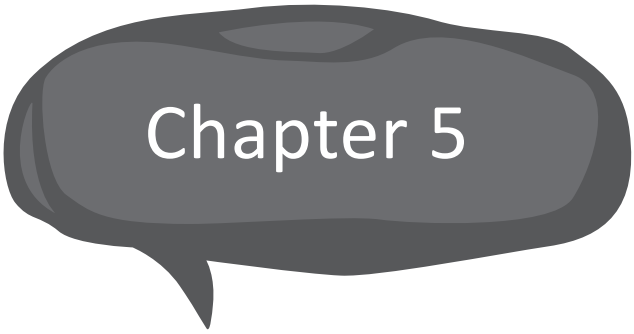

IL-10-producing CD1d^{hi} regulatory B cells from *Schistosoma haematobium*-infected individuals induce IL-10-positive T cells and suppress effector T-cell cytokines

Luciën E.P.M. van der Vlugt[1], Jeannot F. Zinsou[2], Arifa Ozir-Fazalalikhan[1], Peter G. Kremsner[3], Maria Yazdanbakhsh[1], Ayola A. Adegnika[2,3], and Hermelijn H. Smits[1]

[1]Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands
[2]Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon
[3]Institut für Tropenmedizin, Eberhard Karls Universität Tübingen, Tübingen, Germany

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Abstract

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

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

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

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

Introduction

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

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

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

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

Materials and Methods

Study population

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

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

Cell isolation and characterization

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

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

B-cell stimulation and intracellular staining

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

Functional *in vitro* assays

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

Statistical analysis

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

Results

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

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

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

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

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

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

Table I: Characteristics of study population

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

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

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

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

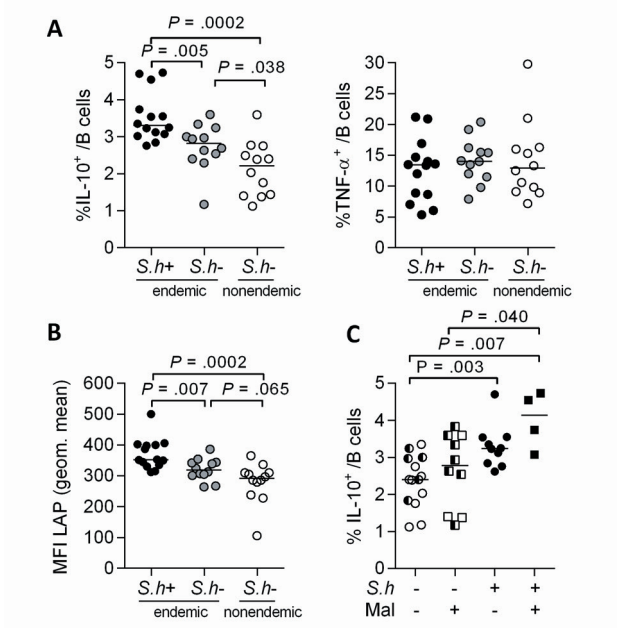

Figure 1. Intracellular interleukin 10 (IL-10) and tumor necrosis factor α (TNF-α) detection in total B cells. 1 × 10^5 blood-derived B cells from *Schistosoma haematobium*–infected individuals from a *S. haematobium*–endemic area (*S.h*+), uninfected individuals from a *S. haematobium*–endemic area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

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

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

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

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

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

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

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

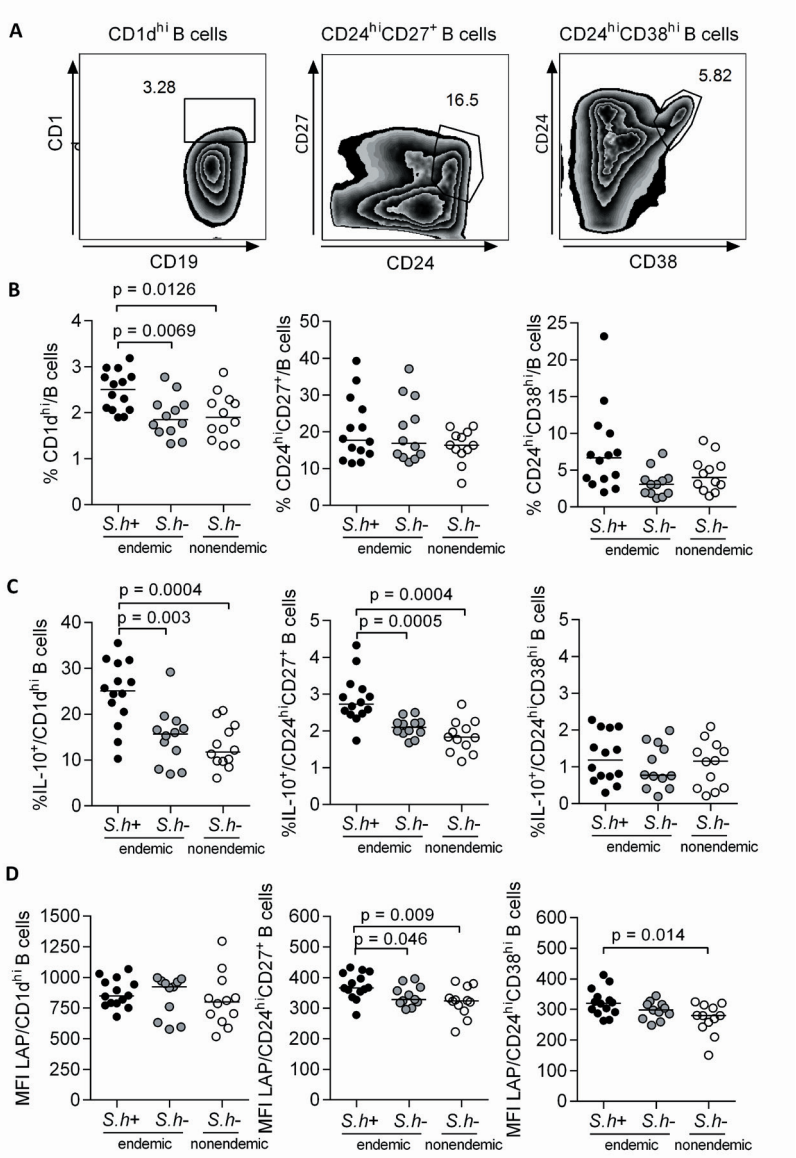

Figure 2. The frequency of different regulatory B cells in the blood specimens from *Schistosoma haematobium*-infected indviduals from a *S. haematobium*- endemic area (*S.h*+), uninfected individuals from a *S. haematobium*- endemic area (*S.h*-) and uninfected individuals from a *S. haematobium*-nonendemic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

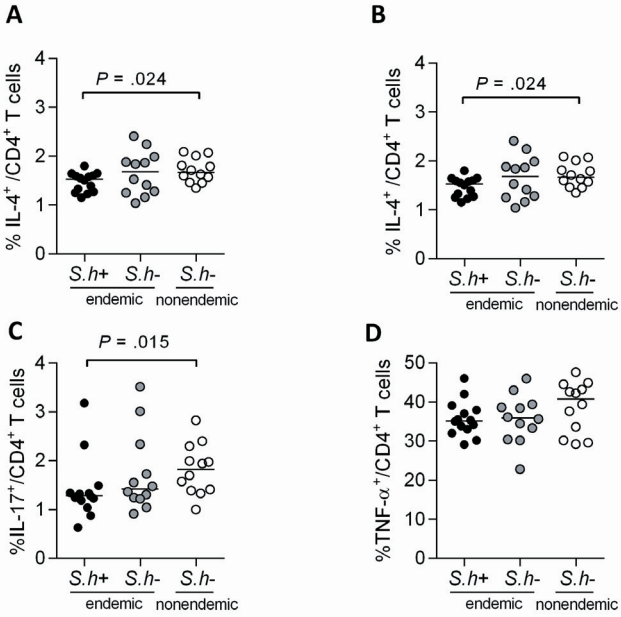

Figure 3. T-cell cytokine expression after coculture with B cells from *S. haematobium*-infected (*S.h*+) and uninfected individuals (*S.h*-) from a *S. haematobium*-endemic area, and uninfected individuals from a *S. haematobium*-nonendemic area (*S.h*-). Isolated B cells were cultured at a ratio of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

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

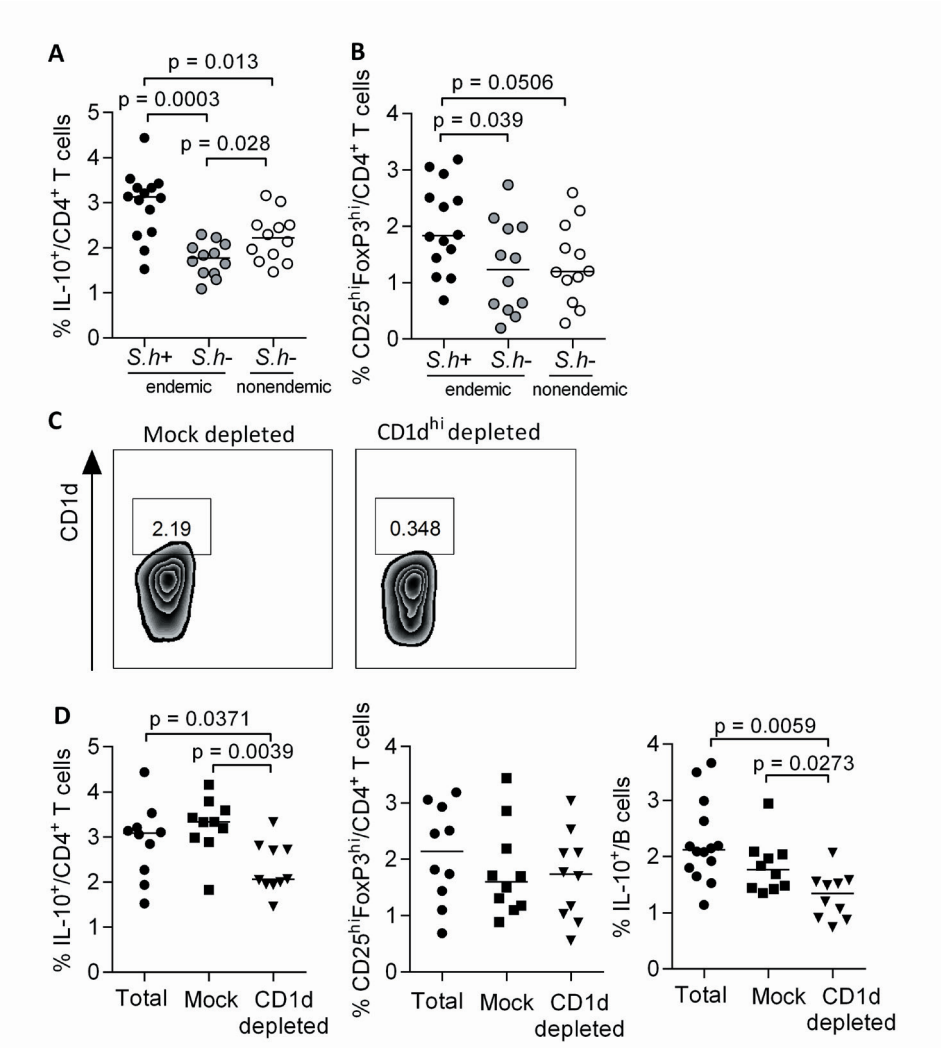

Figure 4. Induction of IL-10$^+$ T and FoxP3$^+$ regulatory (Tregs) cells by B cells. Isolated B cells were cultured at a ratio of 1:1 with allogenic CD4$^+$CD25$^-$ T cells (from 1 European donor) in the presence of soluble anti-CD3 (1 µg/ml) for 6 days. After coculturing, the cells were restimulated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

Discussion

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

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

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

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

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

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

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

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

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Conflict of interest
All authors declare no conflict of interest.

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References

(1) Maizels RM, Bundy DA, Selkirk ME, Smith DF, Anderson RM. Immunological modulation and evasion by helminth parasites in human populations. Nature **1993 Oct 28**;365(6449):797-805.

(2) Pearce EJ, Caspar P, Grzych JM, Lewis FA, Sher A. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. J Exp Med **1991 Jan 1**;173(1):159-66.

(3) Yazdanbakhsh M, Matricardi PM. Parasites and the hygiene hypothesis: regulating the immune system? Clin Rev Allergy Immunol **2004 Feb**;26(1):15-24.

(4) Baumgart M, Tompkins F, Leng J, Hesse M. Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. J Immunol **2006 May 1**;176(9):5374-87.

(5) McSorley HJ, Maizels RM. Helminth infections and host immune regulation. Clin Microbiol Rev **2012 Oct**;25(4):585-608.

(6) Barron L, Wynn TA. Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. Eur J Immunol **2011 Sep**;41(9):2509-14.

(7) Lee JH, Noh J, Noh G, Choi WS, Cho S, Lee SS. Allergen-specific transforming growth factor-beta-producing CD19+CD5+ regulatory B-cell (Br3) responses in human late eczematous allergic reactions to cow's milk. J Interferon Cytokine Res **2011 May**;31(5):441-9.

(8) Natarajan P, Singh A, McNamara JT, et al. Regulatory B cells from hilar lymph nodes of tolerant mice in a murine model of allergic airway disease are CD5+, express TGF-beta, and co-localize with CD4+Foxp3+ T cells. Mucosal Immunol **2012 Nov**;5(6):691-701.

(9) Sattler S, van der Vlugt LE, Hussaarts L, Smits HH, Huang F-P. Regulatory B Cells-Implications in Autoimmune and Allergic Disorders. In: J.R.Kanwar, ed. Recent Advances in Immunology to Target Cancer, Inflammation and Infections. InTech, **2012**. p. 177-200.

(10) Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol **2012**;30:221-41.

(11) Smits HH. B cells in allergic diseases: bad or better? Autoimmunity **2012 Aug**;45(5):415-26.

(12) Klinker MW, Lundy SK. Multiple mechanisms of immune suppression by B lymphocytes. Mol Med **2012**;18:123-37.

(13) Mizoguchi A, Bhan AK. A case for regulatory B cells. J Immunol **2006 Jan 15**;176(2):705-10.

(14) Hussaarts L, van der Vlugt LE, Yazdanbakhsh M, Smits HH. Regulatory B-cell induction by helminths: Implications for allergic disease. J Allergy Clin Immunol **2011 Oct**;128(4):733-9.

(15) Mauri C, Ehrenstein MR. The 'short' history of regulatory B cells. Trends Immunol **2008 Jan**;29(1):34-40.

(16) Kalampokis I, Yoshizaki A, Tedder TF. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res Ther **2013**;15 Suppl 1:S1.

(17) Fillatreau S, Gray D, Anderton SM. Not always the bad guys: B cells as regulators of autoimmune pathology. Nat Rev Immunol **2008 May**;8(5):391-7.

(18) Tian J, Zekzer D, Hanssen L, Lu Y, Olcott A, Kaufman DL. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. J Immunol **2001 Jul 15**;167(2):1081-9.

(19) Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J Allergy Clin Immunol **2010 May**;125(5):1114-24.

(20) van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, et al. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. PLoS One **2012**;7(2):e30883.

(21) Wilson MS, Taylor MD, O'Gorman MT, et al. Helminth-induced CD19+CD23hi B cells modulate experimental allergic and autoimmune inflammation. Eur J Immunol **2010 Jun**;40(6):1682-96.

(22) Das A, Ellis G, Pallant C, et al. IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. J Immunol **2012 Oct 15**;189(8):3925-35.

(23) Blair PA, Norena LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity **2010 Jan 29**;32(1):129-40.

(24) Siewe B, Stapleton JT, Martinson J, et al. Regulatory B cell frequency correlates with markers of HIV disease progression and attenuates anti-HIV CD8(+) T cell function in vitro. J Leukoc Biol **2013 May**;93(5):811-8.

(25) Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood **2011 Jan 13**;117(2):530-41.
(26) Zhang M, Zheng X, Zhang J, et al. CD19(+)CD1d(+)CD5(+) B cell frequencies are increased in patients with tuberculosis and suppress Th17 responses. Cell Immunol **2012**;274(1-2):89-97.
(27) Correale J, Farez M, Razzitte G. Helminth infections associated with multiple sclerosis induce regulatory B cells. Ann Neurol **2008 Aug**;64(2):187-99.
(28) Ateba Ngoa U, Zinsou FJ, Kassa Kassa RF, Ngoune Feugap E. Assessment of the effect of *Schistosoma haematobium* co-infection on malaria parasites and immune responses in rural populations in Gabon: Study protocol. BMC Infectious Diseases **2014**;Submitted.
(29) Adegnika AA, Verweij JJ, Agnandji ST, et al. Microscopic and sub-microscopic Plasmodium falciparum infection, but not inflammation caused by infection, is associated with low birth weight. Am J Trop Med Hyg **2006 Nov**;75(5):798-803.
(30) Akue JP, Nkoghe D, Padilla C, et al. Epidemiology of concomitant infection due to Loa loa and Mansonella perstans in Gabon. PLoS Negl Trop Dis **2011 Oct**;5(10):e1329.
(31) Adegnika AA, Kremsner PG. Epidemiology of malaria and helminth interaction: a review from 2001 to 2011. Curr Opin HIV AIDS **2012 May**;7(3):221-4.
(32) Tran DQ. TGF-beta: the sword, the wand, and the shield of FOXP3(+) regulatory T cells. J Mol Cell Biol **2012 Feb**;4(1):29-37.
(33) Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J Immunol **2009 Jun 15**;182(12):7459-72.
(34) Dilillo DJ, Matsushita T, Tedder TF. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann N Y Acad Sci **2010 Jan**;1183:38-57.
(35) Liu ZQ, Wu Y, Song JP, et al. Tolerogenic CX3CR1+ B cells suppress food allergy-induced intestinal inflammation in mice. Allergy **2013 Oct**;68(10):1241-8.
(36) Noh J, Lee JH, Noh G, et al. Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy. Cell Immunol **2010**;264(2):143-9.
(37) Kessel A, Haj T, Peri R, et al. Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. Autoimmun Rev **2012 Jul**;11(9):670-7.
(38) Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn TA. Immunopathology of schistosomiasis. Immunol Cell Biol **2007 Feb**;85(2):148-54.
(39) Li X, Zhong H, Bao W, et al. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. Blood **2012 Oct 18**;120(16):3318-25.
(40) Flores-Borja F, Bosma A, Ng D, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. Sci Transl Med **2013 Feb 20**;5(173):173ra23.
(41) Palanivel V, Posey C, Horauf AM, Solbach W, Piessens WF, Harn DA. B-cell outgrowth and ligand-specific production of IL-10 correlate with Th2 dominance in certain parasitic diseases. Exp Parasitol **1996 Nov**;84(2):168-77.
(42) Velupillai P, Harn DA. Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosome-infected mice: a mechanism for regulation of CD4+ T-cell subsets. Proc Natl Acad Sci U S A **1994 Jan 4**;91(1):18-22.
(43) Correale J, Farez M. Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. J Immunol **2009 Nov 1**;183(9):5999-6012.
(44) Knippenberg S, Peelen E, Smolders J, et al. Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission. J Neuroimmunol **2011 Oct 28**;239(1-2):80-6.
(45) Wilde B, Thewissen M, Damoiseaux J, et al. Regulatory B cells in ANCA-associated vasculitis. Ann Rheum Dis **2013 Aug**;72(8):1416-9.
(46) van de Veen W, Stanic B, Yaman G, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. J Allergy Clin Immunol **2013 Apr**;131(4):1204-12.

Supplementary figures

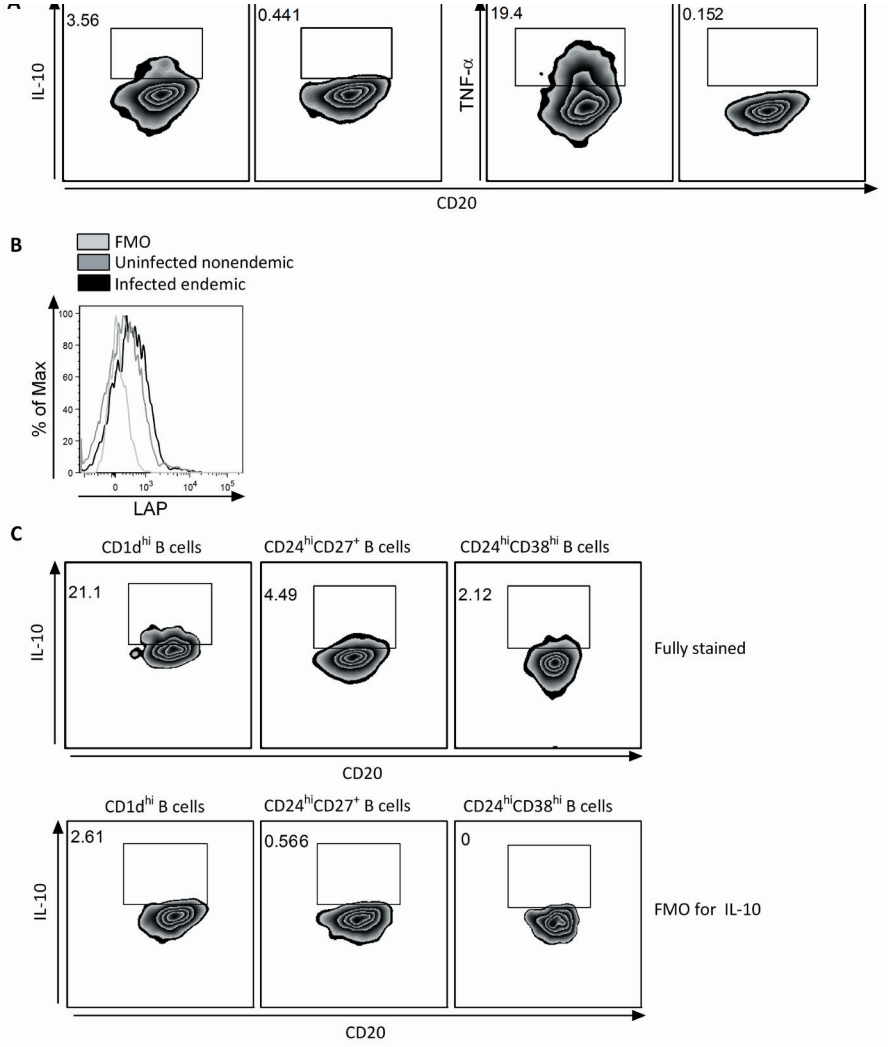

Figure S1. (A) A representative gating example of intracellular IL-10 and TNF-α production upon 5 hours PIB+LPS stimulation in total B cells from a endemic infected (*S.h*+) individual. Fluorescence minus one (FMO) shows background levels of IL-10 and TNF-α. B, A representative gating example for LAP/TGF-β1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

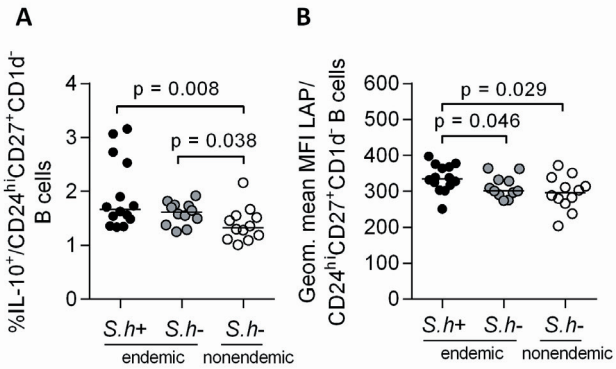

Figure S2. (A) IL-10 production and (B) LAP/ TGF-β1 membrane-bound expression of $CD24^{hi}CD27^{+}$ B cells after gating out the $CD1d^{hi}$ B cells in this population. The Mann-Whitney *U*-test was used for data analysis.

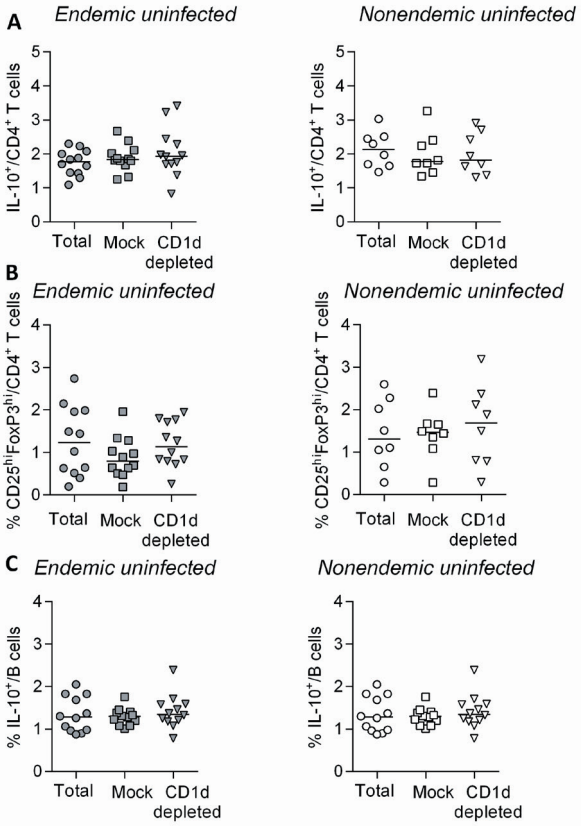

Figure S3. Induction of $IL\text{-}10^{+}$ T and $FoxP3^{+}$ Treg cells by B cells. Isolated B cells were cultured at a ratio of 1:1 with allogenic $CD25^{-}CD4^{+}$ T cells (from 1 European donor) in the presence of soluble anti-CD3 (1 µg/ml) for 6 days. After coculturing, the cells were restimulated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