ontwikkeling van astma in muizen. Momenteel bestaat er nog geen afdoende therapie voor allergisch astma. Wel is duidelijk dat verschillende witte bloedcellen die deel uitmaken van het ontstekingsremmende deel van het afweersysteem, waaronder de Breg cellen, verminderd voorkomen en hun functie verzwakt is in patiënten met allergische astma. Een nieuwe therapeutische strategie gericht op het versterken van het regulatoire deel van het afweersysteem via Breg cellen zou hoopgevend kunnen zijn om allergieën en astma te genezen of te voorkomen (**Hoofdstuk 7**). Daarom is het belangrijk om verder onderzoek te verrichten naar moleculen van wormen of signaaltransductieroutes die specifiek Breg cellen kunnen vermeerderen of activeren om zo een centrale plaats te kunnen innemen in een toekomstige therapie voor allergisch astma.

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Curriculum Vitae

Elisabeth Petronella Maria (given name Luciën) van der Vlugt was born on September 25th 1985 in Ter Aar, the Netherlands. She attended secondary school at the "Scala College" in Alphen aan den Rijn, where she passed her VWO exam in 2003. In that same year she started her study Biomedical Sciences at the University of Leiden. During her education, she carried out three research projects at the departments of Tumor Immunology, Medical Microbiology and Parasitology (LUMC). In December 2008, Luciën received her master degree (M.Sc) in Biomedical Sciences at the University of Leiden, the Netherlands.

After her graduation, her last internship project turned into a PhD program at the department of Parasitology under supervision of Prof. Dr. Maria Yazdanbakhsh and Dr. Hermelijn H. Smits. Her research project was part of a program aiming at understanding interactions between the host and helminth infections and their subsequent role in the protection against allergic disorders. She focused at the role of helminth-induced IL-10-producing regulatory B (Breg) cells in both murine models and in humans. During her training period, she visited the Centre de Recherches Médicales de Lambaréné in Gabon for three months, where she studied Breg cells in peripheral blood of *S. haematobium*-infected and uninfected young adults. The findings obtained during her PhD training are presented in this thesis.

Currently, Luciën works as a postdoctoral researcher at the department of Pulmonology (LUMC) in the group of Prof. Dr. Pieter S. Hiemstra. Here, she is involved in a project investigating how farms can protect against childhood asthma, by studying the effect of farm dust on the airway epithelial cell function.

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