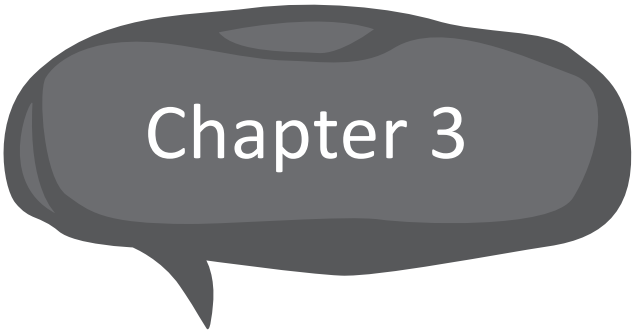

Schistosome-induced pulmonary B cells inhibit allergic airway inflammation and display a reduced Th2-driving function

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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

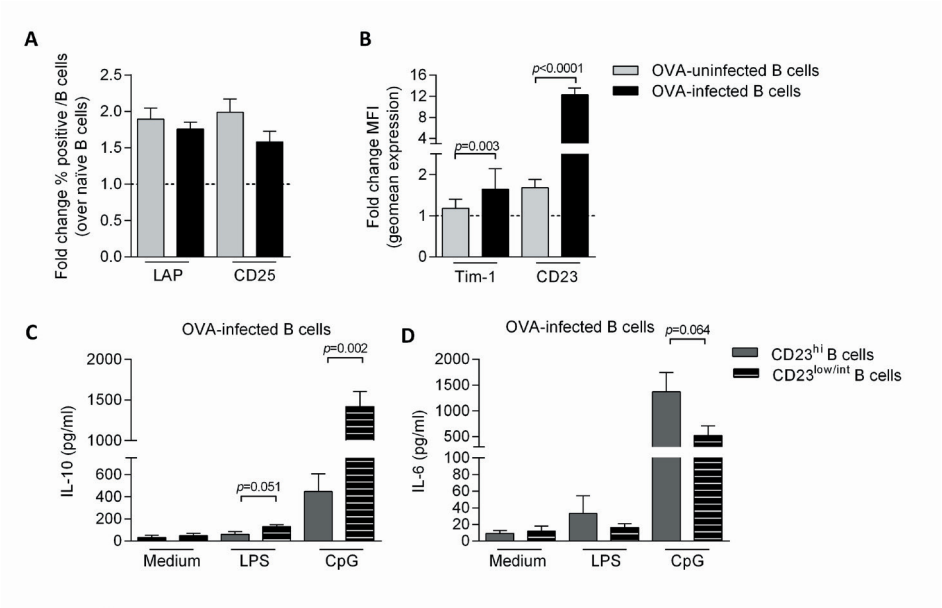

Figure 1. Characterization of Breg cell markers in schistosome-induced pulmonary B cells and inflammatory responses of CD23-sorted B cells after TLR ligation. Mice were infected with *S. mansoni* until the chronic phase (week 15). After sacrifice, the perfused lungs were minced, digested and the single 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.

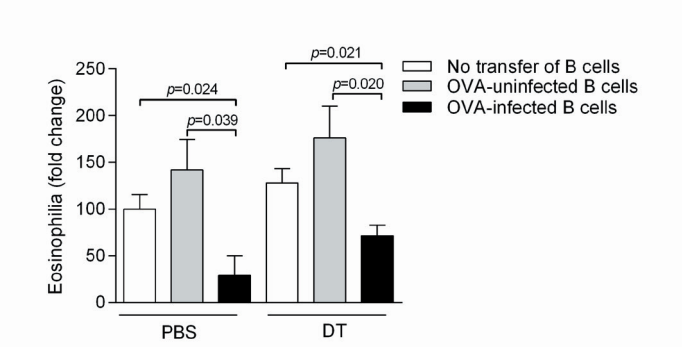

Figure 2. Role of FoxP3$^+$ T cells in pulmonary B cell-induced protection against AAI during schistosomiasis. OVA-sensitized DEREG mice, which carry a diphtheria toxin receptor-eGFP transgene under the control of an additional Foxp3 promoter, were treated with two PBS or diphtheria toxin (DT, 1 µg/ml) 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

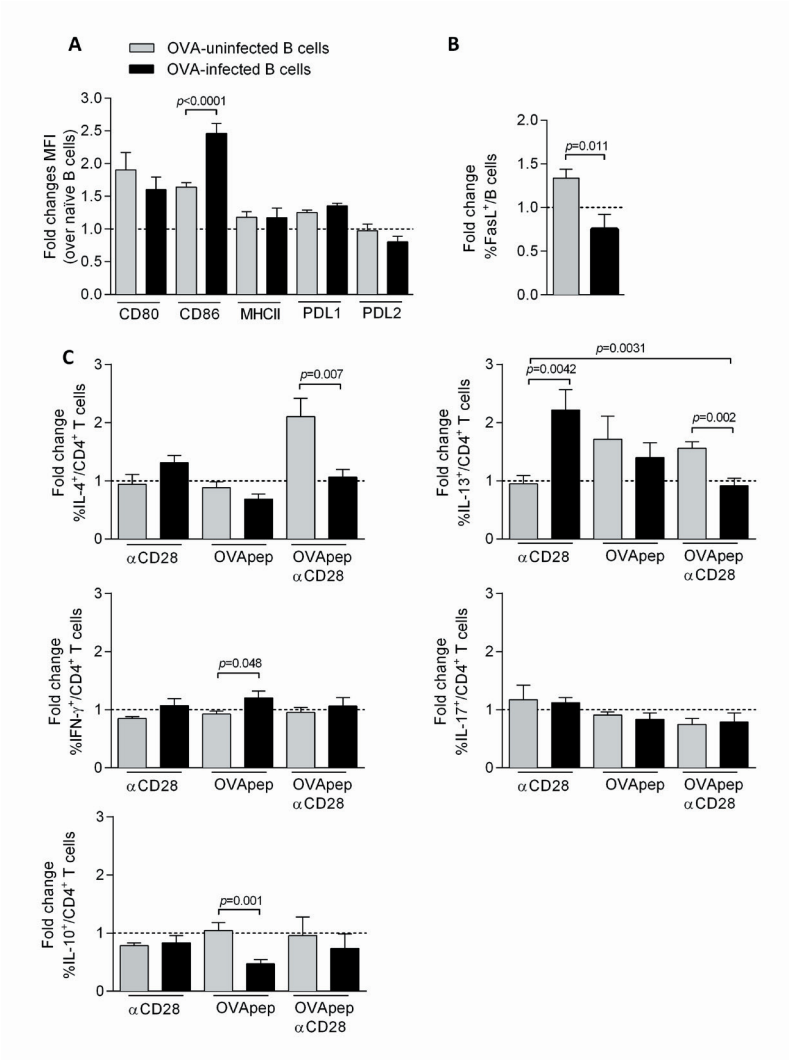

Figure 3. Schistosome-induced pulmonary B cells show elevated expression of CD86 and an impaired capacity to drive Th2 cytokines. Pulmonary B cells were isolated as described in figure 1. For both characterization and co-culture, pulmonary B cells from 2-3 mice were pooled to obtain enough cells for 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.

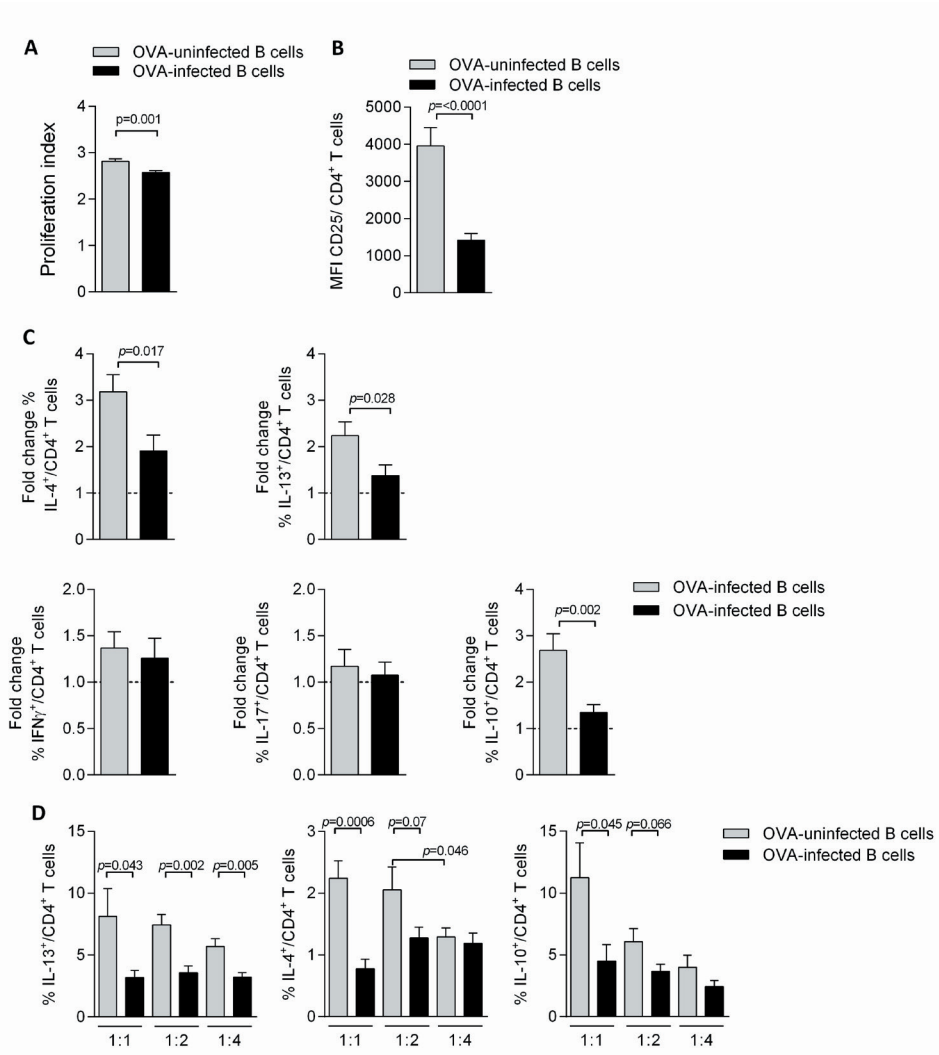

Figure 4. Role of inhibitory molecules in pulmonary B-cell mediated Th2 suppression. (A) Pulmonary B cells (1x10^5), pooled from 2-3 PBS-uninfected, OVA-uninfected, PBS-infected or OVA-infected mice, were cultured with C57BL/6 CD4$^+$ T cells (1x10^5) from naive mice for three days in the presence of 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.

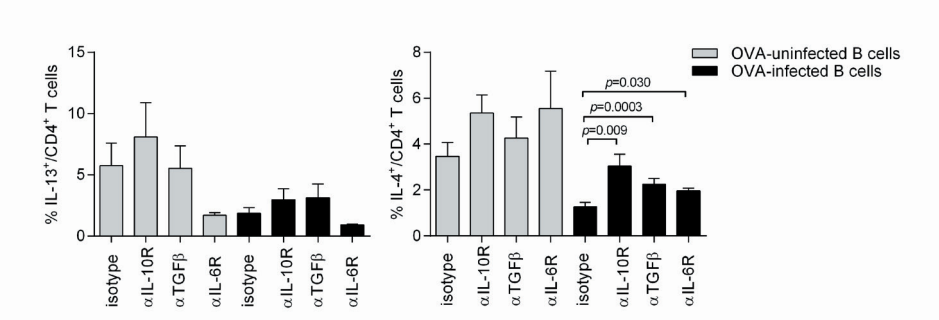

Figure 5. Role of putative inhibitory molecules in pulmonary B cell-mediated Th2 suppression. In vitro co-cultures were performed as described in figure 4 in the presence of blocking anti-IL-10R, anti-TGF-β, anti-IL-6R or isotype control antibodies. Data represents two independent experiment.

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.

Acknowlegments

We thank Louis Boon for kindly providing us isotype control, blocking IL-10R and TGF-β antibodies. We thank Lucja A. Labuda for technical assistance.

Funding

This work was supported by the Lung Foundation (LF grant 3.2.10.072)

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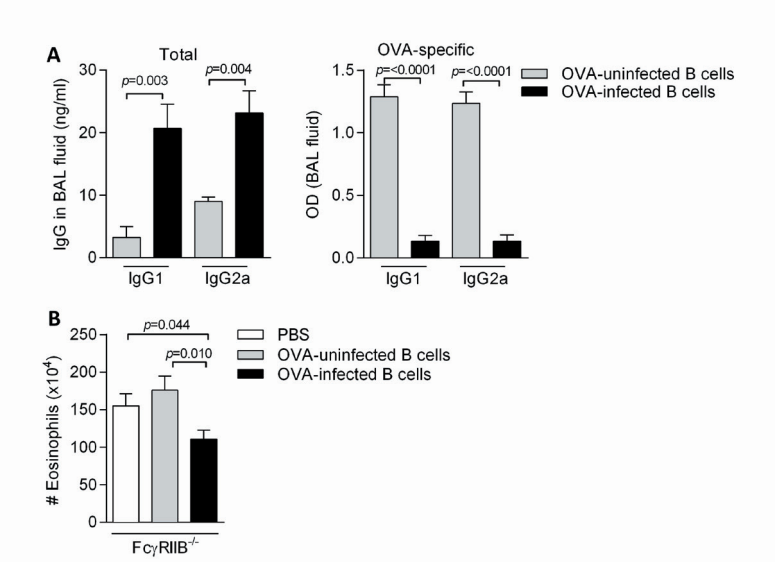
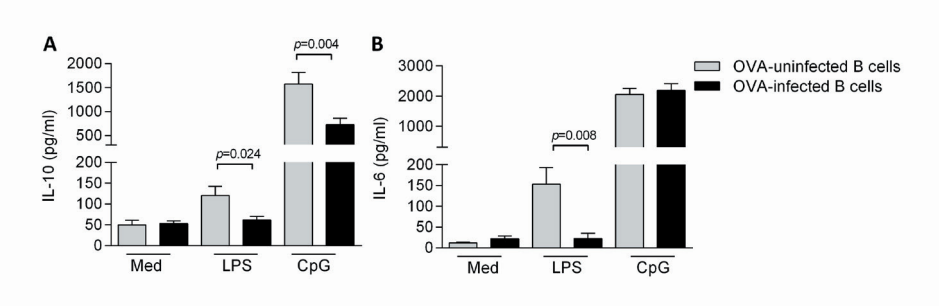
Supplementary figures



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.