Immune modulation by schistosomes: mechanisms of regulatory B cell induction and inhibition of allergic asthma

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Chapter 2

SCHISTOSOME-INDUCED PULMONARY B CELLS INHIBIT ALLERGIC AIRWAY INFLAMMATION AND DISPLAY A REDUCED TH2-DRIVING FUNCTION

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ABSTRACT

Chronic schistosome infections protect against 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 act independently of IL-10. We have now further characterized the phenotype and inhibitory activity of these protective pulmonary B cells. We excluded a role for regulatory T (Treg) cell induction as putative AAI-protective mechanisms. Schistosome-induced B cells showed increased CD86 expression and reduced cytokine expression in response to Toll-like receptor (TLR) ligands compared with control B cells. To investigate the consequences for T cell activation we cultured ovalbumin (OVA)-pulsed, schistosome-induced B cells with OVA-specific transgenic T cells and observed less Th2 cytokine expression and T cell proliferation compared with control conditions. This suppressive effect was preserved even under optimal T cell stimulation by anti-CD3/28. Blocking of the inhibitory cytokines IL-10 or TGF-β only marginally restored Th2 cytokine induction. These data suggest that schistosome-induced pulmonary B cells are impaired in their capacity to produce cytokines to TLR ligands and to induce Th2 cytokine responses independent of their antigen-presenting function. These findings underline the presence of distinct B cell subsets with different stimulatory or inhibitory properties even if induced by the same type of helminth.

INTRODUCTION

Chronic infections with the helminth *Schistosoma mansoni* are associated with immune hyporesponsiveness and an enhanced regulatory network [1]. Regulatory B (Breg) cells, which are predominantly characterized by an enhanced production of IL-10 [2], are part of this network. Their functionality was first demonstrated in mouse models of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), lupus and chronic colitis [3]. Interestingly, helminth-induced Breg cells also can inhibit inflammation and were shown to protect against EAE, systemic fatal anaphylaxis and ovalbumin (OVA)- or house dust mite allergen (Der p1)-induced allergic airway inflammation (AAI) [4-6].

Murine Breg cells were mostly detected within splenic B cell subsets [2]. Breg cells have also been found within the mesenteric lymph node (LN), highly expressing the low-affinity IgE Fc receptor CD23 during *Heligmosomoides polygyrus* infection [6], and within the B-1a B cell compartment of the peritoneal cavity [7]. Various Breg cell-associated markers and suppressive mechanisms have been reported, however most of these studies are based on splenic populations. Apart from their capacity to produce IL-10, Breg cells have been described to induce other members of the regulatory network, e.g. regulatory T (Treg) cells [4, 5, 8], thereby amplifying the protective effect. B cells expressing the membrane-bound T cell immunoglobulin and mucin domain-1 (Tim-1) were able to control auto-immune [9] and allergic diseases [10]. Expression of CD25 by human Breg cells correlated with their IL-10 production [11], and in mice $CD25^+$ Breg cells attenuated inflammatory bowel disease (IBD) [12]. Furthermore, B cells expressing suppressive cytokines other than IL-10 have been described. TGF-β-producing Breg cells controlled inflammation in inhalation tolerance [13] and diabetes models [14], and IL-35-producing B cells regulated immunity during EAE and *Salmonella* infection [15, 16]. B cells also have the capacity to suppress T cell proliferation and cytokine production via cell-cell interactions that involve inhibitory receptors, and result in T cell hypo-responsiveness or the induction of apoptosis. Examples of such inhibitory receptors are T cell-expressed PD-1, Fas as well as CTLA-4, and their respective ligands PD ligand 1 (PD-L1), PD-L2, Fas ligand (FasL) and CD80/CD86 on antigen-presenting cells (APCs) including B cells.

Apart from their role as regulators, B cells play an important role in the induction and maintenance of Th2 immunity. B cells not only produce IgE and IgG1 antibodies, they can also directly interact with Th2 T cells and act as APCs to drive their expansion and cytokine production. Indeed, presentation of allergen by pulmonary B cells has been shown to be required for full Th2 cytokine production in AAI models using μMT and $JH^{-/-}$ mouse models [17, 18], and immunization experiments with the cysteine protease allergen papain suggest that B cells induce T cell/T follicular helper (Tfh) cell IL-4 production in the draining LN [19].

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 to OVA-sensitized mice [5, 20]. Intriguingly, 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 *in vivo* [5]. We hypothesized that schistosome infections support the development of distinct Breg cells in the lungs. Therefore, we further explored the effector mechanism by which pulmonary B cells can protect against AAI. Here, we demonstrate that pulmonary B cells from OVA-allergic mice which were infected with schistosomes are phenotypically and functionally distinct from splenic Breg cells. They have a reduced cytokine response to Toll-like receptor (TLR) ligands and a reduced Th2 cell priming capacity, which seems to be independent of their antigen-presentation function.

RESULTS

Schistosome-induced pulmonary B cells do not share phenotypical characteristics of classical Breg 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 was selectively expanded during schistosome infection compared with 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% of all pulmonary CD19^{+} B cells during infection, data not shown). Therefore, we analyzed several other cell-surface markers as putative markers of Breg cell phenotype and activity, e.g. the membrane-bound marker LAP, as part of a latent TGF-β complex, CD25, Tim-1, CD5 and CD23 [6, 9, 10, 13, 14, 21]. Pulmonary B cells from chronically *S. mansoni*-infected, OVA-allergic (infected/OVA) mice which are known to be protective against AAI [5] had a similar increase in LAP-1^{+} and CD25^{+} cell frequencies over B cells from uninfected, non-allergic mice (uninfected/PBS) as B cells from uninfected, OVA-allergic (uninfected/OVA) mice (**Figure 1A**, **suppl. Figure S1A**), suggesting that TGF-β- or CD25-expressing B cells are not involved in protection against AAI. Although the fold increase in Tim-1^{+} B cell frequencies was significantly higher (**Figure 1A**, **suppl. Figure S1A**) in infected/OVA compared with uninfected/OVA mice, the total percentages of Tim-1^{+} cells remained rather low (less than 7% of all pulmonary CD19^{+} B cells; **suppl. Figure S1A**). CD5 is one of the markers that defines the B-1a subclass of B cells which have been described to have regulatory properties [7]. The fold increase of CD5^{+} B cell frequencies was significantly reduced in infected/OVA compared with uninfected/OVA mice (**Figure 1A**), indicating that this subclass of B cells is probably not important in mediating protection against AAI. The only marker assessed that was clearly enhanced on most pulmonary B cells in infected/OVA mice was CD23 (**Figure 1B**, **suppl. Figure S1B**). As the majority of pulmonary B cells in naïve mice already express CD23, the fold increase in % CD23^{+} cells is only moderate. However, the per cell expression level of CD23 strongly increases on infected/OVA B cells compared with their uninfected/OVA counterparts. The increase in CD23 expression is not surprising as enhanced frequencies of CD23-expressing B cells were already demonstrated for schistosome infections [22] and most probably reflects the general and modified type 2 inflammation present during chronic helminth infection [23]. Furthermore, adoptive transfers of sorted CD23$^{low/intermediate}$ or CD23^{hi} B cells from infected/OVA mice into OVA-sensitized mice remained inconclusive (data not shown), suggesting that the expression level of CD23 on pulmonary B cells does not correlate with suppressive function in our system. Collectively, we found that pulmonary B cells of chronically *S. mansoni*-infected animals do not show enhanced expression of phenotypic markers characteristic to classical Breg cell subsets.

Pulmonary B cell-induced protection against AAI during schistosomiasis is independent of FoxP3^{+} Treg cells

We previously described that pulmonary B cells transfer protection to OVA-sensitized mice in an IL-10-independent manner [24]. We therefore set out to study the involvement of alternative inhibitory mechanisms. 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* [5]. However, this does not exclude the possibility that, despite equal numbers, the activity of Treg cells on a per cell basis had increased. We therefore transferred pulmonary B cells to FoxP3-DTR transgenic DEREG mice, in which Treg cells can be temporarily depleted by DT injections (**Figure 2A**), to investigate the contribution of Treg cell activity during pulmonary B cell-induced protection against AAI. BAL eosinophil numbers remained equally reduced in both PBS- and DT-treated DEREG mice when receiving pulmonary B cells from infected/OVA compared with uninfected/OVA mice (**Figure 2B**). We also did

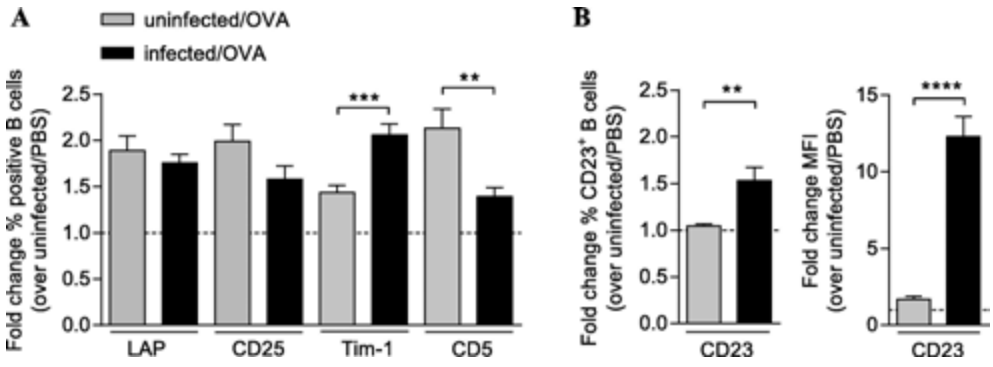


Figure 1. Schistosome-induced pulmonary B cells do not share phenotypical characteristics of classical regulatory B (Breg) cells. Mice were chronically (15 weeks) infected with 36-40 *Schistosoma mansoni* cercariae. Allergic sensitization was induced by two i.p. injections of ovalbumin (OVA)/alum 1 week apart and control mice received PBS. Seven days after the last injection, mice were challenged by OVA aerosol exposure on three consecutive days. Mice were sacrificed 24 h after the last challenge. The perfused lungs were minced, digested and the single cell suspension from 2-3 mice pooled. Next, B cells were purified using anti-CD19 MicroBeads and stained for different Breg cell-associated markers. (**A**) The fold change of percentage surface latency-associated peptide (LAP-), CD25-, T cell immunoglobulin and mucin domain-1 (Tim-1)- and CD5-expressing B cells from uninfected- and infected/OVA mice over control (uninfected/PBS) are shown, representing a summary of 2-4 independent experiments (n=11-17/group). (**B**) Fold change of percentage and geometric mean fluorescence intensity (MFI) of CD23 expression over control (uninfected/PBS). Summary of four independent experiments (n=14-17/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: $^{**}P$ <0.01, $^{***}P$ <0.001, $^{****}P$ <0.0001, as tested by two-tailed unpaired Student's t-test.

not observe an enhanced induction of Treg cells by infected/OVA pulmonary B cells in *in vitro* co-cultures with naïve T cells compared with co-cultures with B cells from allergic control mice (data not shown). These data indicate that AAI is not restored when Treg cell activity is abolished, and suggests that schistosome-induced pulmonary B cells do not drive protection against AAI via enhanced Treg cell activity.

Schistosome-induced pulmonary B cells express elevated levels of CD86 *ex vivo*, and secrete less IL-10 after *in vitro* TLR ligation

T cell-derived Th2 cytokines play a dominant role in the induction and maintenance of AAI [25]. We therefore aimed to investigate the role of pulmonary B cells as APCs and as modulators of effector T cell activation. Important signals that can influence T cell activation/induction of apoptosis, proliferation and cytokine production are, for example, provided by the co-stimulatory molecule CD86, antigen-presentation molecule MHCII, inhibitory receptors such as PD-L1, PD-L2, 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 first analyzed the expression of the above-mentioned molecules. Pulmonary B cells from infected/OVA mice showed a significantly increased CD86 expression compared with B cells from uninfected/OVA mice. Expression levels of MHCII, PD-L1 and PD-L2 were equal between the groups (**Figure 3A**, **suppl. Figure S2**). FasL expression was not induced on pulmonary B cells in response to AAI or *S. mansoni* infection (data not shown). Furthermore, we analyzed the capacity of pulmonary B cells to produce cytokines, which may support or suppress T cell activation, following stimulation by either SEA or TLR-4 ligand LPS and TLR-9 ligand CpG-ODN 1826 as strong B-cell activators. While B cells from infected/OVA mice produced more

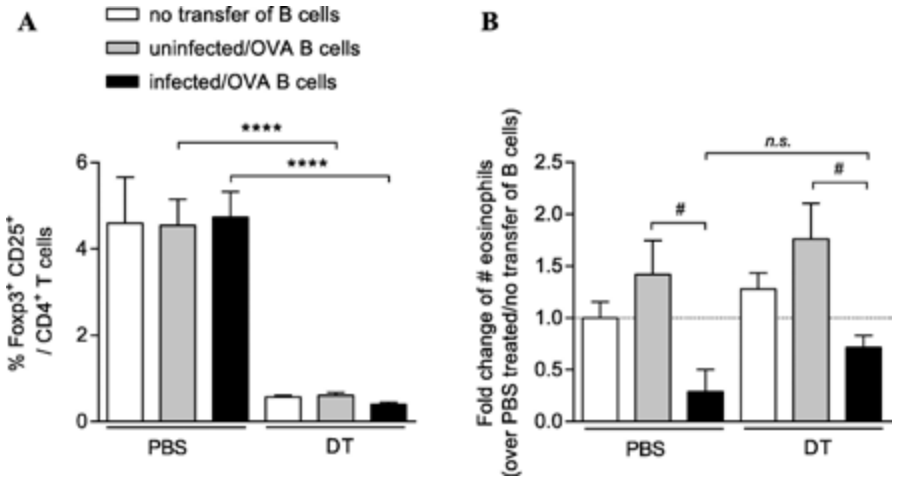


Figure 2. Pulmonary B cell-induced protection against allergic airway inflammation (AAI) during schistosomiasis is independent of Foxp3⁺ regulatory T (Treg) cells. Ovalbumin (OVA)-sensitized DEREG (DEpletion of REGulatory T cells) mice, which carry a diphtheria toxin (DT) receptor-eGFP transgene under the control of an additional Foxp3 promoter, were treated with two PBS or DT (1 µg/mouse) i.p. injections in order to deplete the Foxp3⁺ Treg cells, 1 day before and 2 days after the adoptive transfer of 5 x 10^6 CD19⁺ lung B cells. After 2 days, mice were challenged for three consecutive days and sacrificed 24 h after the last challenge. (**A**) The percentage of Foxp3⁺ CD25⁺ Treg cells in the lungs are shown, representing a summary of 1-2 independent experiments (*n*=2-8/group). (**B**) Fold change of bronchoalveolar lavage (BAL) eosinophil numbers over PBS-injected DEREG mice that did not receive B cell adoptive transfer. A summary of two independent experiments (*n*=3-7/group) is shown. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: ****P <0.0001, as tested by two-tailed unpaired Student's *t*-test (A) or #P <0.05 as tested by One-way ANOVA following Tukey's multiple comparisons test (B). n.s., not significant.

IL-10 in response to SEA as previously described [5], they interestingly secreted significantly less IL-10 and IL-6 in response to LPS or CpG-ODN 1826 compared with B cells from uninfected/OVA mice (**Figure 3B**). Recently, B cells have been reported to secrete the regulatory cytokine IL-35 in response to TLR4 and CD40 ligation [15]. Pulmonary B cells in this study were MACS-sorted for CD19, but they nevertheless contained CD138-expressing plasma cells (**suppl. Figures S3A, B**), which downregulate CD19 expression and have been described as the main IL-35 expressing B cells. We measured a clear gene expression of the IL-35 subunits Ebi3 and IL-12p35 by qPCR after stimulation of pulmonary B cells with LPS/anti-CD40 or CpG-ODN 1826 (Ct-values: 22-25 for *Ebi3* and *IL-12p35* after LPS/aCD40 and *IL-12p35* after CpG-ODN 1826 stimulation; 27-29 for *Ebi3* after CpG-ODN 1826 stimulation), stimuli that were reported to optimally enhance the expression of the separate IL-35 subunits. However, we did not observe an increased expression of either IL-35 subunit by infected/OVA compared with uninfected/OVA pulmonary B cells (**suppl. Figure S3C**), suggesting that it is not likely that IL-35 mediates protection in our model. Taken together, these data show that during chronic schistosomiasis pulmonary B cells have an increased CD86 expression, equal expression levels of MHCII, lower IL-10 and IL-6 production upon TLR ligation and no increase in gene expression of the subunits for IL-35, suggesting that pulmonary B cells do not suppress Th2 cytokine production by an increased expression of those inhibitory molecules or regulatory cytokines.

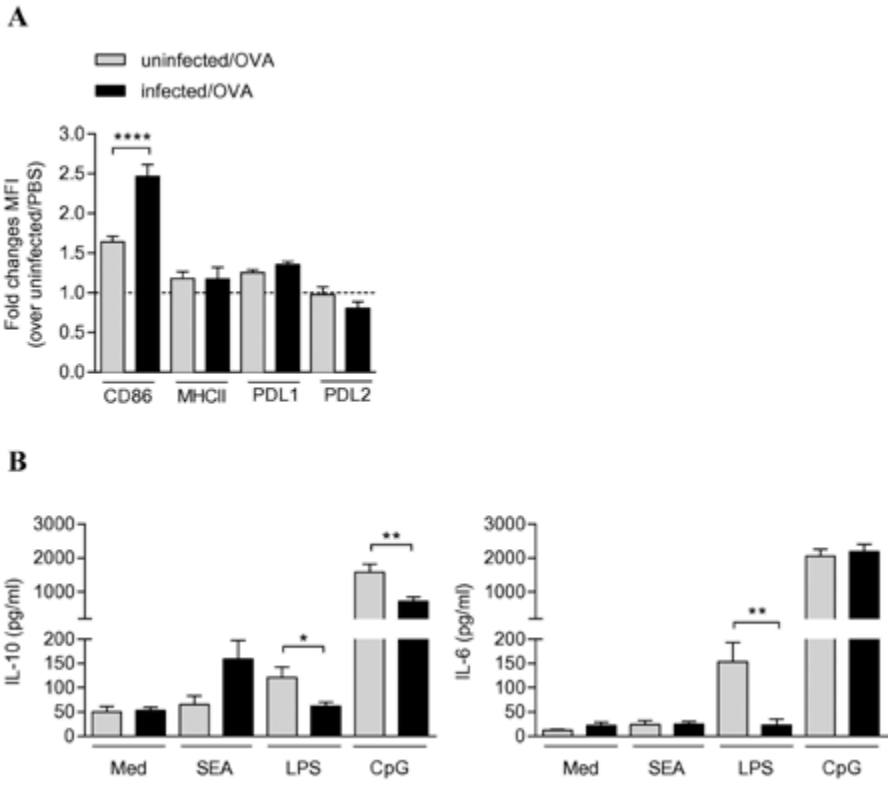


Figure 3. Schistosome-induced pulmonary B cells express elevated levels of CD86 *ex vivo,* and secrete less IL-10 after *in vitro* Toll-like receptor (TLR) ligation. Pulmonary B cells were isolated as described in **Figure 1**. For both phenotypical characterization and co-culture, pulmonary B cells from 2-3 mice were pooled to obtain sufficient cell numbers. (**A**) Fold changes of geometric mean fluorescence intensity (MFI) expression of indicated antigens on B cells from uninfected/ovalbumin (OVA) and infected/OVA mice over control (uninfected/PBS). A summary of 2-4 independent experiments (*n*=6-17/group) is shown. (**B**) Uninfected/OVA and infected/OVA B cells were stimulated with supplemented RPMI medium (Med), schistosomal egg antigen (SEA, 20 µg/ml), lipopolysaccharide (LPS, 100 ng/ml) or CpG-ODN 1826 (5 µg/ml) for 5 days to determine the presence of IL-10 and IL-6 in the supernatant. Data represents a summary of 2-4 independent experiments (*n*=4-12/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: **P* <0.05, ***P* <0.01, *****P* <0.0001, as tested by two-tailed unpaired Student's *t*-test.

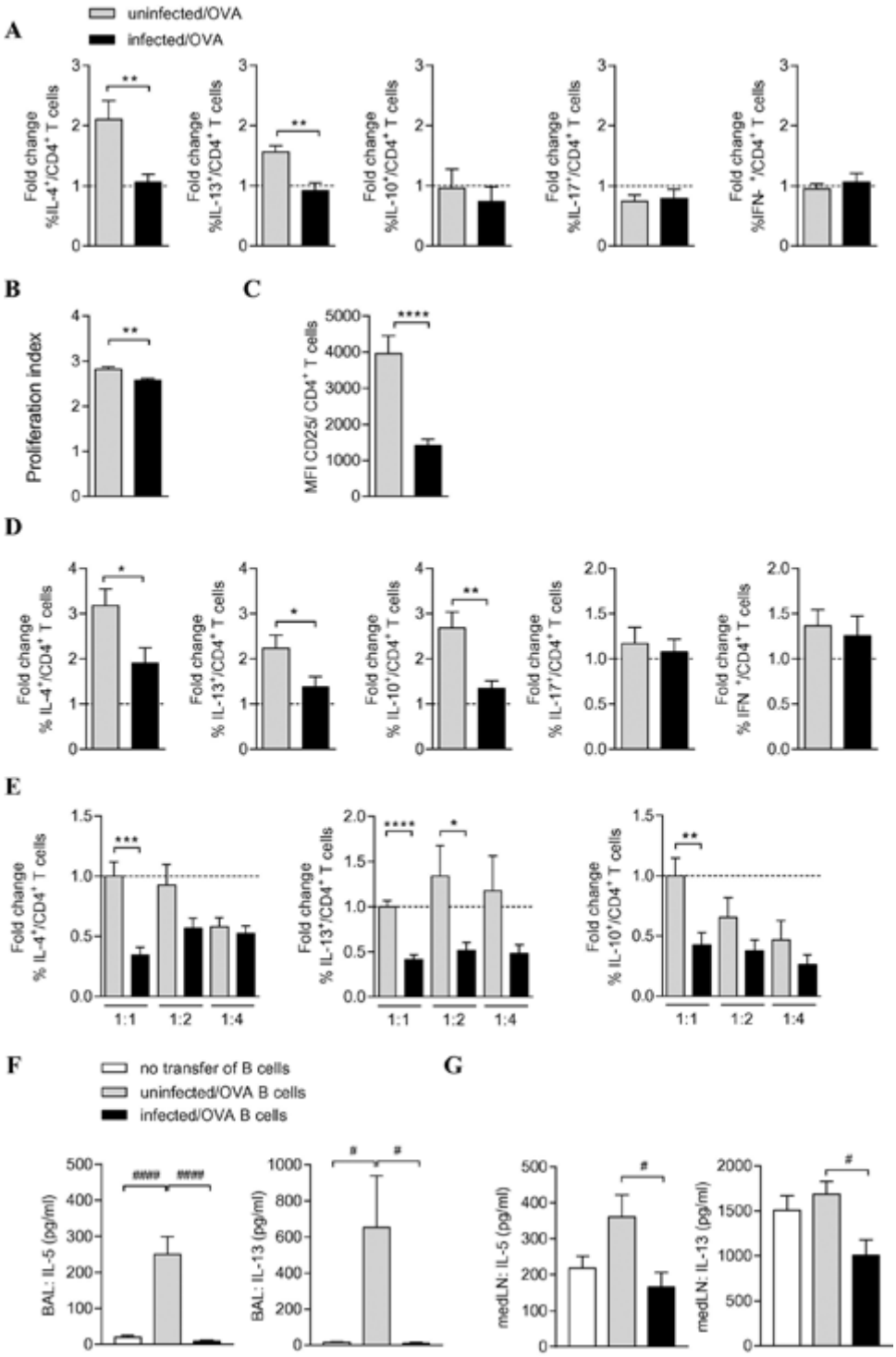
Schistosome-induced pulmonary B cells induce less Th2 cytokine secretion *in vitro*

Because schistosome-induced pulmonary B cells did not show the typical regulatory features of Breg cells such as Treg cell induction or increased production of regulatory cytokines, we next investigated their potential to reduce AAI through a reduced APC function that would lead to less T cell proliferation and/or less T cell cytokines. To this end, T-cell activation was examined under a condition where antigen-presentation by B cells was essential to drive T-cell activation. OVA-specific T-cell activation of $CD4^+$ T cells from OT-II mice was achieved by culture with OVA peptide-pulsed pulmonary B cells. After 3 days, OVA-presentation by B cells from uninfected- or infected/OVA mice did not affect intracellular expression of T-cell IL-4, IL-13 or IL-17 production, whereas IFN-γ seemed to be slightly up- and IL-10 downregulated (**Figure 4A**, **suppl. Figure S4A**). 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

Figure 4. Schistosome-induced pulmonary B cells have an impaired capacity to induce Th2 cytokine secretion *in vitro*. Pulmonary B cells were isolated as described in **Figure 1**. Pulmonary B cells, pooled from 2-3 mice per group were co-cultured at a 1:1 ratio with either naïve, ovalbumin (OVA)-peptide loaded OT-II CD4+ T cells (**A**) or naïve C57BL/6 CD4+ T cells (**B-E**). (**A**) B cells were loaded with OVA_{17} peptide, washed and co-cultured at a 1:1 ratio with OVA-specific $CD4^+$ OTII T cells in the presence of anti-CD28 (1 µg/ml) for 3 days. Intracellular cytokine staining for the Th2 cytokines IL-13, IL-4 and IL-10, Th1 cytokine IFN-γ and Th17 cytokine IL-17 cells was performed following phorbol 12-myristate 13-acetate (PMA)/Ionomycin and Brefeldin A stimulation for the last 4 h. Data are expressed as fold change over co-culture with control B cells (uninfected/PBS). A summary of 2-3 independent experiments (*n*=6-12/group) is shown. Results are expressed as mean ± S.E.M. (**B-E**) B cells were co-cultured at a 1:1 ratio with naïve, CFSE-stained (0.5 uM CFSE, 15 min) C57BL/6 CD4+ T cells in the presence of anti-CD3/anti-CD28 (both (1 µg/ml) for 3 days. (**B**) The proliferation index was calculated within FlowJo. A summary of three independent experiments (*n*=15-20/group) is shown. (**C**) The mean fluorescence intensity (MFI) of the activation marker CD25 on T cells assessed by flow cytometry. (**D**) Fold changes of percentage IL-4, IL-13, IL-10, IL-17 and IFN-γ-producing T cells over control (B cells from uninfected/PBS mice). A summary of four independent experiments (*n*=17/group) is shown. (**E**) Naïve C57BL/6 CD4+ T cells ($1x10^5$) were cultured at ratios of 1:1, 1:2 and 1:4 (B cell:T cell) with B cells from uninfected/OVA and infected/OVA mice. Bar graphs represent fold changes of percentage Th2 cytokines production (with uninfected/OVA 1:1 ratio set to 1). A summary of two independent experiments (*n*=6-7/group) is shown. (**F**, **G**) IL-5 and IL-13 measured by ELISA in supernatants of bronchoalveolar lavage (BAL) fluid (**F**) and OVA-restimulated mediastinal lymph node (medLN) cells (**G**) from recipient mice after adoptive transfer of either uninfected/OVA or infected/OVA B cells. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: $*P$ <0.05, $**P$ <0.01, $***P$ <0.001, $****P$ <0.0001, as tested by two-tailed unpaired Student's *t*-test. $^{\#}P$ <0.05, $^{\#\#\#\#}P$ <0.0001, as tested by One-way ANOVA and Tukey's multiple comparisons test.

co-stimulation. In the presence of anti-CD28, the fold change expression of the Th2 cytokines IL-4 and IL-13 was significantly lower in co-cultures with infected/OVA B cells compared with co-cultures with B cells from uninfected/OVA mice (**Figure 4A**, **suppl. Figure S4A**). The reduced IL-10 production found under stimulation conditions of infected/OVA B cell co-cultures was overcome in the presence of sufficient co-stimulation and may point to an impaired expression during sub-optimal stimulation.

Additionally, we performed co-culture experiments of pulmonary B cells from uninfected/OVA and infected/OVA mice with $CD4^+$ T cells from naive C57BL/6 mice in the presence of anti-CD3/28 to bypass the need for B cell antigen presentation and co-stimulation for T cell activation, and cultured them for 3 days. We still observed higher Th2 cytokine production in co-cultures with B cells from uninfected/OVA mice (**Figure 4D**, **suppl. Figure S4B**), together with slightly more T-cell proliferation (**Figure 4B**) and an enhanced T-cell CD25 expression (**Figure 4C**) compared with co-cultures with infected/OVA B cells. The ability of B cells from uninfected/OVA mice to induce Th2 cytokines was found to be dose-dependent and most effective at a 1:1 ratio (**Figure 4E**), while the percentage of Th2 cytokine-producing T cells induced by infected/OVA B cells was similarly low at all indicated ratios. To evaluate whether B cells could also inhibit Th2 cytokines *in vivo*, we turned to our previous studies, where adoptive transfer of pulmonary B cells from *S. mansoni*-infected, but not uninfected, mice protected recipient mice from AAI[24]. We now analyzed the abundance of Th2 cytokines in both the BAL fluid and mediastinal LN cell cultures of recipient mice after OVA restimulation. While the transfer of uninfected/OVA B cells strongly increased the concentration of Th2 cytokines in the BAL fluid compared with control mice (**Figure 4F**), we found both IL-5 and IL-13 to be significantly reduced in both tissues after transfer of infected/OVA B cells compared with cells transferred from uninfected mice (**Figure 4F, G**). Collectively, we found a lesser induction of Th2 cell proliferation and cytokine production by infected/OVA B cells, which is also evident in a setting independent of B cell antigen-presentation and with optimal co-stimulation. These findings suggest that schistosome-induced pulmonary B cells may not dampen CD4 T cell responses via a reduced APC function, but that this may be the result of both reduced Th2-driving signals and alternative inhibitory factors.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The impaired capacity of schistosome-induced pulmonary B cells to induce Th2 cytokine secretion is largely independent of IL-10 and TGF-β

We next aimed to investigate what other suppressive factors were involved in the reduced Th2 cell driving capacity of schistosome-induced pulmonary B cells. In that context, the roles of IL-10 and TGF-β were investigated by adding anti-IL-10R or anti-TGFβ blocking antibodies to the co-cultures of pulmonary B cells and $CD4^+$ T cells. We focused on the production of IL-4 and IL-13 due to the activity of these Th2 cytokines in boosting allergic responses in the airways. Blocking IL-10 signaling or neutralizing TGF-β only slightly, but significantly, increased the IL-4 production in T cells cultured with pulmonary B cells from infected/OVA mice (**Figure 5**). IL-13 production was only increased upon blocking IL-10 signaling (**Figure 5**). This may suggest that of the two inhibitory cytokines, the influence of IL-10 seemed to be slightly more pronounced. Nevertheless, infected/OVA B cells still induce significantly less IL-4 and IL-13 in co-cultures where IL-10 signaling is ablated or TGF-β neutralized, suggesting that either one of these cytokines, if at all, only play a minor role.

DISCUSSION

Helminths drive strong immunoregulatory processes that limit immunopathology during chronic infection, and Breg cells seem to be important players. Notably, *S. mansoni*-induced splenic and pulmonary B cells also attenuate allergic diseases such as AAI upon adoptive transfer. Splenic Breg cells mediate their suppressive effect through IL-10- and Treg cell-dependent mechanisms [4, 5, 8]. Here, we demonstrate that helminth-induced pulmonary B cells are phenotypically and functionally distinct from splenic Breg cells that we previously studied [5], by showing a reduced capacity to initiate Th2 cytokine responses. The question remains whether this is truly the result of an increased inhibitory, or simply a reduced Th2 stimulatory, activity.

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. While IL-10

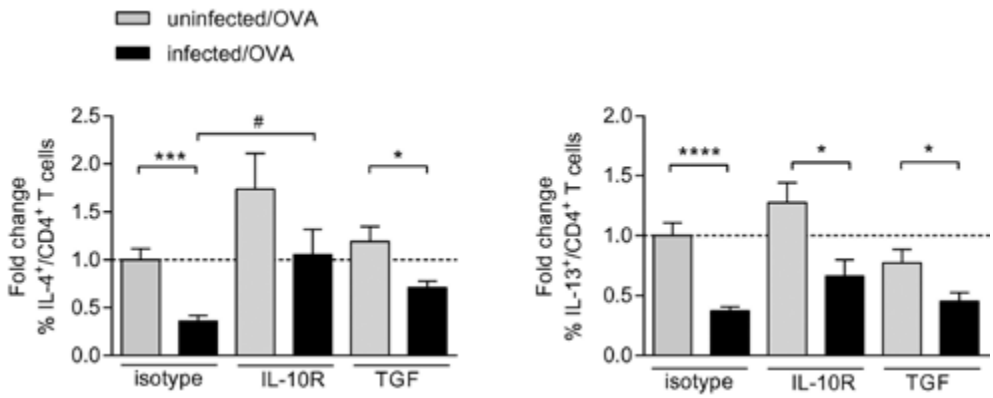

Figure 5. The impaired capacity of schistosome-induced pulmonary B cells to induce Th2 cytokines is independent of IL-10 and TGF-β. *In vitro* co-cultures were performed as described in **Figure 4** in the presence of blocking anti-IL-10R, anti-TGF-β or isotype control antibodies. Bar graphs represent fold changes of IL-4 and IL-13 (with uninfected/ovalbumin (OVA) isotype set to 1). A summary of two independent experiments (n=6-8/group) is shown. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: *P <0.05, ***P <0.001, ****P <0.0001, as tested by two-tailed unpaired Student's t-test, and #P <0.05 as tested by One-way ANOVA following Dunnett's multiple comparisons test.

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 [4, 5, 8]. We adoptively transferred pulmonary B cells from uninfected/OVA and infected/OVA mice into DEREG recipient mice and did not observe a significant change in the protective effect of B cells from infected mice in the absence of Treg cells (**Figure 2**). We have not formally addressed where these adoptively transferred B cells migrate to in recipient mice. However, we have previously examined that i.v. injected splenic B cells migrate to the spleen and lung of allergic mice, and assume that the pulmonary B cells studied here also migrate, at least in part, to the lung (unpublished findings). Nevertheless, we cannot exclude that systemic signals have also contributed to the observed protective effect.

Recently, it has become evident that Breg cells utilize a number of IL-10-independent suppressive mechanisms in order to control inflammation [26]. For example, B cells can contribute to the maintenance of tolerance via the production of TGF-β [13, 14], IL-35 [15, 16] or by induction of T cell hypo-responsiveness [14]. 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, the majority of the B cells in the lungs of infected/OVA mice had elevated levels of CD23 (**Figure 1B**) compared with uninfected/OVA mice. Interestingly, mesenteric LN $CD23^{hi}$ B cells from *H. polygyrus*-infected mice were shown to suppress Der p1-induced airway inflammation by an unknown mechanism, but independently of IL-10 [6]. Although it was unclear whether this suppression was accomplished through CD23, it may point to similarities with the pulmonary B cells in the infected/OVA mice described here. As several adoptive transfer experiments with sorted pulmonary CD23+ B cells, compared with CD23low/int B cells from OVA/infected mice, did not show clear differences in their protection against AAI, the elevated expression of CD23 may merely serve as a proxy for a certain inhibitory phenotype without being actively involved. Alternatively, CD23 expression may not define a specific Breg population but may be more the consequence of the local cytokine milieu [27], as IL-4 and IgE drive CD23 expression [28, 29] on B cells during helminth infections [30]. Indeed, the CD23 expression seemed to be elevated on almost all pulmonary B cells of infected/OVA mice. Interestingly, we found elevated concentrations of total IgG1 and IgG2a in infected/OVA compared with uninfected/OVA mice (data not shown). Earlier studies have suggested an inhibitory role for IgG1 ligating the inhibitory FcγRIIB in OVA-induced AAI [31]. However, adoptive transfers of pulmonary B cells from uninfected- and infected/OVA mice into OVA-sensitized $FcγRIIB^{-/-}$ mice suggested that pulmonary B cell-induced protection against AAI was independent of FcγRIIB ligation (data not shown).

Schistosome-induced pulmonary B cells expressed enhanced levels of CD86, whereas MHC Class II expression levels remained unchanged and in some experiments were even enhanced. Each of these markers has been suggested to affect T cell activation. For example, down-regulation of B cell CD80 and CD86 expression during *Brugia pahangi* larval infection restricted T cell proliferation [32]. Using B cell $B7^{-/-}$ mice, expression of CD86 was shown to be essential for B cell-mediated recovery of EAE by induction of Treg cells and IL-10 [33]. In our study, co-cultures of schistosome-induced pulmonary B cells presenting OVA to OVA-specific T cells resulted in reduced Th2 cytokine production compared with control conditions. However, since similar results were observed in co-cultures of B cells from C57BL/6 mice supplemented with anti-CD3/28 to bypass the role of B cells as APCs, this may suggest the involvement of other molecules or mechanisms.

We additionally examined different inhibitory molecules for their contribution to the protective effect observed. 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 hypo-responsiveness [34, 35], and PD-1-PD-L1/2 interactions have also been reported to be involved in regulating autoimmune and allergic Th2 responses [36, 37]. Other studies however suggested that Th2 hypo-responsiveness during

S. mansoni infection was not related to PD-1-PD-L1/2 interaction [38]. The latter is in agreement with our own data, as we observed that blocking of PD-L1 or PD-L2 expression on infected/OVA B cells did not restore Th2 cytokine production or proliferation in co-cultures with T cells (data not shown). We also investigated a potential role for the enhanced expression of CD86 on the pulmonary B cells from infected/OVA mice. Human $CD25^{hi}$ Breg cells have been reported to increase the expression of the inhibitory receptor CTLA-4 on $FoxP3^{+}$ Treg cells *in vitro* [11], suggesting that the interaction of B cell co-stimulatory molecules CD80/CD86 and CTLA-4 could be important in controlling inflammation. However, blocking of CTLA-4 in *in vitro* co-cultures did not restore Th2 cytokine production in our study (data not shown). Collectively, the pulmonary B cells studied are phenotypically and functionally different from classical Breg cells, and rather show a reduced Th2-driving capacity. It remains to be established whether this is the result of a reduced capacity to drive Th2 stimulation, or rather a suppressive signal by which schistosome-induced pulmonary B cells actively reduce Th2 polarization and inhibit AAI.

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 [39-41]. Secreted helminth products from *Schistosoma* may also directly attenuate the T cell stimulatory capacity of B cells as described for DCs [42]. As pulmonary B cells from infected/OVA mice produced less cytokines after stimulation with TLR ligands (**suppl. Figure S3**), the B cells might become hypo-responsive themselves in the context of chronic *S. mansoni* infection, resulting in a reduced capacity to stimulate T cells. In addition to their role as APCs and cytokine producers, B cells have also been reported to remodel the LN architecture to facilitate DC-dependent Th2 induction and be required for the formation of a Tfh cell response in the context of nematode infections [43, 44].

Very little is known with regard to B cell migration to the respiratory system, and the permanence of their residence in the tissue, in contrast to other mucosal sites such as the gut or the peritoneal cavity. The lung B cell population in chronically *S. mansoni*-infected mice that are subjected to AAI is likely to be heterogeneous, consisting both of resident and infiltrating cells. Further studies are needed to examine the origin and migration pattern of the protective pulmonary B cells in *S. mansoni* infection.

Recently, various Breg cell populations have been identified in peripheral blood of humans infected with schistosomiasis or other helminths [5, 45]. Furthermore, in several inflammatory diseases, Breg cells were impaired in terms of their number and/or their regulatory function, i.e. in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) or allergic asthma [46-48]. Since most human studies are restricted to peripheral blood B cells, these results might not fully reflect the processes that occur in inflamed organs. Therefore, further studies on 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. Collectively, we identified that murine infection with schistosomes induces potent IL-10-producing suppressive Breg cells in the spleen [5] and impaired Th2-driving pulmonary B cells in the inflamed tissue (this study) which both contribute to the suppression of AAI. Identifying the mechanisms that influence pulmonary B cell function and impair their capacity to induce Th2 cytokines may be an interesting novel strategy to prevent or control allergic inflammatory responses.

MATERIAL AND METHODS

Animals

Six week-old female C57BL/6 OlaHsd mice were purchased from Harlan Sprague Dawley Inc. (USA). DEREG (depletion of regulatory T cells) mice [49] were kindly provided by Dr. T. Sparwasser (Twincore/ Centre for Experimental and Clinical Infection Research, Germany) and bred in the animal facilities of the Leiden University Medical Center (LUMC) Leiden, The Netherlands. Mice were housed under

specific-pathogen-free (SPF) conditions in the animal facilities of the LUMC. 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 36-40 *S. mansoni* cercariae and kept until the chronic phase of infection (15 weeks). For AAI induction, mice were sensitized twice by i.p. injections of OVA (10 µg/mL, Worthington Biochemical Corp, USA) in Imject Alum (2 mg/ml; Pierce, USA) at weeks 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 h after the last challenge. Bronchoalveolar lavage (BAL) fluids were collected and phenotyped by flow cytometry [5, 20].

Cell purification

Perfused lungs were minced to ~1 mm pieces and digested by collagenase III (Worthington) and DNase (Sigma-Aldrich, USA) for 1 h. 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, Germany). Next, B cells were purified using anti-CD19 MicroBeads (Miltenyi Biotec). Untouched splenic $CD4^+$ T cells were enriched using negative selection with MicroBeads (Miltenyi Biotec) and were ~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 in Section 2.2. Ten days after the last injection, the OVA-sensitized animals received an i.v. injection of 5 x 10^6 $CD19^+$ lung B cells from uninfected- or infected/OVA 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, 1 day before and 2 days after the adoptive transfer of B cells in order to deplete the $FoxP3^+$ Treg cells. After 2 days, mice were challenged for three consecutive days and sacrificed 24 h after the last challenge.

Phenotypic characterization

Ex vivo pulmonary B cells were characterized using CD25-FITC (clone: 3C7; BD Biosciences), Tim-1-PE (RMT1-4), B220-V510 (RA3-6B2), PD-L1-Pe-Cy7 (10F.9G2; all Biolegend, USA), B220-APC-eF780 (RA3-6B2), B220 eF450 (RA3-6B2), LAP-1-PerCP-eFluor710 (TW7-16B4), CD5-APC (53-7.3), CD23-PeCy7 (B3B4), CD86-PE-Cy5 (GL1), FasL-APC (MFL3), major histocompatibility complex Class II (MHCII)-APC-eF780 (M5/114.15.2), PD-L2 Biotin (TY25; all eBioscience, USA) combined with streptavidin-Qdot525 (Life Technologies, USA) and LIVE/DEAD fixable Violet or Aqua stain (eBioscience). For all flow cytometric measurements, FcγR-binding inhibitor (2.4G2) was added and fluorescence minus one (FMO) controls were used for gate setting for all surface markers and cytokines. All antibody stainings were performed as surface stainings for 30 min at 4ºC.

Cytometric bead array

Cytokines were measured in BAL fluid and in cell culture supernatant of mesenteric lymph node (LN) cells (0.3x10^6 cells) restimulated for 3 days with 10 µg/mL of OVA using BD cytometric bead array Flex-set kits (BD Biosciences, USA).

***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, USA), containing 5% heat-inactivated FBS (Greiner Bio-One, Austria), 5×10^{-5} M 2-Mercaptoethanol (Sigma-Aldrich) and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin; Invitrogen). Cells were cultured either in the presence of schistosomal egg antigen (SEA; 20 µg/ml), lipopolysaccharide (LPS; 100 ng/ml) or CpG-oligodeoxynucleotides (CpG-ODN) 1826 (5 µg/ml) for 5 days for the detection of IL-10 and IL-6 in culture supernatants by ELISA (BD Biosciences), or in the presence of LPS (1 µg/ml) and anti-CD40 (clone 1C10; 10 µg/ml) or CpG-ODN 1826 (1 µg/mL) for the detection of IL-35 mRNA expression.

Immunoglobulin measurements

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

***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 of OVA_{17} peptide ($OVA_{323-339}$: ISQAVHAAHAEINEAGR, kindly provided by M.G.M. Camps (Leiden University Medical Center, The Netherlands) for 1 h at 37 ° C, washed, and subsequently co-cultured with OT-II $CD4^+$ T cells ($1x10^5$ cells/well) at a 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 ratios, 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 carboxy- fluorescein diacetate, succinimidyl ester (CFSE; 0.5 µM) for 15 min. Under some conditions, the following blocking antibodies were added to the cultures: 10 µg/ml of isotype control anti-βGal and anti-TGF-β (kindly provided by L. Boon, Bioceros, The Netherlands). T cells were incubated for 30 min at 37 °C with 10 µg/ml of anti-IL-10 receptor (kindly provided by L. Boon). After 3 days, CFSE-labelled T cell co-cultures were stained with anti-CD3-eFluor450 (17A2), CD25-PE (PC61.5), B220-eFluor780 (RA3-6B2), 7-AAD, CD4-biotin (GK1.5) (all eBioscience) with streptavidin-Qdot525 to measure proliferation of activated T cells. The proliferation index, being the total number of cell divisions divided by the number of cells that went into division, was calculated within the FlowJo proliferation platform. For cytokine analysis, the cells were restimulated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml of ionomycin for 6 h in the presence of 10 µg/ml of Brefeldin A (all Sigma-Aldrich) for the last 4 h, followed by fixation using 1.9% paraformaldehyde (PFA; Sigma-Aldrich). Next, the cells were stained for viability (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, ThermoFisher Scientific, USA) IL-4-PE (BVD4-1D11; BD Biosciences), CD3-eFluor710 (17A2), IFN-γ-FITC (XMG1.2), IL-17-PeCy7 (eBio17B7), IL-10-APC (JES5-16E3), IL-13-eFluor450 (eBio13A), and B220-eFluor780 (RA3-6B2; all eBioscience).

Quantitative PCR (qPCR)

Isolated pulmonary B cells were snap-frozen in liquid nitrogen, and RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. cDNA was synthetized and quantitative real-time PCR performed on a C1000 Thermal Cycler (BioRad, USA) using GoTaq qPCR MasterMix (Promega, USA). Transcripts were quantified using the following forward (FP) and reverse (RP) primers (Eurogentec, Belgium): β-2-microglobulin FP: 5'-CACTGAATTCACCCCCACTGA -3', β-2-microglobulin RP: 5'-TGTCTCGATCCCAGTAGACGG-3'; Ebi3 FP: 5'-CATTGCCACTTACAGGCTCG-3', Ebi3 RP: 5'-TGATGATTCGCTCAGCCACA-3'; IL-12p35 FP: 5'-GGTGAAGACGGCCAGAGAAA-3', IL-12p35 RP: 5'-GTAGCCAGGCAACTCTCGTT-3'. mRNA expression was normalized to the reference gene β-2-microglobulin, and expressed as the fold change to B cells from uninfected, non-allergic mice by using the ΔΔ comparative threshold (ΔΔCt) method.

Statistical analysis

All results were analyzed using GraphPad Prism (version 5.00/6.05 for Windows, GraphPad Software, La Jolla USA) and are expressed as mean ± S.E.M. Statistical analysis was performed using the two-tailed unpaired or paired Student's *t*-test for comparison of two experimental groups, and One-Way ANOVA followed by Tukey's multiple comparisons test for comparisons between more than two groups. Differences between groups were considered significant at $P < 0.05$.

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SUPPLEMENTARY MATERIAL

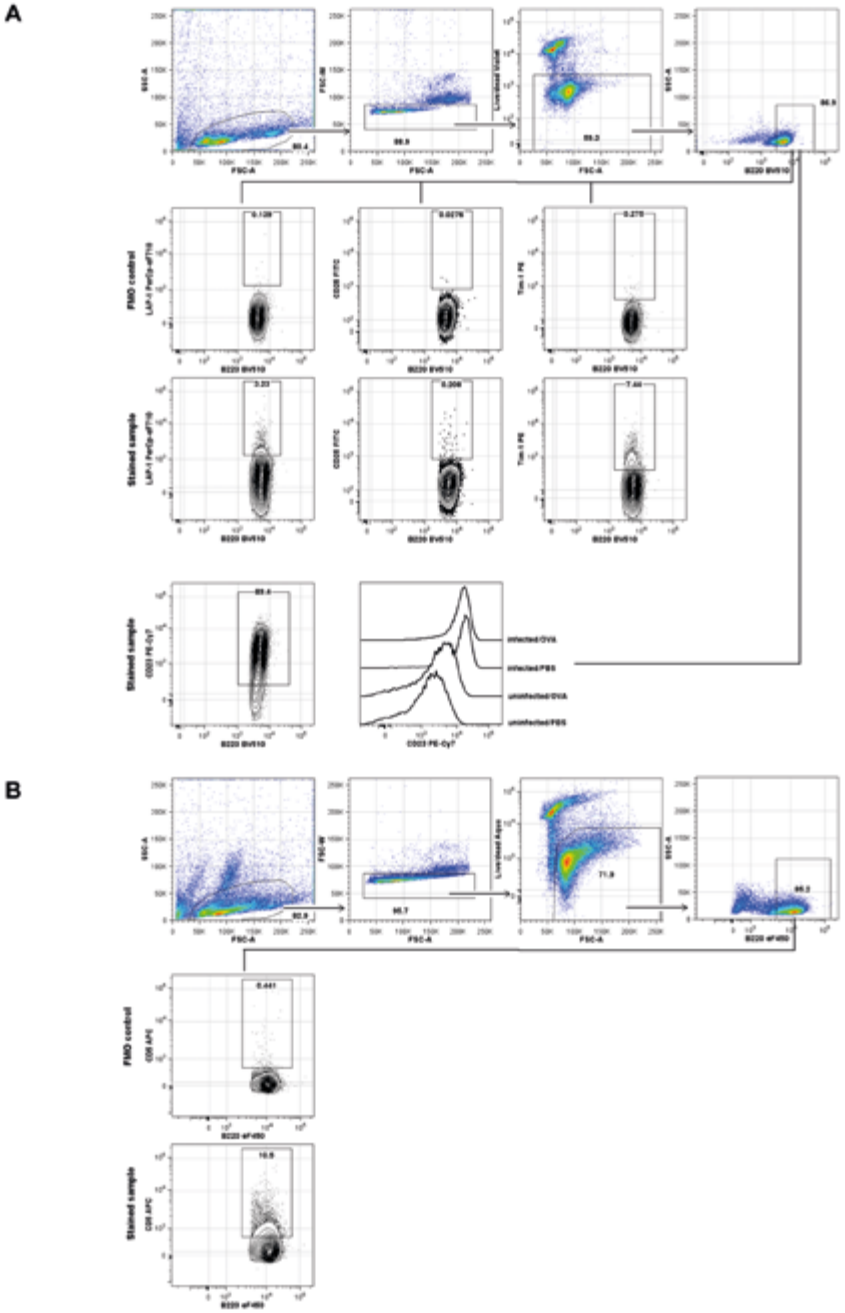

Figure S1. Gating strategy and representative flow cytometry plots (relating to **Figure 1**). (**A**) Percentage of latency-associated peptide (LAP$^+$), CD25$^+$, T cell immunoglobulin and mucin domain-1 (Tim-1$^+$) and CD23$^+$ pulmonary B cells, as well as mean fluorescence intensity (MFI) of CD23. (**B**) Percentage of CD5$^+$ pulmonary B cells. OVA, ovalbumin; FMO, fluorescence minus one control.

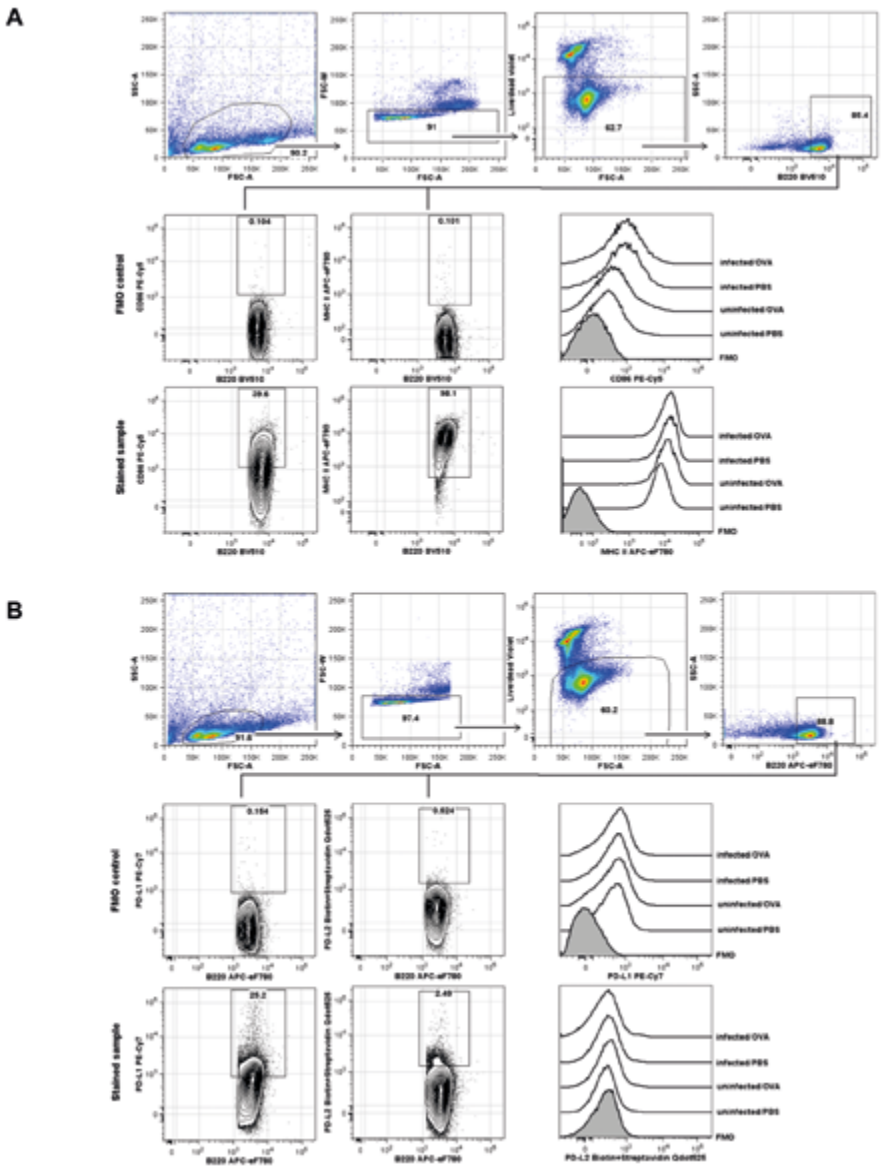

Figure S2. Gating strategy and representative flow cytometry plots (relating to **Figure 3A**). (**A**) Percentage of CD86^{+}, MHCII^{+}, and (**B**) PD-L1^{+} and PD-L2^{+} pulmonary B cells in addition to the mean fluorescence intensities (MFIs) presented in **Figure 3A**. OVA, ovalbumin; FMO, fluorescence minus one control.

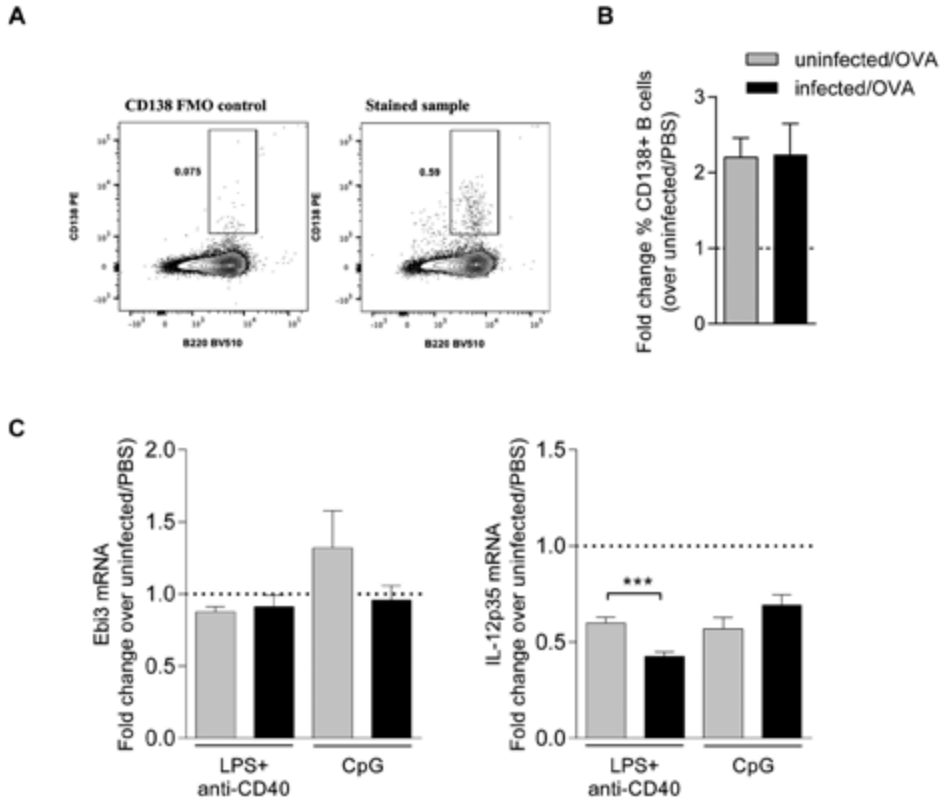

Figure S3. Pulmonary B cells were isolated as described in **Figure 1**. Cells from 2-3 mice were pooled to obtain sufficient cell numbers. (**A**) Representative flow cytometry plots showing the expression of the plasma cell marker CD138 on pulmonary B cells. (**B**) Fold change of percentage CD138-expressing B cells from uninfected/ovalbumin (OVA) and infected/OVA mice over control (uninfected/PBS). A summary of three independent experiments (n=11-14/group) is shown. (**C**) Fold change of mRNA expression of the IL-35 subunits Ebi3 and IL-12p35 as analyzed by quantitative PCR using β-2-microglobulin as a reference gene and the ΔΔ comparative threshold method. Data represent one experiment (n=3-6/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: ***P <0.001, as tested by a two-tailed unpaired Student's t-test. FMO, fluorescence minus one control; LPS, lipopolysaccharide.

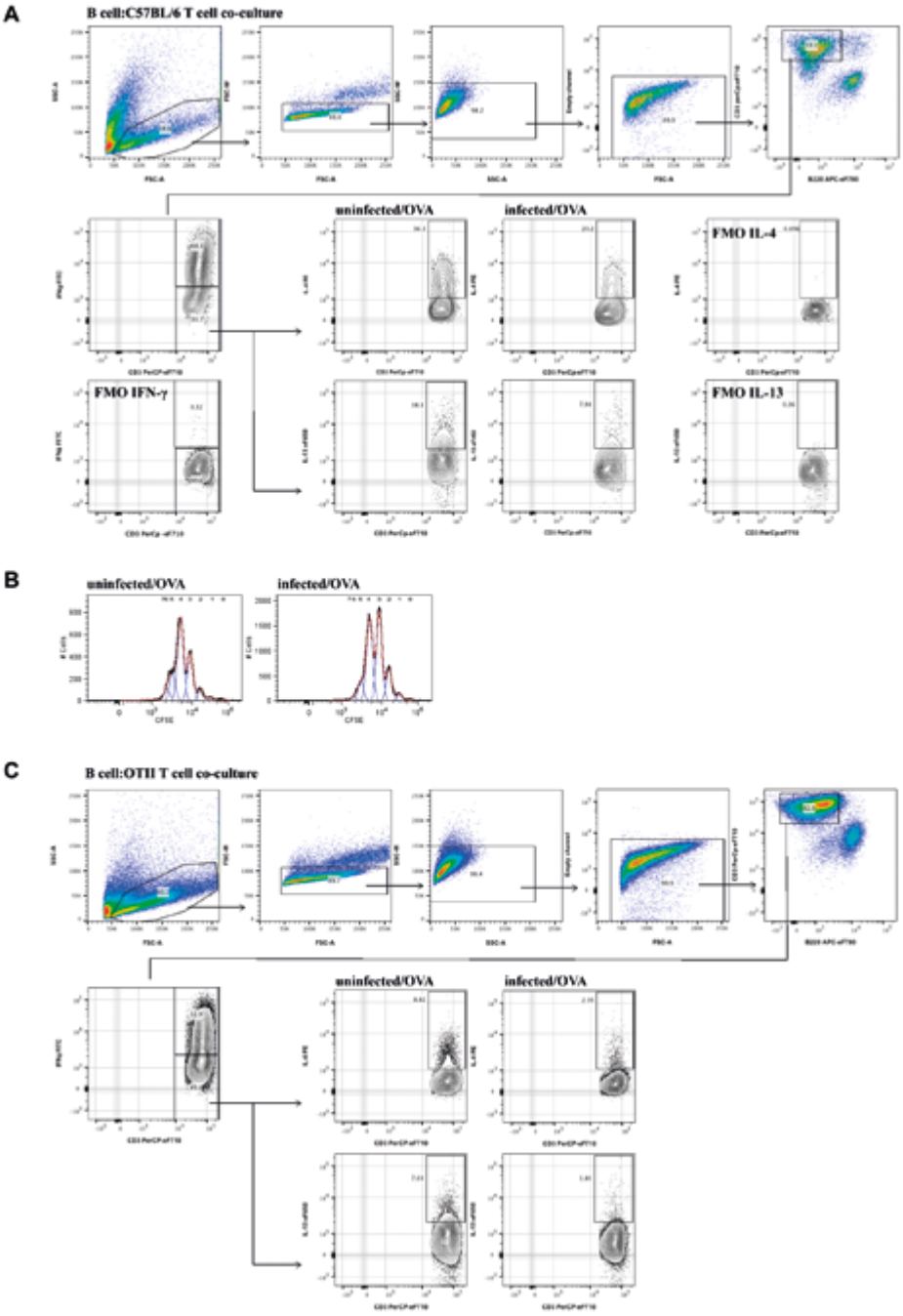

Figure S4. Gating strategy and representative flow cytometry plots (relating to **Figure 4**), showing (**A**) the expression of IL-4 and IL-13 by C57BL/6 T cells after co-culture with pulmonary B cells in the presence of anti-CD3 and anti-CD28, (**B**) the proliferation as assessed by CFSE dilution, and (**C**) the expression of IL-4 and IL-13 by OTII T cells after co-culture with ovalbumin $(OVA)_{17}$ peptide-loaded pulmonary B cells in the presence of anti-CD28. FMO, fluorescence minus one control.