

Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model

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Background: Parasitic helminth infections of humans have been shown to suppress the immune response to allergens. Experimentally, infection of mice with the helminth *Schistosoma mansoni* prevents allergic airway inflammation and anaphylaxis via IL-10 and B cells.

Objective: To identify and characterize the specific helminth-induced regulatory B-cell subpopulation and determine the mechanism by which these regulatory B cells suppress allergic airway inflammation.

Methods: IL-10–producing B cells from the spleens of helminth-infected mice were phenotyped, isolated, and transferred to ovalbumin-sensitized mice, and their ability to modulate allergic airway inflammation was analyzed.

Results: *S mansoni* infection induced IL-10–producing CD1d^{high} regulatory B cells that could prevent ovalbumin-induced allergic airway inflammation following passive transfer to ovalbumin-sensitized recipients. The capacity of regulatory B cells to suppress allergic airway inflammation was dependent on the expression of CD1d, and they functioned via an IL-10–mediated mechanism. Regulatory B cells induced pulmonary infiltration of CD4⁺CD25⁺ forkhead box protein 3⁺ regulatory T cells, independent of TGF- β , thereby suppressing allergic airway inflammation. Regulatory B cells that were generated *ex vivo* also suppressed the development of allergic airway inflammation. Furthermore, the transfer of regulatory B cells reversed established airway inflammation in ovalbumin-sensitized mice.

Conclusion: We have generated *in vivo* and *ex vivo* a regulatory B cell that can prevent or reverse allergen-induced airway inflammation via regulatory T cells. (J Allergy Clin Immunol 2010;■■■:■■■–■■■.)

Key words: Mouse, helminth, inflammation, asthma, regulatory B cell, IL-10, regulatory T cell, CD1d

In recent decades, the global incidence of allergic diseases, such as asthma, has increased dramatically,¹ with the decrease in parasitic helminth infections implicated as a causal reason for this rise.^{2,3} Allergic asthma is a chronic inflammatory disease of the airways associated with airway hyperresponsiveness (AHR) in response to inhaled allergens and dysregulated type 2 immunity.⁴ Dysregulated type 2 immunity in allergic asthma is characterized by an expansion of CD4⁺ T_H2-cell populations, with increased production of T_H2 cytokines (IL-4, IL-5, and IL-13), elevated total and allergen-specific IgE, eosinophilia, and airway inflammation. Despite helminth-infected individuals displaying the characteristic type 2 responses seen in patients with allergy, helminths have evolved multiple mechanisms to regulate inflammatory responses in the infected host.^{2,3,5,6} To identify the mechanisms helminths use to suppress allergic inflammation, specific interest has focused on the helminth induction of CD4⁺ T-cell populations that produce the regulatory cytokines TGF- β and IL-10. Regulatory T (Treg) cells that are implicated in the modulation of allergic asthma include thymus-selected naturally occurring CD4⁺CD25⁺ forkhead box protein 3 (FoxP3)⁺ Treg cells and the inducible IL-10–secreting Treg type 1 cells (T_R1).^{7,8}

We have previously demonstrated a role for infection with *Schistosoma mansoni* worms in the suppression of acute (anaphylaxis) and chronic (allergen-induced AHR) experimental murine models of allergic disease.^{9,10} These earlier studies identified that *S mansoni*–mediated protection from anaphylaxis and allergic airway inflammation was dependent on the induction of both IL-10 and B cells.^{9,10} IL-10–producing B cells have been shown to downregulate inflammation in various mouse disease models such as inflammatory bowel disease,¹¹ T-cell–mediated inflammation,¹² experimental autoimmune encephalomyelitis (EAE),^{13–15} anaphylaxis,⁹ AHR,¹⁰ and collagen-induced arthritis.^{16,17} Phenotypical studies have revealed a specific B-cell subset, termed regulatory B (Breg) or B10 cells, characterized by CD19⁺ expression and IL-10⁺; with different groups using various combinations of surface markers, such as IgM, IgD, CD1d, CD5, CD21, CD23, CD24, CD43, and CD93, to describe such cells.^{12,17,18} Although the production of IL-10 is considered to be involved in the regulatory activity of Breg cells, the exact mechanisms of action of Breg-cell suppression of inflammation remains unknown.

In this study, we used the propensity of *S mansoni* worm infection to induce the expansion of IL-10–producing B cells, which

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Abbreviations used

AHR: Airway hyperresponsiveness
 BAL: Bronchoalveolar lavage
 Breg: Regulatory B
 EAE: Experimental autoimmune encephalomyelitis
 FoxP3: Forkhead box protein 3
 iNKT: Invariant natural killer T
 MZ: Marginal zone
 NKT: Natural killer T
 Treg: Regulatory T

can suppress allergic inflammation,^{9,10} to identify and characterize the specific B-cell population involved. We have identified a distinct Breg-cell subpopulation, CD19⁺IL-10⁺CD1d^{high}CD5⁺CD21^{high}CD23⁺IgD⁺IgM^{high} cells, that suppresses or reverses allergic airway inflammation *in vivo*. Further, we have demonstrated that Breg cells function via a mechanism of action mediated by IL-10 and dependent on the expression of CD1d. Breg cells suppress airway inflammation by inducing the recruitment of natural Treg (CD4⁺CD25⁺FoxP3⁺) cells to the lungs in a TGF- β -independent manner, with these Treg cells mediating the suppression of lung inflammation. These data define a mechanism of action for the suppression of asthma-like allergic airway inflammation in a mouse model via a distinct Breg-cell population.

METHODS**Mice**

Female BALB/c and C57BL/6J mice, 6 to 12 weeks of age, were bred in-house. IL-10^{-/-}, CD1d^{-/-}, FoxP3-enhanced Green Fluorescent Protein (eGFP) reporter mice, IL-10-eGFP reporter mice, and CD4-dominant-negative(dn) TGF β RII mice, all backcrossed to a BALB/c background, were from Jackson Laboratories (Bar Harbor, Maine) and bred in-house. J α 18^{-/-} mice, on a C57BL/6 background, were kindly provided by Dr Carsten Wiethe (University of Erlangen, Germany). Mice were housed in a pathogen-free facility in individually ventilated and filtered cages under positive pressure (Tecniplast, Northants, United Kingdom). All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin BioResources Ethical Review Committee.

***S. mansoni* infections**

Infection of mice with *S. mansoni* male worms was performed as described previously.^{9,10,19} In all experiments, infected mice were analyzed or first exposed to ovalbumin (Sigma-Aldrich St. Louis, Mo) at 8 weeks postinfection. Information on *in vitro* modulation of cells with live worms is provided in this article's Methods section in the Online Repository at www.jacionline.org.

Experimental allergic airway inflammation and analysis of AHR

Mice were sensitized with ovalbumin to induce allergic airway inflammation, as described previously,¹⁰ in an acute and established model (see this article's Fig E1 in the Online Repository at www.jacionline.org). In an acute sensitization model, mice were administered 20 μ g ovalbumin in alum (Pierce) intraperitoneal (i.p.) on days 0 and 14 and subjected to airway exposure with 1% ovalbumin in Dulbecco's Phosphate Buffered Saline (DPBS) for 20 minutes on days 21 to 24 (Fig E1, A). A model of established airway inflammation²⁰ was used to test the therapeutic effect of Breg-cell transfer on existing airway inflammation. Mice were sensitized i.p. on days 0 and 12 with 20 μ g ovalbumin in alum. Airway challenge with 5% ovalbumin aerosol was for 20 minutes on days 16 to 19 and days 25 to 28 to establish airway inflammation. On days 36 to 39, 5% ovalbumin aerosol was administered concurrent with cell transfer, with an additional aerosol challenge on day 44 (Fig E1, B). Twenty-four hours after the last ovalbumin aerosol challenge, lung

function was analyzed by unrestrained, single-chamber barometric plethysmography (EMKA, Paris, France) to determine enhance pause, or by an invasive method in which mice were tracheostomized and ventilated using a whole-body plethysmography with a pneumotachograph linked to a transducer (EMMS, Hants, United Kingdom), to determine changes in lung resistance in response to increasing doses of inhaled methacholine (10, 30, 60, and 100 mg/mL; Sigma-Aldrich). In some studies, area under the curve was determined for individual mice for statistical analysis.²¹ In compliance with Journal policy, lung resistance determined by invasive techniques is presented.²²

Cell preparation for flow cytometry

Spleens, lung draining mediastinal lymph nodes, mesenteric lymph nodes, and lungs were collected and cells isolated for culture or flow cytometry analyses, as described in the Methods section in the Online Repository.

Antibody depletion studies

Anti-IL-10 receptor (clone 1B1.3a), anti-TGF- β (clone 1D11.16.8), anti-CD25 (clone PC61), and as a control, an anti-trinitrophenyl mAb (clone 1B7.11) were all purchased from American Type Culture Collection (Manassas, Va). Anti-CD1d (clone 1B1) mAb depletion was as described.²³ Hybridomas were grown, and mAb was isolated and assayed to ensure endotoxin levels of <0.5 endotoxin units per milligram (Chromogenic LAL, Bio-whittaker, Md), as described.^{9,24} In all cell depletion experiments, depleting antibodies were used at dose of 1 mg mAb per mouse. Mice treated with mAb were checked by ELISA or flow cytometry to ensure efficient and specific depletion of the target cells. To confirm efficacy of anti-IL-10 receptor or anti-TGF- β mAb treatment, serum and lungs were removed from mAb-treated mice, and levels of IL-10 and TGF- β were quantified by ELISA.

Sorting and adoptive transfer of B and Breg cells

Spleen cells from infected or uninfected wild-type, IL-10^{-/-}, or CD1d^{-/-} mice were stained for CD19 and CD1d. Cells from CD1d^{-/-} mice were stained for CD19 alone. Because a subset of dendritic cells coexpress CD19,^{25,26} cells were stained for CD11c expression to ensure sorted cells did not contain this cell population. Cells were sorted by using a single-cell gate on a MoFlo DXP (Beckman Coulter, Brea, Calif). Ovalbumin-sensitized BALB/c, CD4-dnTGF β RII, or FoxP3-eGFP mice received 2×10^6 sorted B cells i.p. daily for 4 consecutive days. B cells were transferred concurrent with ovalbumin aerosol challenge on days 21 to 24 or days 36 to 39 postsensitization in mice with acute or established airway inflammation models, respectively (Fig E1).

Cytokine ELISA

ELISA was performed to quantify levels of specific cytokines in serum, bronchoalveolar lavage (BAL) fluid, or lung homogenates. Reagents for quantification of IL-4, IL-5, and IL-13 were from BD Biosciences (San Jose, Calif), and eotaxin, IL-17, IL-10, and IFN- γ were purchased as the DuoSet ELISA development system from R&D Systems (Minneapolis, Minn). Total TGF- β (acidified samples) was measured by ELISA according to the manufacturer's protocol (Promega, Madison, Wis).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0 and GraphPad Instat software (GraphPad Software Inc, La Jolla, Calif). The data from *in vivo* studies are from 2 to 4 separate experiments with 5 to 8 mice per group. Results represent the means $\pm$ SEMs unless stated otherwise. Differences were considered significant when $P < .05$ as assessed by the Student *t* test or the Mann-Whitney test.

RESULTS***S. mansoni* worm infection prevents allergic airway inflammation via an IL-10 and B-cell-dependent mechanism**

We have previously reported that mice infected with *S. mansoni* male worms are refractory to anaphylaxis and allergic airway inflammation.^{9,10} The mechanism by which worms mediate

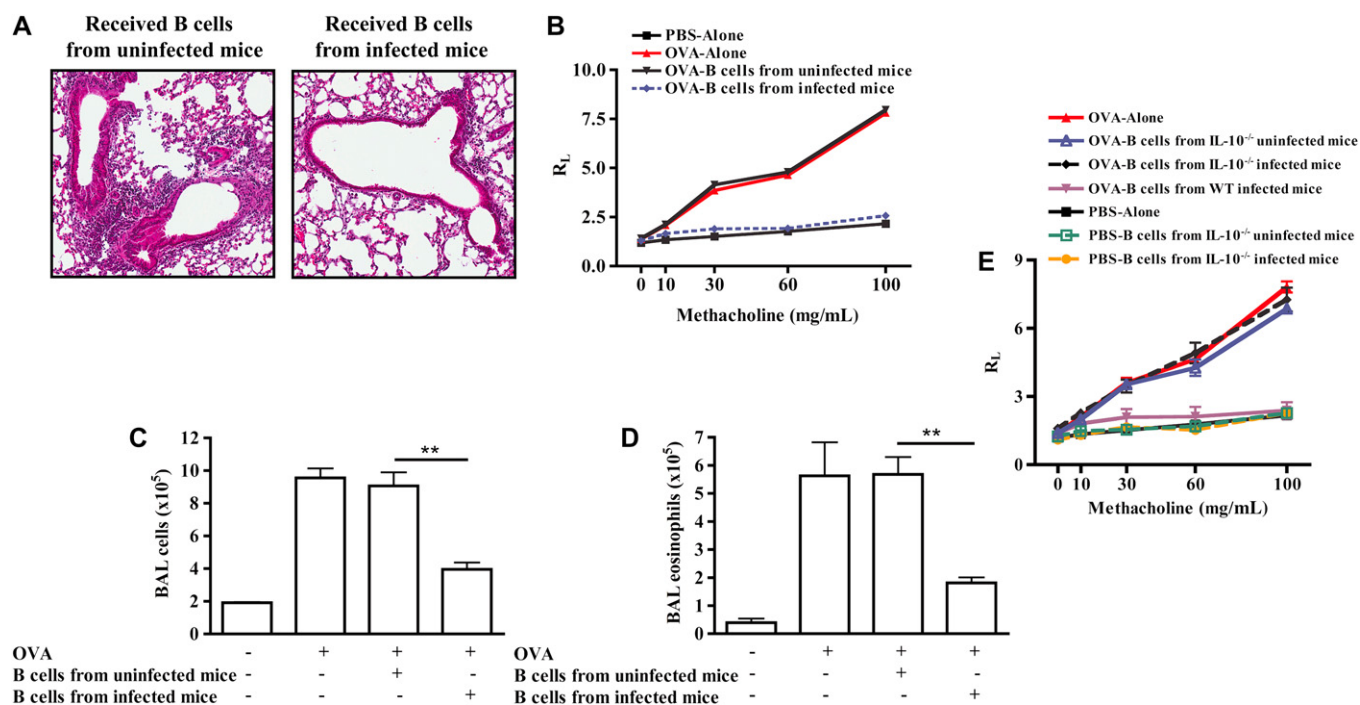


FIG 1. Administration of B cells isolated from *S. mansoni*-infected mice prevents the development of acute allergic airway inflammation. **A**, Representative hematoxylin and eosin–stained lung sections of ovalbumin (OVA)–sensitized mice. **B**, Methacholine-induced lung resistance (R_L) in PBS-sensitized or OVA-sensitized mice. Total cells (**C**) and eosinophil counts (**D**) in the BAL. **E**, Methacholine-induced R_L in PBS-sensitized or OVA-sensitized uninfected mice from groups as shown. Mice were treated with OVA in an acute sensitization model (Fig E1, A). Data represent the mean change from baseline values for each group. The Student *t* test was used to test for statistical difference between groups: ***P* < .01.

protection against allergic airway inflammation was shown to be dependent on both IL-10 and B cells,^{9,10} but the IL-10–producing B-cell population is as yet uncharacterized. To determine whether CD19⁺ B cells from the spleens of worm-infected mice were functional *in vivo*, these cells were isolated by cell sorting and transferred to uninfected ovalbumin-sensitized recipients concurrent with airway challenge, and allergic airway inflammation was analyzed (Fig E1, A). Recipients of splenic CD19⁺ B cells from worm-infected mice were protected from ovalbumin-induced airway inflammation (Fig 1, A), with a negligible methacholine-induced increase in lung resistance (Fig 1, B) and limited pulmonary infiltration of cells, including eosinophils (*P* < .01; Fig 1, C and D). In contrast, CD19⁺ B cells from the spleens of uninfected mice did not alter the course of ovalbumin-induced allergic airway inflammation, with pulmonary inflammation comparable to ovalbumin-sensitized uninfected mice (Fig 1, A–D). To address formally whether IL-10 production by splenic B cells from infected mice was involved in conferring protection from allergic airway inflammation, we transferred B cells from uninfected or infected IL-10^{−/−} mice to uninfected ovalbumin-sensitized recipients. Although transfer of B cells from infected wild-type mice prevented allergic airway inflammation, B cells from infected or uninfected IL-10^{−/−} animals were not protective (Fig 1, E). Collectively, these data show that *S. mansoni* worm infection induces the expansion of a spleen IL-10–producing B-cell population that can transfer suppression of ovalbumin-induced allergic airway inflammation via an IL-10–mediated mechanism.

IL-10–producing Breg cells are CD1d^{high}

We sought to exploit the capacity of *S. mansoni* infection to induce IL-10–producing B cells that can prevent allergic airway inflammation *in vivo*, to define the B-cell subpopulation involved, and to dissect their mechanism of suppression of allergic airway inflammation. Initially, to identify whether a specific B-cell population was selectively expanded by worm infection, we phenotyped surface markers on a range of B-cell subpopulations from the spleens of uninfected and infected mice. Worm infection caused a generalized expansion in all B-cell subpopulations analyzed (see this article's Fig E2 in Online Repository at www.jacionline.org), precluding the identification of a defined B-cell subpopulation uniquely induced by infection. Therefore, to specifically identify IL-10–producing B-cell subpopulations, we initially used intracellular detection of IL-10⁺CD19⁺ cells in wild-type mice and subsequently used IL-10–eGFP reporter mice²⁷ to detect IL-10–eGFP⁺–expressing B cells. All data herein are from IL-10–eGFP reporter mice with comparable data obtained with intracellular staining for IL-10⁺ B cells in wild-type mice. Worm infection evokes an expansion of IL-10⁺ cells, in particular CD19⁺ cells that are CD4⁺ (data not shown), with 1-fold more IL-10⁺ CD19⁺ cells in infected mice relative to uninfected (Fig 2, A, and data not shown). The majority (82%) of IL-10⁺CD19⁺ spleen cells were CD1d^{high} (Fig 2, A). Further surface phenotyping of CD19⁺CD1d^{high} IL-10–producing B cells demonstrated that they were CD5⁺, CD21^{high}, CD23⁺, IgD⁺, and IgM^{high} (Fig 2, B). We have termed worm-induced CD19⁺, IL-10⁺, CD1d^{high} B cells as Breg cells. Quantification

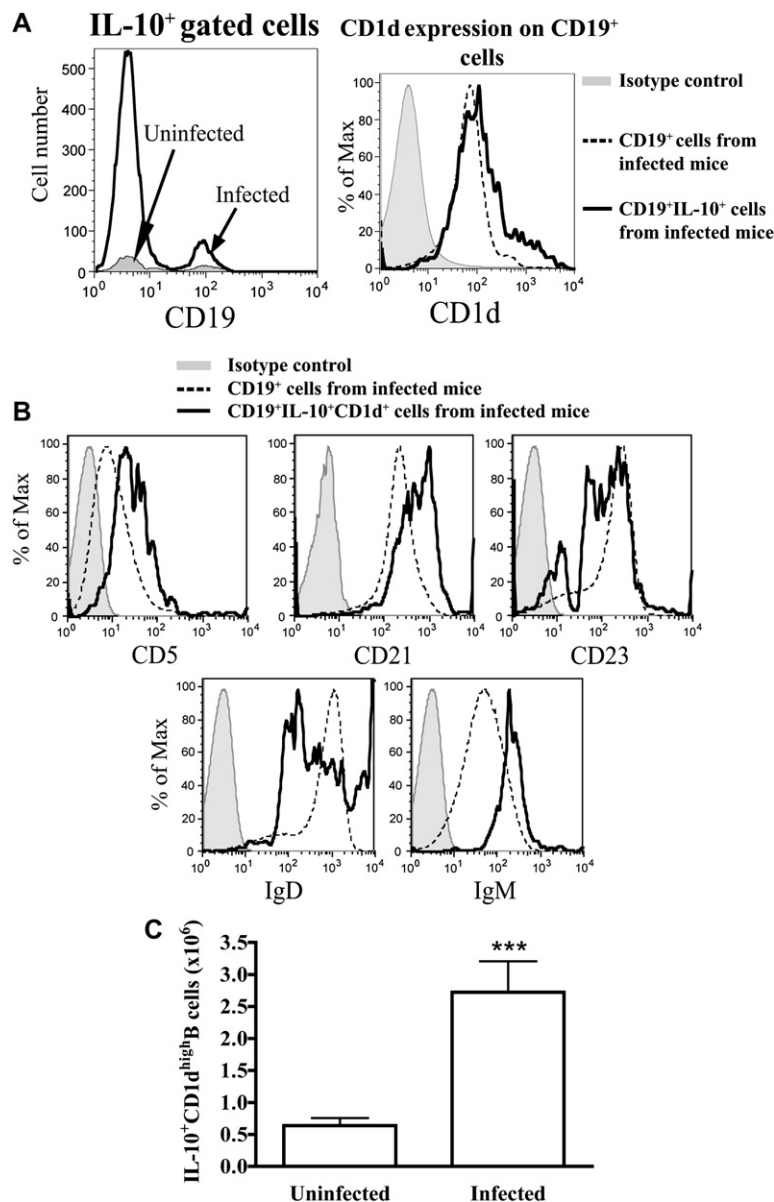


FIG 2. Characterization of IL-10-producing Breg cells. **A**, Representative histograms of spleen IL-10⁺ cells from infected (black histogram) and uninfected (gray histogram) IL-10-eGFP mice that express CD19. CD1d expression on CD19⁺ or CD19⁺IL-10⁺ spleen cells in infected IL-10-eGFP mice, and isotype control. **B**, CD5, CD21, CD23, IgD, and IgM expression on CD19⁺IL-10⁺CD1d^{high} Breg cells. Surface expression of markers on whole CD19⁺ cells and isotype control is shown. **C**, Numbers of Breg cells in the spleen of uninfected and infected mice. Data are from analysis of cells from individual mice and representative of 6 to 8 experiments. The Student *t* test was used to test for statistical difference between groups: ****P* < .001. Max, Maximum.

of the frequency of Breg cells in spleens of infected mice relative to uninfected mice illustrated the significant (*P* < .001) 5-fold expansion of these cells after infection (Fig 2, C). These data identify a distinct Breg-cell population induced by helminth infection.

Worm protection from allergic airway inflammation is CD1d-dependent

Although the presence of IL-10-producing B cells has been described previously in naive mice and during murine disease models,^{13,14,16,28} the mechanisms of action of these cells has not yet been fully dissected. To address whether the expression of

CD1d on Breg cells was relevant to worm-mediated protection from allergic airway inflammation, we first used an anti-CD1d mAb (1B1)²⁹ to block CD1d *in vivo*. In support of a potential role for CD1d expression in worm-mediated resistance to allergic responses, treatment of mice with the blocking anti-CD1d antibody abrogated the protection from ovalbumin-induced allergic airway inflammation afforded by worm infection (see this article's Fig E3, A, in the Online Repository at www.jacionline.org). Furthermore, allergen-sensitized *S mansoni* worm-infected mice treated with anti-CD1d mAb develop Penicillin-V-induced anaphylaxis, whereas control infected-mice, as reported previously,⁹ are resistant to anaphylaxis (Fig E3, B).

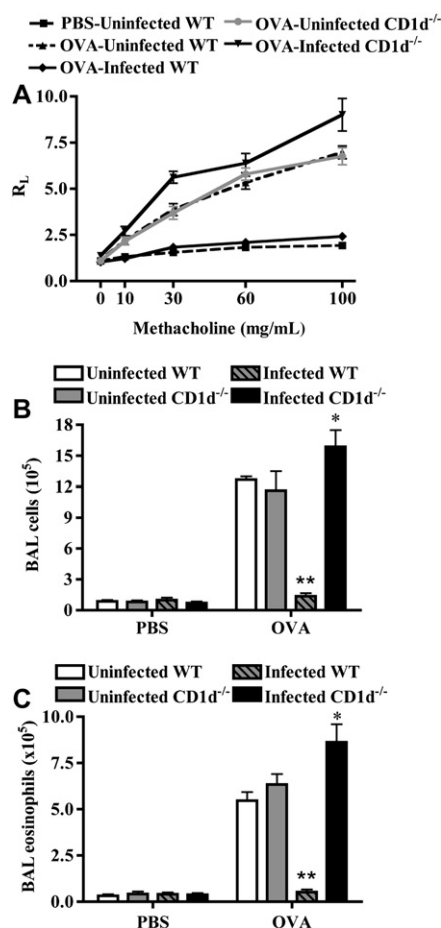


FIG 3. *S. mansoni*-infected $CD1d^{-/-}$ mice are susceptible to acute allergic airway inflammation. **A**, Methacholine-induced lung resistance (R_L) in ovalbumin (OVA)-sensitized uninfected and infected $CD1d^{-/-}$ or wild-type (WT) mice. Total cells (**B**) and eosinophils (**C**) in BAL. Mice were treated with OVA in an acute sensitization model (Fig E1, A). The Student *t* test was used to test for statistical difference between groups: * $P < .05$; ** $P < .01$.

To confirm further a role for CD1d in the worm-mediated mechanism of resistance to allergic airway inflammation, we infected $CD1d^{-/-}$ mice with *S. mansoni* and analyzed ovalbumin-induced AHR. Infected $CD1d^{-/-}$ mice are highly susceptible to AHR (Fig 3, A). Indeed, infected ovalbumin-sensitized $CD1d^{-/-}$ mice demonstrated higher levels of lung resistance relative to ovalbumin-sensitized wild-type or uninfected $CD1d^{-/-}$ mice, with mortalities of infected $CD1d^{-/-}$ mice during methacholine challenge (Fig 3, A). Furthermore, infected wild-type mice had negligible total cell or eosinophil infiltration in the BAL, whereas infected ovalbumin-sensitized $CD1d^{-/-}$ mice had a significant increase ($P < .01$) in total cell counts and eosinophils in BAL (Fig 3, B and C) relative to ovalbumin-sensitized uninfected mice.

Because CD1d engages with invariant natural killer T (iNKT) cells³⁰ and iNKT cells are known to play a role in helminth infection^{31,32} and regulation of lung inflammation,³³ it was possible that worm infection induced $CD1d^{high}$ Breg cells leading to the expansion of iNKT cells that mediated resistance to allergic airway inflammation. To address the induction of iNKT cells, α -Gal-Cer-tetramer staining was used to quantify the levels of iNKT cells both in the lungs and spleens in PBS or ovalbumin-sensitized uninfected and worm-infected mice. Although there

was a difference in basal levels of iNKT cells between uninfected and infected mice, there were fewer iNKT cells induced by ovalbumin in worm-infected mice (see this article's Fig E4, A and B, in the Online Repository at www.jacionline.org). To confirm formally that worm-mediated protection was not iNKT-cell-mediated, $J\alpha 18^{-/-}$ mice deficient in iNKT cells³⁴ were infected and evaluated for ovalbumin-induced AHR. Infected $J\alpha 18^{-/-}$ mice were refractory to ovalbumin-induced AHR (Fig E4, C). These data confirm that protection mediated by worm infection of mice is CD1d-dependent but does not involve iNKT cells.

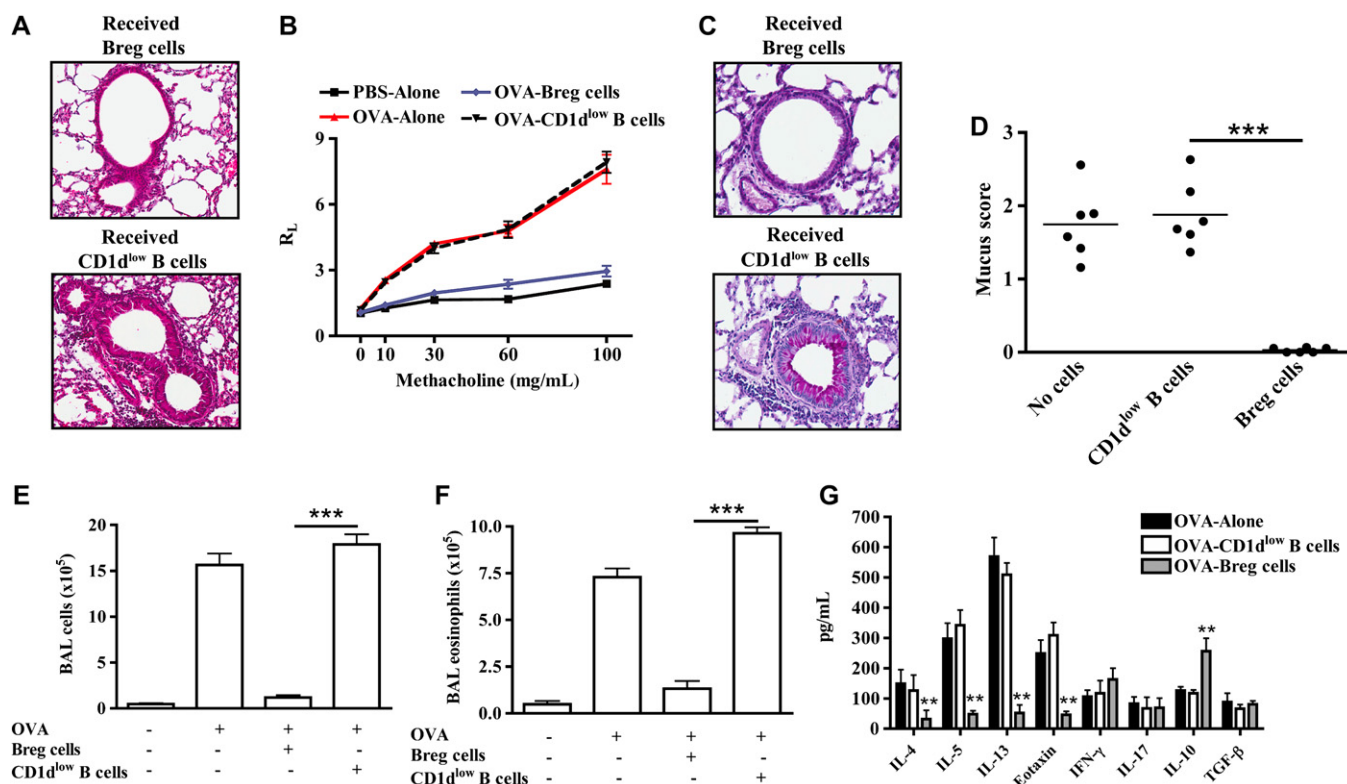
Breg cells prevent the development of allergic airway inflammation

To investigate the functional role of $CD1d^{high}$ Breg cells in the protection against allergic airway inflammation, these cells were sorted from the spleens of infected mice and transferred to recipient ovalbumin-primed mice during pulmonary sensitization in an acute model (Fig E1, A). As a control, $CD19^{+}$ cells that were not $CD1d^{high}$, termed $CD1d^{low}$, were also sorted from infected mice and transferred to ovalbumin-sensitized animals. The transfer of Breg cells from infected mice induced significant protection from allergen-induced airway inflammation relative to ovalbumin-alone or ovalbumin- $CD1d^{low}$ B-cell recipient mice (Fig 4). Breg-cell recipients had less airway inflammation (Fig 4, A) with reduced airway resistance (Fig 4, B). Furthermore, bronchial goblet cell hyperplasia was significantly reduced ($P < .001$) in Breg-cell recipients relative to other ovalbumin-challenged groups (Fig 4, C and D). In addition, mice receiving Breg cells had significantly reduced ($P < .001$) BAL infiltration of cells, specifically eosinophils (Fig 4, E and F). The absence of lung inflammation in Breg-cell recipients was associated with significantly lower ($P < .01$) levels of IL-4, IL-5, IL-13, and eotaxin in the BAL fluid, whereas the levels of IL-10 were increased relative to other groups (Fig 4, G). BAL levels of other cytokines tested, IFN- γ , IL-17, and TGF- β , were comparable between groups. These data confirm that Breg-cell transfer protects mice from allergic airway inflammation.

In $CD19^{+}$ B-cell surface phenotyping analysis, a marked increase in a $CD1d^{-}$ cell population in the spleens of infected mice was observed (see this article's Fig E5 in the Online Repository at www.jacionline.org). This B-cell population was very small or absent in naive or ovalbumin-sensitized uninfected mice (Fig E2 and Fig E5, A). These $CD1d^{-}CD19^{+}$ B cells were $CD23^{+}$, $CD21^{low}$, $CD5^{+}$, IgD^{+} , and IgM^{low} (Fig E5, B). To investigate the functionality of $CD1d^{-}$ B cells, they were sorted from spleens of infected mice and transferred to ovalbumin-primed recipients during aerosol challenge (Fig E1, A). $CD1d^{-}$ B cells from spleens of infected mice exacerbated AHR in recipients, leading to mortalities in response to methacholine challenge, with marked lung inflammation (Fig E5, C-E). Therefore, *S. mansoni* worm infection induces Breg cells that can prevent ovalbumin-induced airway inflammation and, conversely, elicits a $CD1d^{-}$ B-cell population that can exacerbate airway inflammation.

Breg cells prevent allergic airway inflammation via T regulatory cells

Having shown a role for Breg cells in the *in vivo* suppression of allergic airway inflammation, we sought to determine the mechanism of action of these cells. Because Treg ($CD4^{+}CD25^{+}FoxP3^{+}$) cells are widely implicated in the downregulation of allergic



inflammation,⁸ we investigated whether Breg cells altered Treg cell levels *in vivo* during inflammation. Breg cells, or CD1d^{low} B cells as controls, were sorted from infected mice and transferred into ovalbumin-sensitized FoxP3-eGFP reporter mice concurrent with ovalbumin aerosol challenge, and the levels of Treg cells were analyzed. There were significantly more (*P* < .05) Treg cells in the lung digests, lung draining mediastinal lymph nodes (*P* < .05), and BAL (*P* < .01) in ovalbumin-sensitized FoxP3-eGFP reporter mice after receiving Breg cells relative to control groups (Fig 5, A–C). Furthermore, similar to earlier data (Fig 4), Breg-cell recipients were protected from allergic airway inflammation (data not shown). Interestingly, the levels of Treg cells in the spleen of all 3 groups of mice were comparable (Fig 5, D), suggesting a lung-specific expansion of Treg cells after Breg-cell transfer during pulmonary allergen sensitization. Adoptive transfer of CD1d^{high} B cells from IL-10^{−/−} mice to ovalbumin-sensitized recipients did not increase FoxP3⁺ Treg cells in the BAL (Fig 5, E) or confer protection from allergic airway inflammation (data not shown). Therefore, the induction of pulmonary Treg cells by Breg cells is IL-10-dependent.

Because TGF- β is implicated in the generation of Treg cells³⁵ and Treg-cell suppression of allergic airway inflammation is potentially mediated by TGF- β ,⁸ recipients of Breg cells were treated with an anti-TGF- β mAb or an anti-IL-10-receptor mAb.

ELISA on lung homogenates confirmed the efficacy of mAb treatment, with significantly reduced TGF- β and increased IL-10 in anti-TGF- β and anti-IL-10 receptor-mAb-treated groups, respectively (data not shown). Following mAb depletion, the induction of Treg cells in the lungs of Breg-cell recipients, and protection from allergic airway inflammation (data not shown), were shown to be independent of TGF- β but dependent on IL-10 (Fig 6, A). To validate further that Breg cells induce Treg cells in the lungs of mice independently of TGF- β , we transferred Breg cells to CD4-dnTGF β R2 mice, which have TGF- β -activated signaling specifically blocked in T cells, facilitating the analysis of immunologic responses in the absence of TGF- β signaling in CD4⁺ cells.³⁶ The numbers of Treg cells in the lungs of mice after Breg-cell transfer were similar in ovalbumin-sensitized wild-type and CD4-dnTGF β R2 mice, further confirming that Breg cells induce Treg cells independent of TGF- β (Fig 6, B). To validate whether the increase in Treg cells in the lungs of mice receiving transferred Breg cells was relevant to suppression of ovalbumin-induced airway inflammation, we treated Breg-cell recipients with anti-CD25 mAb to deplete CD25-expressing cells, including Treg cells. Ovalbumin-sensitized mice treated with anti-CD25 mAb had comparable ovalbumin-induced AHR to ovalbumin-alone mice, whereas mice receiving Breg cells were protected from ovalbumin-induced allergic airway inflammation

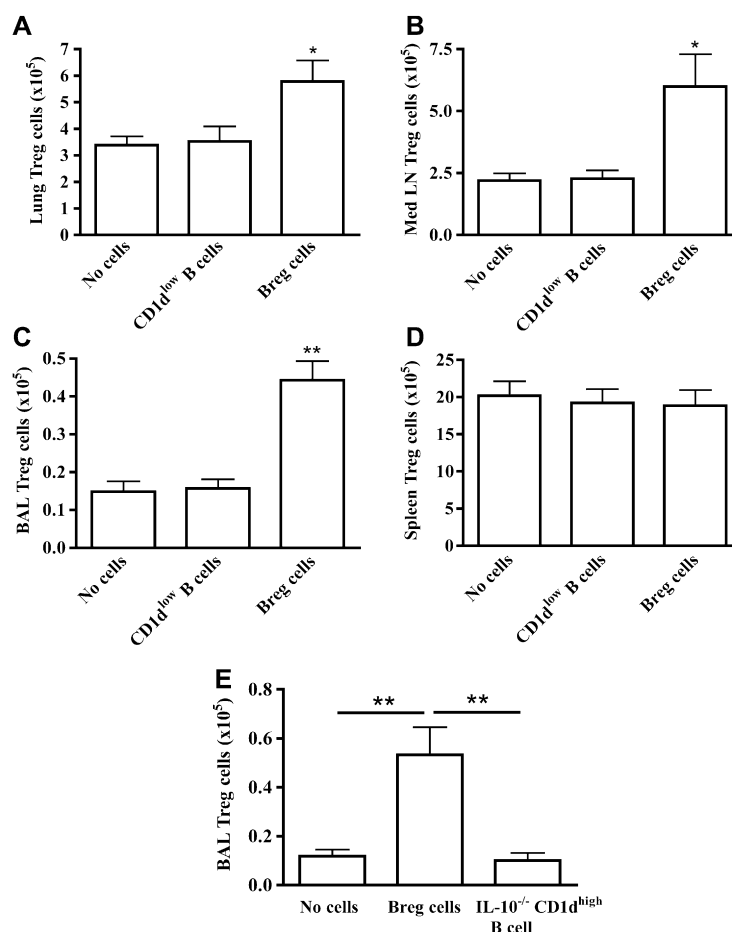


FIG 5. Administration of Breg cells expands pulmonary Treg cells. *In vivo*-generated Breg or CD1d^{low} B cells were transferred to ovalbumin-sensitized FoxP3-eGFP reporter mice, and the number of Treg cells (CD4⁺CD25⁺FoxP3⁺) was measured in lung digests (A), mediastinal lymph nodes (Med LN) (B), BAL (C), and spleen (D). E, Number of Treg cells in BAL of FoxP3-eGFP reporter mice OVA-sensitized receiving no cells, *in vivo*-generated Breg cells from wild-type mice, or CD1d^{high} B cells from IL-10^{-/-}-infected mice. Mice were treated with ovalbumin and cells transferred in an acute sensitization model (Fig E1, A). The Student t test was used to test for statistical difference between groups: **P* < .05; ***P* < .01.

(Fig 6, C). In contrast, mAb-mediated depletion of CD25⁺ cells ablated the protection conferred by transfer of Breg cells (Fig 6, C). These data confirm that helminth-induced Breg cells prevent allergic airway inflammation via a Treg-cell-mediated and TGF- β -independent mechanism.

Breg cells generated *ex vivo* prevent the development of allergic airway inflammation

Rather than using *in vivo* worm infection to induce Breg cells, an *ex vivo* system was used involving exposure of spleen cells from IL-10-eGFP reporter mice to live worms, which allows the worm excretory/secretory molecules to modulate the cells.¹⁹ This has been previously shown to induce IL-10-producing B cells.⁹ Incubation of spleen cells from uninfected mice with live worms induced an increase in IL-10-eGFP⁺ B cells that were CD1d⁺ (see this article's Fig E6, A, in the Online Repository at www.jacionline.org). Indeed, *ex vivo*-generated Breg cells have a comparable cell surface marker profile to that seen in Breg cells obtained from spleens of infected mice (Fig 2, A; data not shown). Furthermore, Breg cells were sorted from spleen cells cultured *in vitro* with worms or in media alone and transferred to ovalbumin-

sensitized mice. The transferred worm-modulated Breg cells reduced airway inflammation, mediated protection from allergic airway inflammation, and induced an increase in FoxP3⁺ Treg cells in BAL of recipients (Fig E6, B-D). Therefore, Breg cells generated by worms *in vivo* and *ex vivo* can induce Treg cells in the lung and prevent the development of allergic airway inflammation.

Breg cells reverse established allergic airway inflammation

Although we have demonstrated that Breg cells can prevent the development of allergic airway inflammation (Fig 4), a more relevant question in the context of therapeutic intervention for human beings with asthma is whether such cells reverse established disease. To address this, animals were ovalbumin-sensitized and treated with repeated aerosol exposure with ovalbumin to induce established allergic airway inflammation before Breg cells were transferred (Fig E1, B). The transfer of Breg cells to mice with established airway inflammation reversed lung resistance (Fig 7, A), with a decrease in cell infiltration in the BAL (*P* < .01), specifically eosinophils (*P* < .05; Fig 7, B and C).

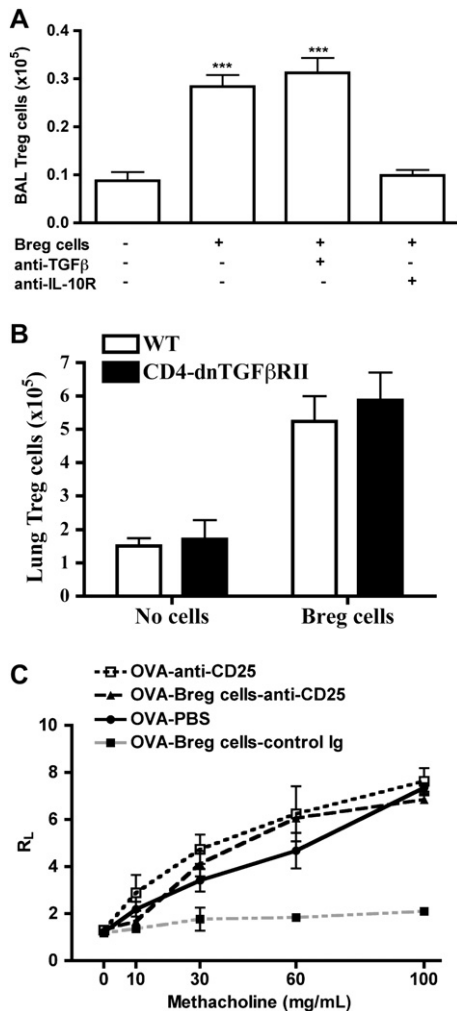


FIG 6. Administration of *in vivo*-generated Breg cells prevents the development of acute allergic airway inflammation via a Treg-dependent and TGF-β-independent mechanism. **A**, Number of Treg cells in BAL from ovalbumin (OVA)-sensitized FoxP3-eGFP reporter mice receiving no cells, Breg cells, or Breg cells and anti-TGF-β or anti-IL-10-receptor(R) mAb. **B**, Treg-cell numbers in the lung digests from OVA-sensitized wild-type (WT) and CD4-dnTGFβRII mice after receiving no cells or Breg cells. **C**, Methacholine-induced lung resistance (R_L) in OVA-sensitized uninfected mice, that were transferred Breg cells and treated with anti-CD25 or control mAb. Control mice not receiving Breg cells and untreated (OVA-alone) or treated with anti-CD25 mAb are shown. Mice were treated with OVA and Breg cells transferred in an acute sensitization model (Fig E1, A). Data represent the means $\pm$ SDs. The Student *t* test was used to test for groups with statistical difference from OVA-alone-treated mice: ****P* < .001.

Using FoxP3-eGFP reporter mice, it was demonstrated that the lower AHR after Breg-cell transfer was associated with a significant increase in Treg-cell infiltration in the BAL (*P* < .01) relative to control mice (Fig 7, D). These data indicate that transfer of Breg cells reverses airway inflammation in mice with established AHR.

DISCUSSION

In the current study, we used the known propensity of *S mansoni* worms to expand an IL-10-producing B-cell subpopulation *in vivo* and *ex vivo*, to allow characterization of these regulatory B cells, and identify their potential mechanism of action. In the

spleens of infected mice, the preferential production of IL-10 by CD19⁺ B cells was from a CD1d^{high} population, termed here Breg cells. Transfer of Breg cells to ovalbumin-sensitized recipients suppressed and reversed allergic airway inflammation, AHR, eosinophil infiltration, goblet cell hyperplasia, and allergen-induced increase in T_H2 cytokines in the lungs. We have further identified that worm-induced Breg cells suppress allergic airway inflammation via a mechanism of action that is IL-10-dependent and mediated by pulmonary infiltration of CD4⁺CD25⁺FoxP3⁺ Treg cells.

A major role for IL-10-producing B cells has been previously described in the downregulation of inflammation in various mouse disease models, including EAE, inflammatory bowel disease, collagen-induced arthritis, lupus, anaphylaxis, and AHR.^{9,11,13,14,16,18} Despite recent attempts to phenotype these B cells, a definitive marker characterization has remained elusive.³⁷ Thus far, the phenotype of Breg cells, or B10 cells, shares characteristics with transitional 2 marginal zone (MZ) precursor B cells.^{17,28} Furthermore, no defined universal mechanism of action for these cells has been elucidated.³⁷ We show here that CD1d expression on worm-induced Breg cells is functionally relevant to the suppression and reversal of allergic airway inflammation. CD1d^{high} B cells, which are known to produce IL-10, have been shown to exert regulatory function in chronic intestinal inflammation,¹¹ experimental arthritis,¹⁷ and T-cell-mediated inflammation.¹²

Given that CD1d⁺ and natural killer T (NKT) cells have been shown to suppress or induce inflammation in mouse models and human studies of allergic lung disease,^{33,38} we considered a potential mechanistic involvement for NKT cells in mediating the protective function of CD1d^{high}-expressing Breg cells in allergic airway inflammation. NKT cells are divided into 3 subtypes based on their T-cell receptor structure. Types I and II are CD1d-dependent, and type III is CD1d-independent.³⁹ Type I NKT cells have been shown to be important for the induction of AHR,^{33,40-42} with suppressive ability if activated with α-Gal-Cer.⁴³⁻⁴⁷ Previous studies have used CD1d^{-/-} mice lacking type I and II NKT cells, and Jα18^{-/-} mice lacking the type I NKT cells, for the dissection of NKT cell subset *in vivo* function,^{40,48} although discrepancies exist between data obtained in the inflammatory response in various pulmonary models using CD1d^{-/-} or Jα18^{-/-} mice.³⁸ Indeed, in the ovalbumin airway model used herein, both uninfected CD1d^{-/-} and Jα18^{-/-} mice develop AHR, whereas in other variations of the ovalbumin model, such mice do not develop lung inflammation.^{33,41,42} However, with respect to helminth infection, we now show that infected CD1d^{-/-} mice are susceptible to allergen-induced AHR, whereas infected Jα18^{-/-} mice are resistant. This indicates schistosome infection prevents AHR independent of iNKT cells but suggests a potential role for type II NKT cells. Little is known about the role of type II NKT cells in asthma, but these cells have demonstrated a suppressive function in EAE, type I diabetes, and tumor models,^{49,50} and consequently, further work is required in elucidating the potential interactions between helminths and type II NKT cells.

The CD1d^{high} Breg cells reported herein express MZ B-cell markers, similar to other studies.^{12,17,28,51} CD1d-expressing MZ B cells have been shown to activate iNKT cells in the presence of dendritic cells⁵² and are important for the iNKT-cell-dependent development of Treg cells during the generation of peripheral tolerance.⁵³ The interplay between MZ B cells and iNKT cells is mediated by the ability of the B-cell receptor to capture and present CD1d-restricted glycolipids and antigenic lipids.^{54,55}

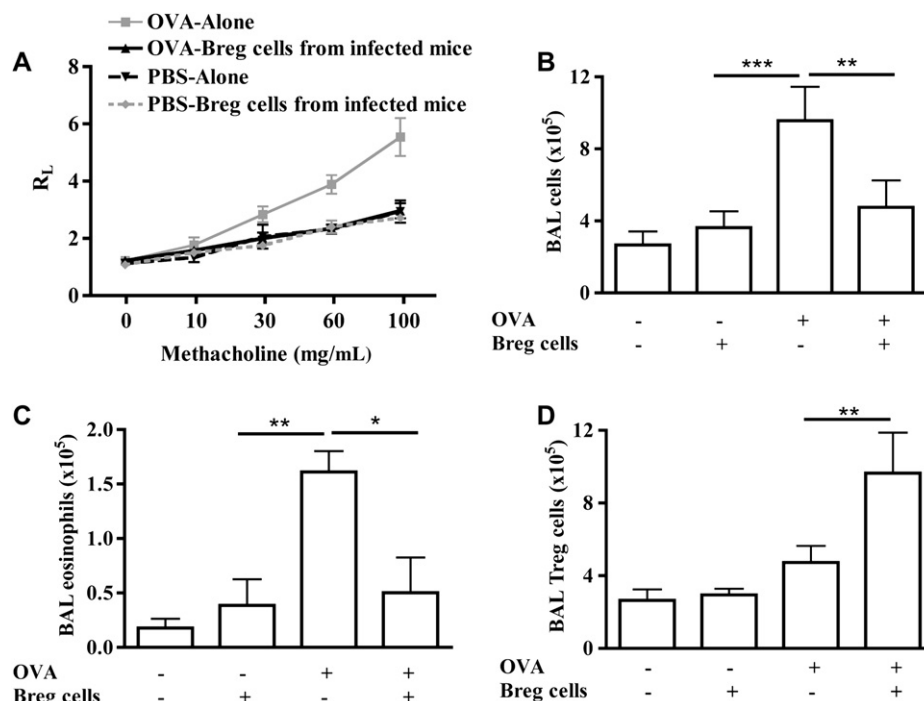


FIG 7. Administration of *in vivo*-generated Breg cells reverses established allergic airway inflammation. **A**, Lung resistance (R_L), in response to methacholine, of mice with established airway inflammation receiving *in vivo*-generated Breg cells. Total cells (**B**) and eosinophil cells (**C**) in BAL. **D**, Number of Treg cells in BAL. Data represent the means $\pm$ SDs. Mice were treated with ovalbumin (OVA) and Breg cells transferred in a model of established airway inflammation (Fig E1, B). The Student *t* test was used to test for statistical difference between groups: **P* < .05; ***P* < .01; ****P* < .001.

This interplay results in MZ B-cell proliferation *in vivo* and antigen-specific antibody production by these cells.^{55,56} These previous studies show a direct interaction between MZ B cells and iNKT cells with resulting immune modulation. Therefore, Breg cells may, in addition to the IL-10-dependent induction of Treg cells as described herein, suppress AHR via the type II NKT cell subset. Indeed, Breg cells may regulate inflammation by other mechanisms. For example, in a chronic intestinal inflammatory condition, CD1d-expressing IL-10-producing B cells downregulate inflammation via suppression of IL-1 β production and signal transducer and activator of transcription 3 (STAT3) activation.¹¹ Alternatively, Breg cells may suppress allergic inflammation via antibody production, including IgA-mediated mechanisms.⁵⁷

B cells have been shown to promote Treg-cell expansion *in vitro*^{58,59} and to influence Treg cells in an EAE mouse model.⁶⁰ The role for Treg cells in downregulation of inflammation in allergic AHR is well established,²⁰ with the suggestion that regulation is mediated by TGF- β ⁶¹ or IL-10.^{62,63} We did not see any role for TGF- β in the Breg-cell-mediated suppression of allergic airway inflammation, as reported previously,⁶⁴ and there was no detection of TGF- β production by Breg cells (data not shown), as recently described by others.⁵¹ In contrast, Breg cells from worm-infected mice blocked allergic airway inflammation via an IL-10-mediated mechanism involving the expansion of Treg cells in the lung. It is most noteworthy that when Breg cells were transferred in acutely sensitized mice, Treg cells were detected in the lungs and not the spleen of recipients, whereas in mice chronically sensitized with ovalbumin, there was an increase in the number of Treg cells in the lungs but also the spleen (data

not shown). Breg cells may have a more systemic effect on Treg expansion in chronic sensitized mice relative to the compartmentalized lung-specific upregulation of Treg cells in acute airway inflammation. This is the first formal *in vivo* demonstration of Breg cells inducing Treg cells to suppress allergic airway inflammation in a mouse model.

Clinical studies have highlighted the link between schistosome infection of human beings and allergic inflammation.^{65,66} Previously we have shown that *S mansoni* worms increased IL-10-producing B cells, and transfer of these B cells from infected mice protected recipient mice against experimentally induced anaphylaxis.⁹ In addition, B cells from *S mansoni* male and female worm-infected mice mediate protection against airway inflammation in an IL-10-dependent manner.⁶⁷ Indeed, a recent study involving helminth-infected patients with multiple sclerosis also highlighted a role for B-cell-derived IL-10 in ameliorating disease symptoms.⁶⁸ The demonstration of a distinct Breg-cell population evoked by *S mansoni* *in vivo* and *ex vivo* supports a role for such cells in the downregulation of allergic inflammation. It is intriguing that we also described worm-induced CD1d⁺ B cells that exacerbated airway inflammation when transferred to ovalbumin-sensitized mice. However, although we show that these helminth-induced CD1d⁺ B cells do not express IL-10 and are not upregulated in uninfected naive or ovalbumin-sensitized mice, we currently have no insights about how these cells can exacerbate AHR. This highlights the delicate balance helminths have evolved to modulate immunity in their infected hosts, with B-cell subpopulations that can differentially regulate or potentiate allergic type 2 immunity. Indeed, although we have shown in

ovalbumin-sensitized mice that IL-10-producing B cells altered T_H2 cytokines in the lungs, a more recent study using an anti-mouse IgD *in vivo* model showed that B-cell-specific IL-10 production does not affect T_H2 polarization.⁶⁹ A possible partial explanation for this difference may be the model used and type of inflammatory response.

Helminth parasites modulate multiple cell types in the immune system, resulting in the generation of an anti-inflammatory environment favorable for their survival, facilitating the suppression of unrelated allergic-like inflammation.³ With the urgent need for new therapies for the treatment of allergic disease,⁷⁰ insights such as those reported herein into the novel mechanisms helminths have evolved to suppress allergic inflammation may open new strategies for allergic disease therapy. The potential for use of helminth molecules as new anti-inflammatory therapies is postulated,⁷¹ with the use of worm molecules to generate Breg cells, as described here, an option for further investigation. The reversal of established airway inflammation by Breg cells highlights their clinical potential as a novel cellular therapy for patients with chronic pulmonary inflammation, in particular allergic asthma.

We have demonstrated the *in vivo* and *ex vivo* generation of a defined Breg-cell population that can prevent and reverse asthma-like allergic airway inflammation. We also demonstrate the mechanism of action by which Breg cells stimulate the IL-10-dependent recruitment of Treg cells to the allergic lung, with the suppression of airway inflammation mediated by the Treg cells.

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Clinical implications: Breg cells induced by *S mansoni* *in vivo* and *ex vivo* prevent and reverse airway inflammation in a mouse model via an IL-10 and Treg-cell-dependent mechanism.

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METHODS

In vitro modulation of spleen cells by live schistosome worms

A Puerto Rican strain of *S. mansoni* was maintained by passage in albino *Biomphalaria glabrata* snails. BALB/c mice were infected percutaneously with 200 male and female cercariae to produce worms. Worms were isolated by portal perfusion of infected mice using conditions optimized to reduce damage or stress to worms. Spleen cells from IL-10-eGFP mice (1×10^7 per well) were seeded into 12-well transwell culture plates (Costar, Vernon Hills, Ill). Five male worms were then placed in the transwell insert. The cells/worms were incubated at 37°C, 5% CO₂ for 48 hours. As a control, cells were cultured in transwell plates with media alone. The worms were removed and the cells harvested and washed 3 times with fresh media and checked for viability.^{E1,E2} B-cell populations were isolated by cell sorting (see the Methods section in the article).

Lung immunology

Processing of lung tissue and immunologic analysis was as described.^{E1,E3,E4} In brief, the following was carried out.

BAL. Bronchoalveolar lavage fluids were collected by cannulating the trachea and lavaging the lungs twice with 0.8 mL ice-cold PBS. BAL cells were pelleted, washed, and counted. BAL fluid was stored at -80°C for cytokine/chemokine ELISAs. BAL cells were used for cytopins or flow cytometry. For cytopins, the numbers of eosinophils, neutrophils, macrophages, and lymphocytes was determined by performing a differential count, with at least 400 cells per slide, on Giemsa-stained cytocentrifuge preparations.

Lung homogenates and digests. Whole lungs were removed from mice and snap-frozen, placed in 10% formaldehyde-saline for histology, or digested with collagenase-D (Roche) for flow-cytometry analysis.

Lung histology. Lungs were fixed in 10% formaldehyde-saline for histology. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, and eosinophil infiltration was counted on Giemsa-stained sections. Airway mucus occlusion was analyzed on periodic acid-Schiff-stained lung sections. A semiquantitative score was used for airway occlusion as follows: 0, 0% to 10% occlusion; 1, 10% to 30% occlusion; 2, 30% to 60% occlusion; 3, 60% to 90% occlusion; and 4, 90% to 100% occlusion, as described.^{E5} For each mouse, the mean score was determined from at least 10 individual airways.

Flow cytometry on BAL cells. BAL cells were prepared for surface staining as described previously.^{E4} Cell surface marker expression was assessed by flow cytometry using a CyAn (Beckman Coulter, Brea, Calif). Data were analyzed by using FlowJo (Tree Star, Ashland, Ore) software. Cells were stained with BD Biosciences (San Jose, Calif) mAbs; peridinin chlorophyll-protein complex (PerCP) anti-CD4 (RM4-5), PerCP-Cy5.5 anti-CD19 (1D3), PerCP anti-CD8a (53-6.7), and phycoerythrin (PE) anti-Siglec-F (E50-2440). Caltag mAb; allophycocyanin anti-CD25 (PC61 5.3). R&D Systems mAb; PE anti-CCR3 (83101). Flow-cytometry buffer (PBS, 2% FCS, 0.05% sodium azide, and 0.5 μ mol/L EDTA) contained EDTA to exclude doublets. In brief, BAL cells were first gated on CD19, CD4, and CD8 versus forward scatter (FSC). Lymphocytes were identified as FSC^{lo}, Side scatter (SSC)^{lo} CD19⁺, CD4⁺, and CD8⁺. Eosinophils were distinguished as SSC^{hi}, CD19⁻, CD4⁻, CD8⁻, nonautofluorescent granulocytes that stained positive for CCR3, and/or SiglecF. Mononuclear cells were characterized as large (FSC^{hi}), granular (SSC^{hi}), autofluorescent cells that were CD4, CD8, and CD19-negative—that is, not in the lymphocyte gate. Macrophages were characterized as large, highly autofluorescent mononuclear cells that were confirmed to be CD11b (Mac-1)⁺ or F4/80^{hi}.

Cell preparation and flow cytometry for spleen, lung, lung draining mediastinal lymph nodes, and mesenteric lymph nodes

Spleen, lungs, lung draining mediastinal lymph nodes, and mesenteric lymph nodes were collected and cells isolated for culture or flow-cytometry analyses. Single-cell suspensions were prepared from all the organs and

erythrocytes lysed with 0.78% ammonium chloride solution where needed. For surface staining, single-cell suspensions were prepared in flow-cytometry buffer (PBS, 2% FCS, 0.05% sodium azide, and 0.5 μ mol/L EDTA). Cells were blocked by using Fc-block CD16/CD32 (2.4G2; BD Biosciences). Biotin or directly conjugated antibodies with fluorescein isothiocyanate (FITC), r-phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP), allophycocyanin (APC), or Pacific Blue (PB) were used. Anti-CD1d (1B1), anti-CD5 (53-7.3), anti-CD62L (MEL-14), anti-IgM (11/41), and anti-IgD (11-26c.2a) were from BD Biosciences, and anti-CD4 (RM4-5), anti-CD19 (1D3), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD25 (PC61), anti-CD43 (R2/60), and anti-CD138 (281-2) were from eBioscience. All antibodies were used at optimal concentration after titration experiments. Cells were acquired by using a Cyan (ADP Analyzer; Beckman Coulter). Gating of cells was based on the specific isotype control values as well as fluorochrome minus 1 setting when needed.^{E6} All analyses were performed by using FlowJo software (Tree Star Inc). For intracellular FoxP3 staining, cells were first stained for surface CD4 and CD25 expression followed by fixation, permeabilization, and intracellular staining by using a Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscience) according to the manufacturer's protocol. Antibodies used were PE-conjugated anti-FoxP3 (NRRF-30) and its matched isotype control rat IgG_{2a,k} (eBiosciences). For intracellular IL-10, cells were cultured for 8 hours at 1×10^6 cells/mL in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Labtech), 2 mmol/L L-glutamine (Invitrogen), and 50 U/mL penicillin and 50 μ g/mL streptomycin (Invitrogen), stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin 2.5 ng/mL and 250 ng/mL, respectively, or cultured with phorbol or medium in the presence of 10 μ g/mL brefeldin A (Sigma-Aldrich). In the cultures with worms, the worms were taken out before brefeldin A was added. Surface markers were stained first, followed by intracellular IL-10 (JES5-2A5) or matched isotype control IgG₁ staining (Caltag).

Tetramer staining. PE-conjugated CD1d-tetramers loaded with α -galactosylceramide (α GalCer) were as described.^{E7} Streptavidin-PE-labeled PBS-57-loaded tetramers specific for mouse CD1d^{E8} were obtained from Dr May Stout at the National Institutes of Health Tetramer Core Facility, Atlanta, Ga. Empty tetramers were used as an isotype control. In all experiments, cells from CD1d^{-/-} or J α 18^{-/-} mice were included as additional controls for gating. First, cells were blocked with Fc-block and neutravidin (Molecular Probes; 0.4 μ g/10⁶ cells) for 15 minutes at room temperature to block nonspecific binding. Cells were then incubated with FITC-conjugated anti-TCR β and PE-loaded or unloaded tetramers for 30 minutes on ice. Stained cells with unloaded tetramers were used to set appropriate gates for CD1d-tetramer-positive cells.

Active systemic anaphylaxis model. For the induction of active systemic anaphylaxis, penicillin V (Pen V)—OVA and Pen V-BSA conjugates were used, as described.^{E1} In brief, mice were sensitized by i.p. injection of 500 μ g penicillin V-OVA with 2×10^9 *Bordetella pertussis* (Wako Pure Chemical) and 1.0 mg Imject alum (Pierce). On day 14, anaphylaxis was elicited by i.v. challenge with 100 μ g Pen V-BSA. Temperature transponders (Bio Medic Data Systems) were implanted s.c. into recipient mice and temperatures recorded electronically (DAS-6007; Bio Medic Data Systems) just before challenge and subsequently every 10 minutes for the next 60 minutes.

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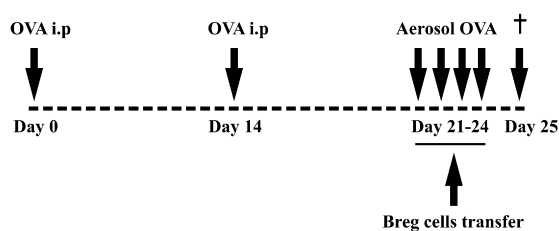
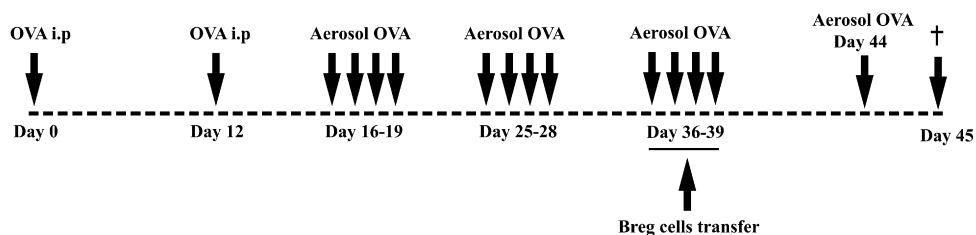
A Acute model**B** Established model

FIG E1. Ovalbumin(OVA) sensitization protocol for acute and established airway inflammation. **A**, Acute sensitization model: mice were sensitized with 20 μ g OVA in alum i.p. on days 0 and 14. Airway challenge with 1% OVA was performed on days 21 to 24, concurrent with transfer of Breg cells. Lung analysis was performed on day 25. **B**, Established model: mice were sensitized with 20 μ g OVA in alum i.p. on days 0 and 12. Mice received airway challenge with 5% OVA on days 16 to 19 and days 25 to 28. On days 36 to 39, mice received Breg cells and 5% OVA aerosol. On day 44, mice received 5 % OVA aerosol challenge. Lung analysis was performed on day 45.

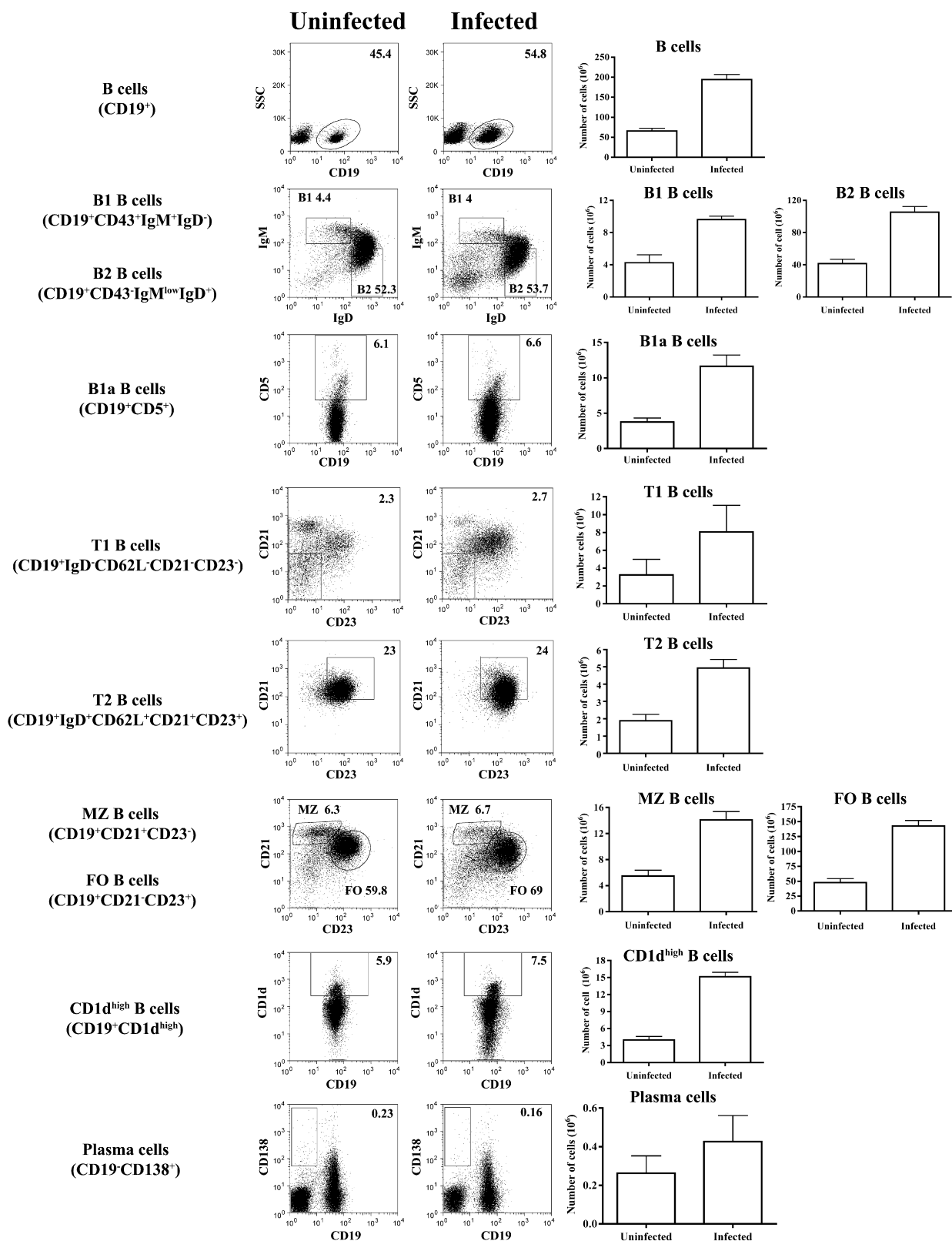


FIG E2. Characterization of different B-cell subpopulations in spleens of uninfected and worm-infected mice. Representative flow-cytometry plots for uninfected and infected mice and the absolute numbers for each subpopulation are shown: B cells (CD19⁺), B1 B cells (CD19⁺IgM⁺CD43⁺IgD⁻), B2 B cells (CD19⁺IgM^{low}IgD⁺CD43⁺), B1a B cells (CD19⁺CD5⁺), T1 B cells (CD19⁺IgD⁻CD62L⁻CD21⁻CD23⁻), T2 B cells (CD19⁺IgD⁺CD62L⁺CD21⁺CD23⁺), MZ B cells (CD19⁺CD21⁺CD23⁻), follicular zone B cells (FO B cells; CD19⁺CD21⁻CD23⁺), CD1d^{high} B cells (CD19⁺CD1d^{high}), and plasma cells (CD19⁺CD138⁺). Cell populations are gated on live cells first and then on CD19⁺ B cells with 20,000 cells shown. Plasma cells are gated on live cells and then CD19⁺ cells.

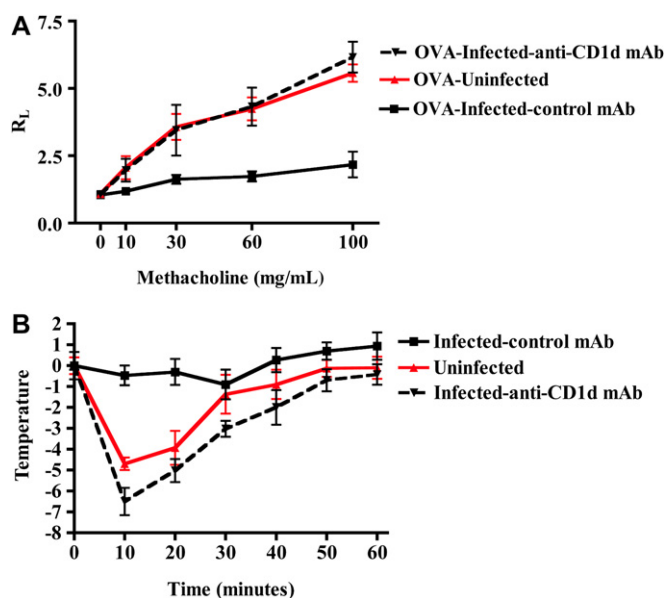


FIG E3. Role for CD1d in *S mansoni*-mediated protection against allergic airway inflammation and anaphylaxis. **A**, Lung resistance (R_L) in response to methacholine of ovalbumin (OVA)-sensitized uninfected mice, and infected mice treated with an anti-CD1d mAb or a control mAb. Data represent the mean $\pm$ SEM change from baseline values for each group. **B**, Drop in temperature in Pen V-sensitized uninfected mice and infected mice treated with an anti-CD1d mAb or a control mAb. Anaphylaxis was induced by i.v. injection of Pen V. Data are mean $\pm$ SEM change in temperature.

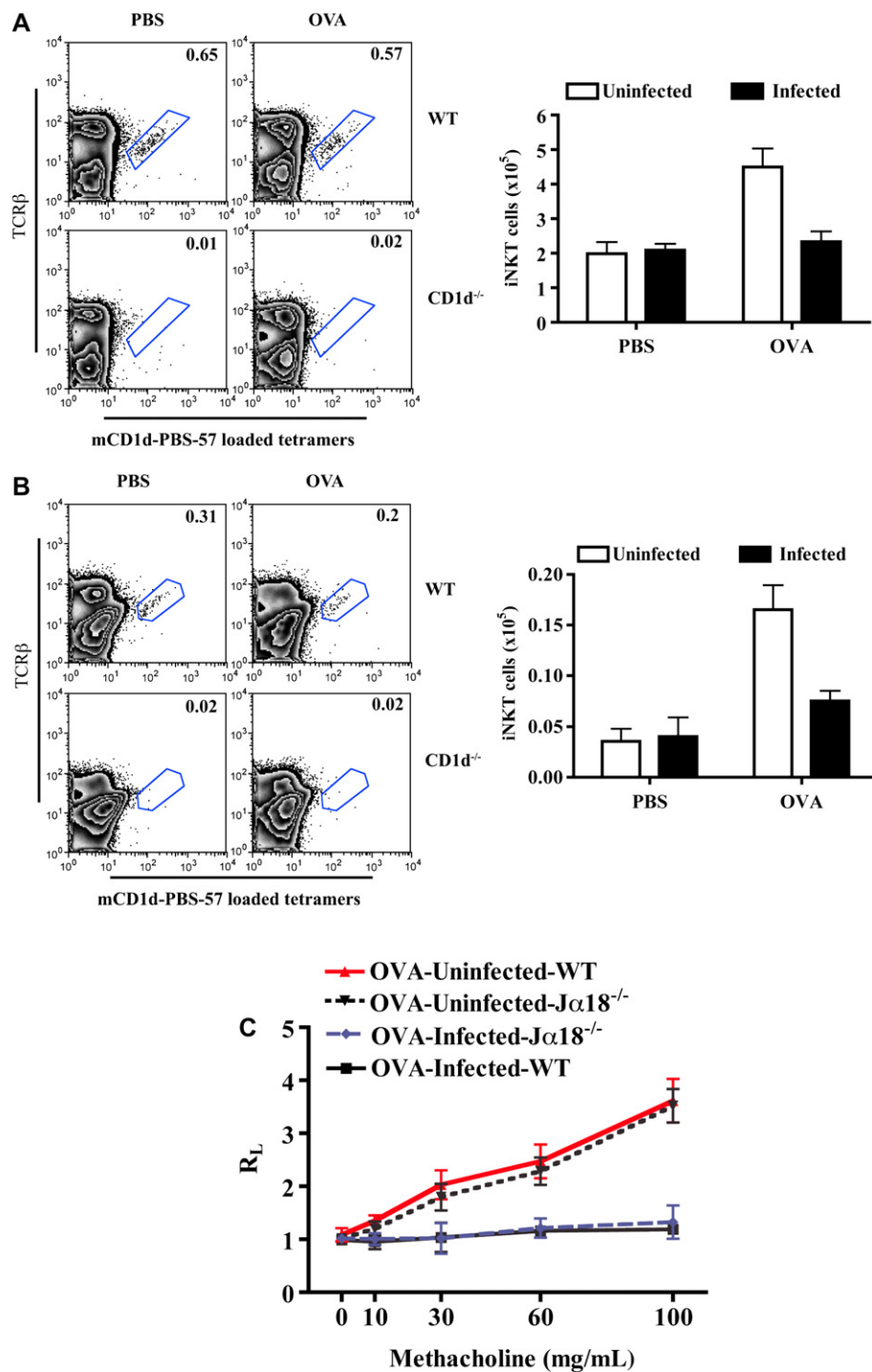


FIG E4. *S. mansoni*-infected mice are refractory to allergic airway inflammation independent of iNKT cells. Flow-cytometry plots and absolute numbers of iNKT cells, identified by staining with mCD1d-PBS056 loaded tetramer and anti-TCR β mAb, in spleens (**A**) and lungs (**B**) of uninfected and infected wild type (WT) and CD1d^{-/-} mice that were sensitized to ovalbumin (OVA) or PBS. **C**, Lung resistance (R_L) in response to methacholine of OVA-sensitized uninfected and infected WT and J α 18^{-/-} mice.

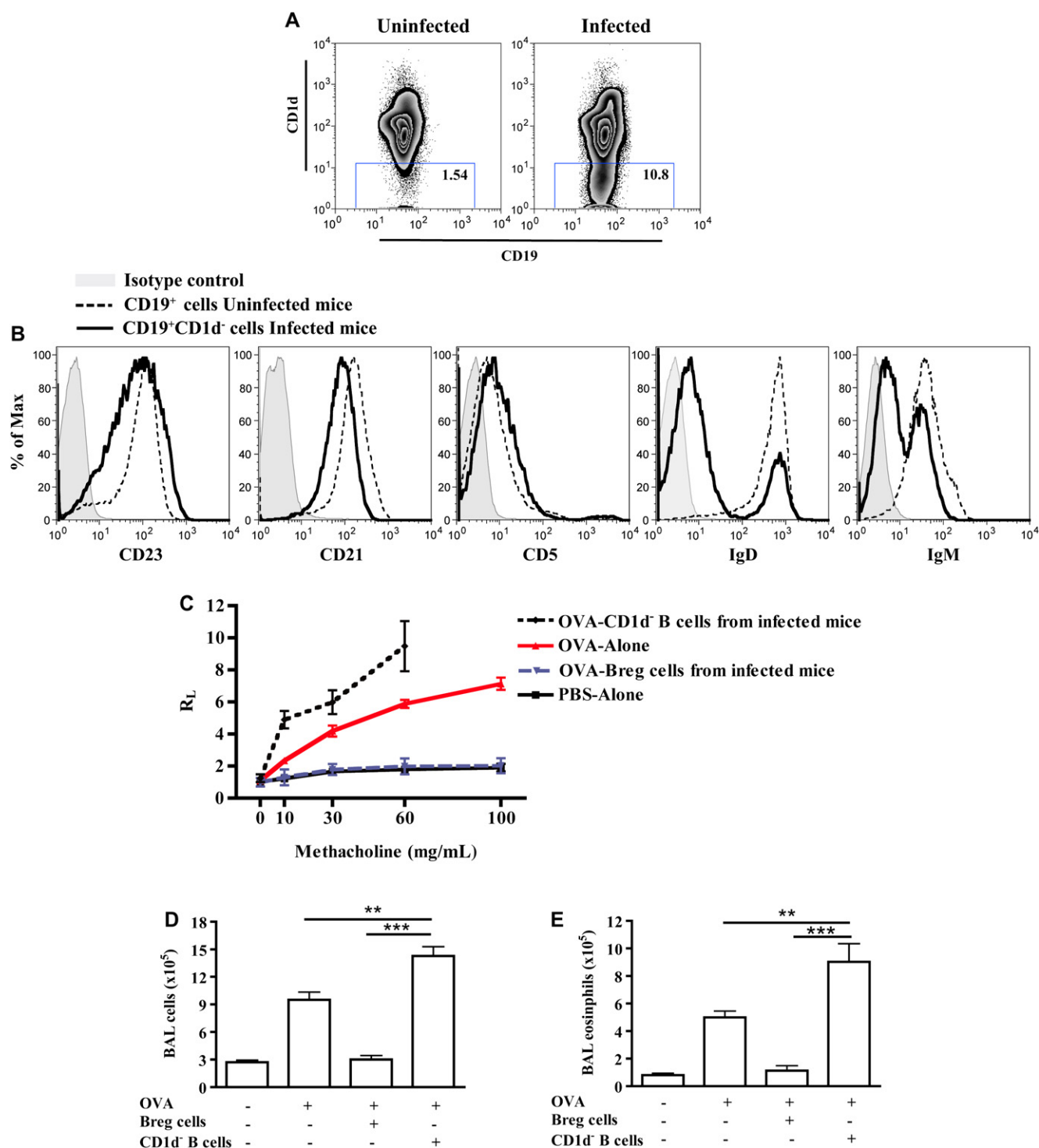


FIG E5. Expansion of a CD1d⁺ B-cell population that exacerbates allergic airway inflammation in the spleens of worm-infected mice. **A**, Representative flow-cytometry images of increased CD1d⁺ B cells in spleen cells from infected mice compared to uninfected mice. **B**, Flow-cytometry histograms showing gated CD1d⁺CD19⁺ B cells from infected mice compared with the whole CD19⁺ population from uninfected mice and isotype control for expression of CD23, CD21, CD5, IgD, and IgM. **C**, Lung resistance (R_L) in response to methacholine of ovalbumin (OVA)-sensitized uninfected mice transferred Breg cells or CD1d⁺ B cells sorted from the spleens of infected mice. Recipients of CD1d⁺ B cells died from 30 to 60 mg/mL methacholine exposure. Total cell (**D**) and eosinophil (**E**) counts in BAL from mice treated as described. Data are the means $\pm$ SEMs. The Student *t* test was used to test for statistical difference between groups as indicated: ***P* < .01; ****P* < .001. Max, Maximum.

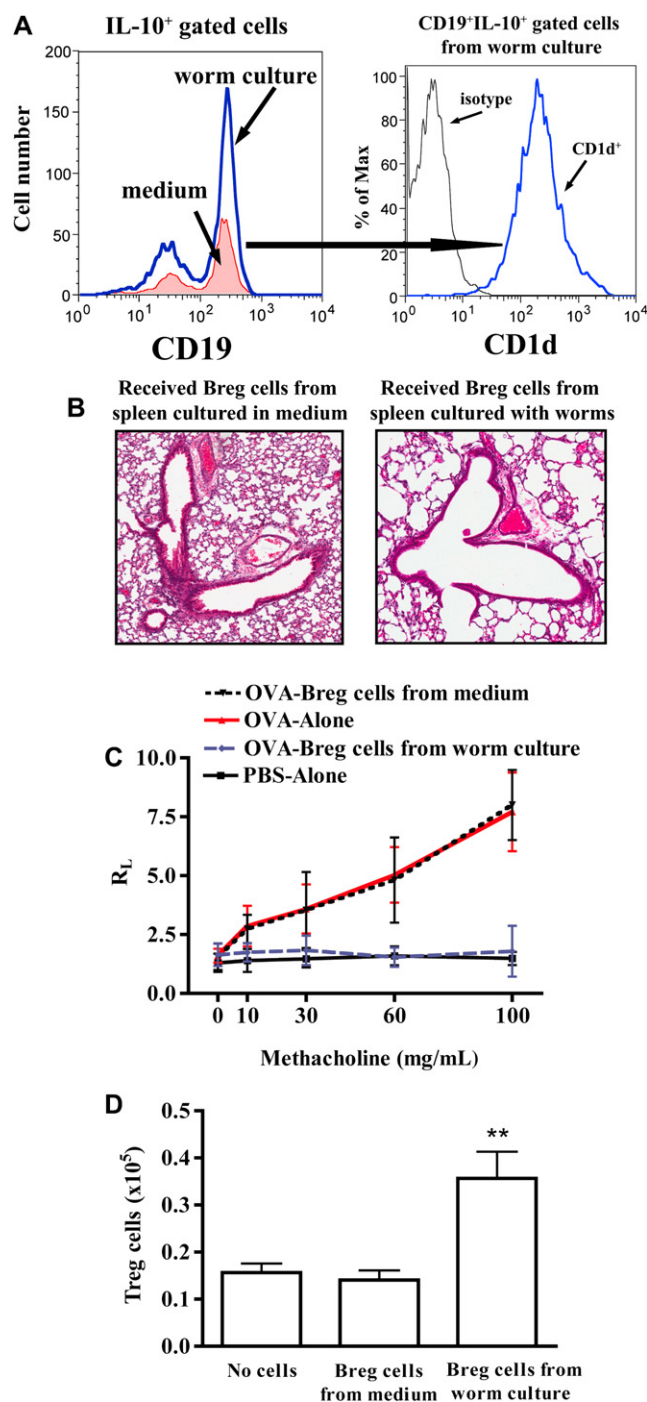


FIG E6. Breg cells generated *ex vivo* by worms mediate protection against allergic airway inflammation and induce Treg cells *in vivo*. Spleen cells from uninfected mice were cultured for 48 hours with 5 worms or in medium. **A**, Spleen cells from IL-10-eGFP mice cultured in media or with worms for flow cytometry. After culture, the IL-10⁺-eGFP spleen cells were gated and the numbers of CD19⁺ B cells plotted. CD19⁺IL-10⁺ B cells from worm cultures (blue histogram) or media (red histogram) are shown. CD19⁺IL-10⁺ cells induced by worms express CD1d (blue histogram); isotype controls (black histogram). **B**, Representative hematoxylin and eosin-stained sections of lungs from ovalbumin (OVA)-sensitized mice that received *ex vivo* generated Breg cells. **C**, Lung resistance (R_L) in response to methacholine of OVA-primed mice receiving *ex vivo* generated Breg cells. Untreated mice (PBS-Alone) or OVA-sensitized mice (OVA-Alone) were used as controls. **D**, Total number of Treg cells (CD4⁺CD25⁺Foxp3⁺) detected in BAL of mice sensitized with OVA and receiving no cells, Breg

cells from medium cultures, and *ex vivo* worm-generated Breg cells. The Student *t* test was used to test for statistical difference between groups as indicated: ***P* < .01.