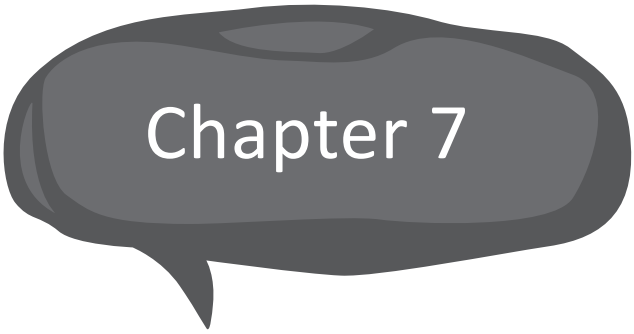

Summarizing discussion

Modified from:

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

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Journal of Allergy and Clinical Immunology 2011;128:733-9

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

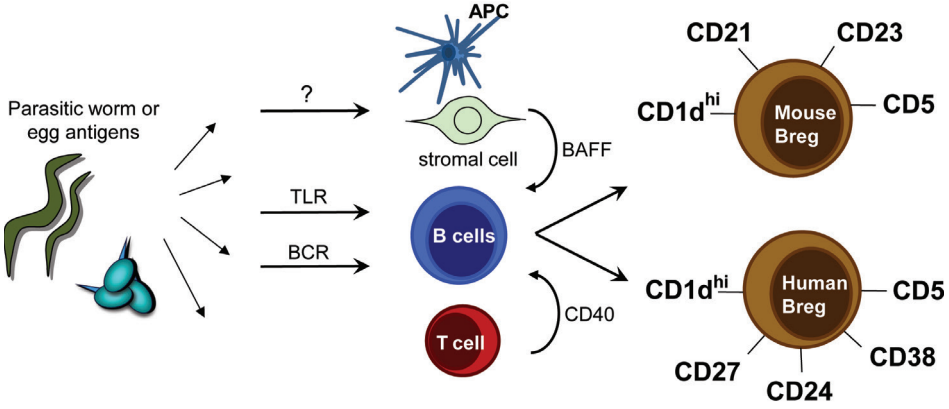

Figure 1. Signals for Breg cell development. Helminth antigens support Breg cell development either directly via TLRs and/or BCR-crosslinking plus CD40 ligation or indirectly via BAFF, produced by stromal cells or local APC. Typical mouse Breg cell markers are CD1d^{hi}, CD5, CD21 and/or CD23. Typical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

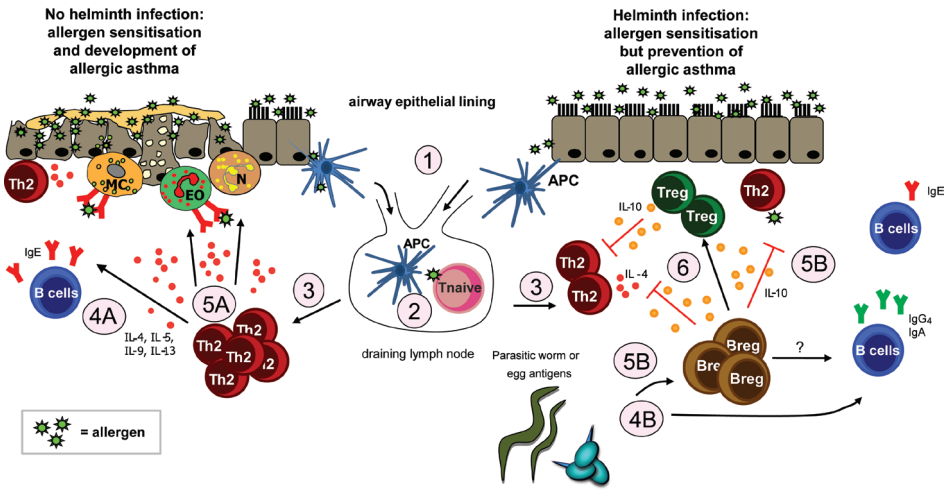

Figure 2. Breg cell-induced protection against allergic airway inflammation. In non-infected individuals, allergic sensitization leads to allergen-loaded airway DCs, driving polarized Th2 cells (1-3). These Th2 cells will subsequently drive the allergic effector cascade in the lung (4a,5a). In helminth-infected 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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