

Critical roles of regulatory B and T cells in helminth parasite-induced protection against allergic airway inflammation

X. Gao,^{*†} X. Ren,[‡] Q. Wang,^{*}
Z. Yang,[‡] Y. Li,[§] Z. Su,[¶] and J. Li[‡]

^{*}State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, [†]University of Chinese Academy of Sciences, Beijing, [‡]Department of Allergy and Clinical Immunology, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, and [§]Department of Public Health, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

Accepted for publication 7 August 2019

Correspondence: J. Li, Department of Allergy and Clinical Immunology, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, 151 Yanjiang Road, Guangzhou, 510120, China.

E-mail: lijing@gird.cn

Z. Su, State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Road, Guangzhou 510530, China.

E-mail: su_zhong@gibh.ac.cn

Summary

The prevalence of allergic asthma and incidences of helminth infections in humans are inversely correlated. Although experimental studies have established the causal relation between parasite infection and allergic asthma, the mechanism of the parasite-associated immunomodulation is not fully elucidated. Using a murine model of asthma and nematode parasite *Heligmosomoides polygyrus*, we investigated the roles of regulatory B cells (B_{reg}) and T cells (T_{reg}) in mediation of the protection against allergic asthma by parasite. *H. polygyrus* infection significantly suppressed ovalbumin (OVA)-induced allergic airway inflammation (AAI) evidenced by alleviated lung histopathology and reduced numbers of bronchoalveolar inflammatory cell infiltration, and induced significant responses of interleukin (IL)- $10^{+} B_{reg}$, IL- $10^{+} T_{reg}$ and forkhead box protein 3 (FoxP3) $^{+} T_{reg}$ in mesenteric lymph node and spleen of the mice. Adoptive transfer of IL- $10^{+} B_{reg}$ and IL- $10^{+} T_{reg}$ cell prevented the lung immunopathology in AAI mice. Depletion of FoxP3 $^{+} T_{reg}$ cells in FoxP3-diphtheria toxin (DT) receptor transgenic mice by diphtheria toxin (DT) treatment exacerbated airway inflammation in parasite-free AAI mice and partially abrogated the parasite-induced protection against AAI. IL- $10^{+} B_{reg}$ cells were able to promote IL- $10^{+} T_{reg}$ expansion and maintain FoxP3 $^{+} T_{reg}$ cell population. These two types of T_{reg} s failed to induce CD19 $^{+} B$ cells to transform into IL- $10^{+} B_{reg}$ cells. These results demonstrate that B_{reg} , IL- $10^{+} T_{reg}$ and FoxP3 $^{+} T_{reg}$ cells contribute in a discrepant manner to the protection against allergic airway immunopathology by parasite. B_{reg} cell might be a key upstream regulatory cell that induces IL- $10^{+} T_{reg}$ response and supports FoxP3 $^{+} T_{reg}$ cell population which, in turn, mediate the parasite-imposed immunosuppression of allergic airway inflammation. These results provide insight into the immunological relationship between parasite infection and allergic asthma.

Keywords: asthma, immunoregulation, parasite, regulatory B cell, regulatory T cell

Introduction

Asthma and allergic diseases have become increasingly prevalent during the past few decades in children in developed countries and in populations undergoing urbanization [1]. Analysis of the epidemiological data reveals that the increased prevalence of asthma in developed countries is associated with the decreased frequencies of microbial infections during the same period of time [2–4],

suggesting that microbial infections modulate the host immune response to protect against the immunopathologies. Helminth parasites are known to modulate the host's immune response and induce immunosuppression [5]. Human studies and meta-analyses on epidemiological data provide evidence to support the inverse association between helminth infection and asthma and other atopic symptoms [6–8]. Laboratory animal models offer useful tools for

study of the immunological relationship between parasite infection and asthma. Acute and chronic phases of *Trichinella spiralis* infection suppress ovalbumin (OVA)-induced allergic airway inflammation (AAI) [9]. Infection of mice with intestinal nematode parasite *Heligmosomoides polygyrus* suppressed allergen-induced airway eosinophilia and bronchial hyper-reactivity [10,11]. Similar protection against allergen-induced airway inflammation and hyper-responsiveness is also observed in mice infected with the male *Schistosoma mansoni* parasite [12]. These studies with animal models provide direct evidence for the causal relationship of helminth infection and allergic asthma.

Allergic asthma is a T helper type 2 (Th2)-driving immunopathology in the respiratory system [13]. The observations that infection with Th2 response-inducing helminth prevents or alleviates the asthma manifestation suggest that immunoregulatory mechanisms, other than Th1/Th2 reciprocal suppression, might be involved in protection against asthma by parasites. Nematode parasite infections in mice induced interleukin (IL)-10 production, and protection against OVA-induced AAI by parasite was abrogated in IL-10-deficient mice [10], indicating that the protective effect is IL-10-dependent. Adoptive transfer of CD4⁺CD25⁺forkhead box P3 (FoxP3)⁺ regulatory T cells from parasite-infected mice conferred protection against the OVA-induced inflammation in the lung [9–11,14]. A study by Wilson *et al.* showed that CD19⁺ B cells from *H. polygyrus*-infected IL-10-deficient C57BL/6 mice were able to suppress allergen-induced airway eosinophilia, and the suppressive effect was not observed in the B cells from uninfected donor mice [15]. These findings demonstrate that multiple factors of the host's immune regulatory arm are involved in the modulation of allergic immunopathology by helminth parasites.

In the present study, we used the murine gastrointestinal parasite *H. polygyrus* and OVA-induced AAI asthma model to investigate the immunological relationship between helminth parasite infection and allergic asthma. In agreement with the findings by others, *H. polygyris* infection strongly ameliorates the inflammatory pathology in AAI animals. More importantly, IL-10⁺CD19⁺ regulatory B cells (B_{reg}), IL-10⁺CD4⁺ regulatory T cells (IL-10⁺ T_{reg}) and FoxP3⁺CD4⁺ regulatory T cells (FoxP3⁺T_{reg}) induced in the gut-associated lymphoid tissue during parasite infection contribute, in a sequential manner, to the regulation of allergic inflammatory response of lung by intestinal parasite infection.

Materials and methods

Animals and parasites

Female C57BL/6 mice, 6–8 weeks old, were purchased from Vital River Laboratories (Beijing, China). IL-10 green fluorescent protein (GFP) reporter mice [16] and Depletion

of Regulatory T cells (DEREG) mice, that co-express diphtheria toxin (DT) receptor and GFP protein under the control of the *foxp3* gene locus [17], were obtained from Jackson Laboratory (Bar Harbor, ME, USA). *H. polygyrus* parasites were maintained in the laboratory via passage in BALB/c mice. For *H. polygyrus* infection, mice were given 300 third-stage larvae (L3) by oral gavage. *H. polygyrus* antigen was prepared following the procedure described previously [18]. Briefly, 3 weeks after infection with 500 *H. polygyrus* L3, adult worms were collected from the small intestine of mice and washed extensively with sterile phosphate-buffered saline (PBS) containing antibiotics. The adult worms were homogenized with a glass tissue grinder, and the homogenate was centrifuged (3000 g, 10 min). The soluble fraction of the homogenate was collected, the protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and the material was stored at –20°C. Mice were housed in the specific pathogen-free animal facility of the Guangzhou Institutes of Biomedicine and Health. All experiments were carried out in accordance with the National Animal Protection Guidelines of China and approved by the Institutional Animal Care and Use Committee.

Induction of allergic airway inflammation (AAI)

AAI was induced in mice using the OVA sensitizing/challenging protocol [19,20], with some modifications. Briefly, groups of mice were sensitized on days 0 and 7 by intraperitoneal (i.p.) administration of 25 µg OVA (Sigma-Aldrich, St Louis, MO, USA) with 2 mg aluminum hydroxide (Thermo Scientific, Rockford, IL, USA) in 100 µl PBS. Mice were challenged with aerosolized OVA (1% solution, 30 min) three times on days 14, 15 and 16, and euthanized on day 18. To determine the effect of parasite infection on AAI, mice were infected with 300 *H. polygyrus* L3s 14 days before the first OVA-sensitization.

Bronchoalveolar lavage (BAL) cell analysis

Forty-eight hours after final challenge, mice were anesthetized, the tracheas were cannulated and airspaces of lung were lavaged with 600 µl PBS, followed by two 600 µl washes. BAL cell suspensions were centrifuged at 300 g and pellets were collected and suspended in medium. BAL fluids from the first lavage were collected analysis of cytokines by enzyme-linked immunosorbent assay (ELISA). Cell counts were performed on individual samples. In some experiments, lung-draining lymph nodes [21] were collected, a single-cell suspension was made and cell numbers were determined. To differentiate the cell types, BAL cells were stained with anti-CD11c-allophycocyanin (APC) (clone N418; eBioscience, San Diego, CA, USA) and anti-Siglec-F-phycoerythrin (PE)

(clone E50-2440BD; BD Biosciences, San Jose, CA, USA) fluorescent monoclonal antibodies (mAbs). Alveolar macrophage was defined as CD11c⁺SiglecF⁺ and eosinophils as SiglecF⁺CD11c⁻ [22,23]. Flow cytometry analysis was performed on a fluorescence activator cell sorting (FACS) Calibur™ (BD Biosciences).

Histopathology

The mice were euthanized and lungs were removed, fixed in a 4% formaldehyde solution for 48 h and embedded in paraffin. Tissue sections (3 µm thick) were prepared and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich) following the standard procedures. Alcian blue periodic acid-Schiff (AB-PAS) staining was also performed on sections to identify mucus-containing goblet cells.

Cell culture

Spleens and mesenteric lymph nodes (MLN) single-cell suspensions were prepared in RPMI-1640 supplemented with 25 mM HEPES, 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 200 µg/ml penicillin and 200 U/ml streptomycin (HyClone) (complete medium). Red blood cells (RBCs) were lysed with 0.175 M NH₄Cl. Cell viability was determined by trypan blue (Invitrogen) exclusion and was always >95%. For cell culture, 0.5 ml cell suspensions in complete medium (1 × 10⁷ cells/ml) were cultured in 48-well plates (Greiner Bio-One, Frickenhausen, Germany) with OVA (20 µg/ml) or *H. polygyrus* antigen (20 µg/ml) and incubated for 48 h at 37°C in a humidified CO₂ incubator. Supernatants were collected and the cytokine levels were determined by ELISA. In some experiments, spleen and MLN cells were stained with fluorescent mAb and sorted for adoptive transfer.

For cell co-culture, spleen cell suspensions were made from IL-10-GFP and DERE mice. Cells were stained with anti-CD4-PE (clone GK1.5; eBioscience) or anti-CD19-APC (clone 1D3; eBioscience) mAbs and sorted on FACS Aria II U (BD Biosciences) to collect IL-10⁺ T_{reg}, FoxP3⁺ T_{reg} or IL-10⁺ B_{reg} cells. Purified target (2 × 10⁵) and effector cells (2 × 10⁵) in 0.2 ml complete medium were co-cultured in U-bottomed 96-well plates for 48 h. The cultured cells were harvested, stained for surface markers and analyzed by flow cytometry.

Cytokine and antibody ELISA

Levels of cytokines in sera, BAL fluids and cell culture supernatants and total IgE in sera were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Cytokine and antibody levels in samples were calculated by linear regression against the standards.

Transfer of IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells

Spleens were collected from *H. polygyrus*-infected or uninfected IL-10-GFP reporter mice and single-cell suspension was made as described in the previous section. Total B or T cells were first enriched by negative selection using magnetic bead columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched B and T cells were stained with anti-CD19-APC or anti-CD4-PE antibodies, respectively, sorted and CD19⁺GFP⁺ B cells (B_{reg}) and CD4⁺GFP⁺ T cells (IL-10⁺ T_{reg}) were collected. The cells were washed with PBS twice and aliquots of 0.1 ml cell suspension (5 × 10⁵) were injected to mice intravenously. To examine the *in-vivo* induction of IL-10⁺ T_{regs} and FoxP3⁺ T_{regs} by B_{reg}, the sorted IL-10⁺ B_{reg} cells and IL-10⁻ B cells (3 × 10⁶ in 0.1 ml) were injected to mice i.v. The recipient mice were euthanized 48 h later, the spleen cells were stained and the frequencies of IL-10⁺ T_{reg} and FoxP3⁺ T_{reg} cells in CD4⁺ T cell population were determined by flow cytometry.

In vivo depletion of FoxP3⁺ T_{reg} cells in DERE mice

To deplete FoxP3⁺ T_{reg} cells in DERE mice, 1 µg of DT (Merck Millipore, Darmstadt, Germany) was injected to mice i.p. four times during the experiment. Depletion efficiency was evaluated by examination of CD4⁺ FoxP3⁺ cells in spleen cells from the DT-treated mice by flow cytometry 24 h after the final DT injection.

Statistical analysis

All experiments were performed two to three times and each experimental group consists of three to five mice, as indicated in the figure legends. Data were shown as means [± standard error of the mean (s.e.m.)]. Statistical analysis was performed using GraphPad Prism (GraphPad software Inc., San Diego, CA, USA). Statistical significance of the differences between experimental groups was determined by two-tailed Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Results

Suppression of airway inflammation and Th2 immune response by *H. polygyrus* infection

To test whether the intestinal parasite infection modulates the airway inflammation induced by OVA, a group of naive mice and a group of *H. polygyrus*-infected mice (day 14 of infection) were treated with OVA to induce AAI. Naive untreated and *H. polygyrus*-infected mice were included as control groups. Two days after the final OVA challenge, lung histology and cellular infiltration were examined (Supporting information, Fig. S1). Examination

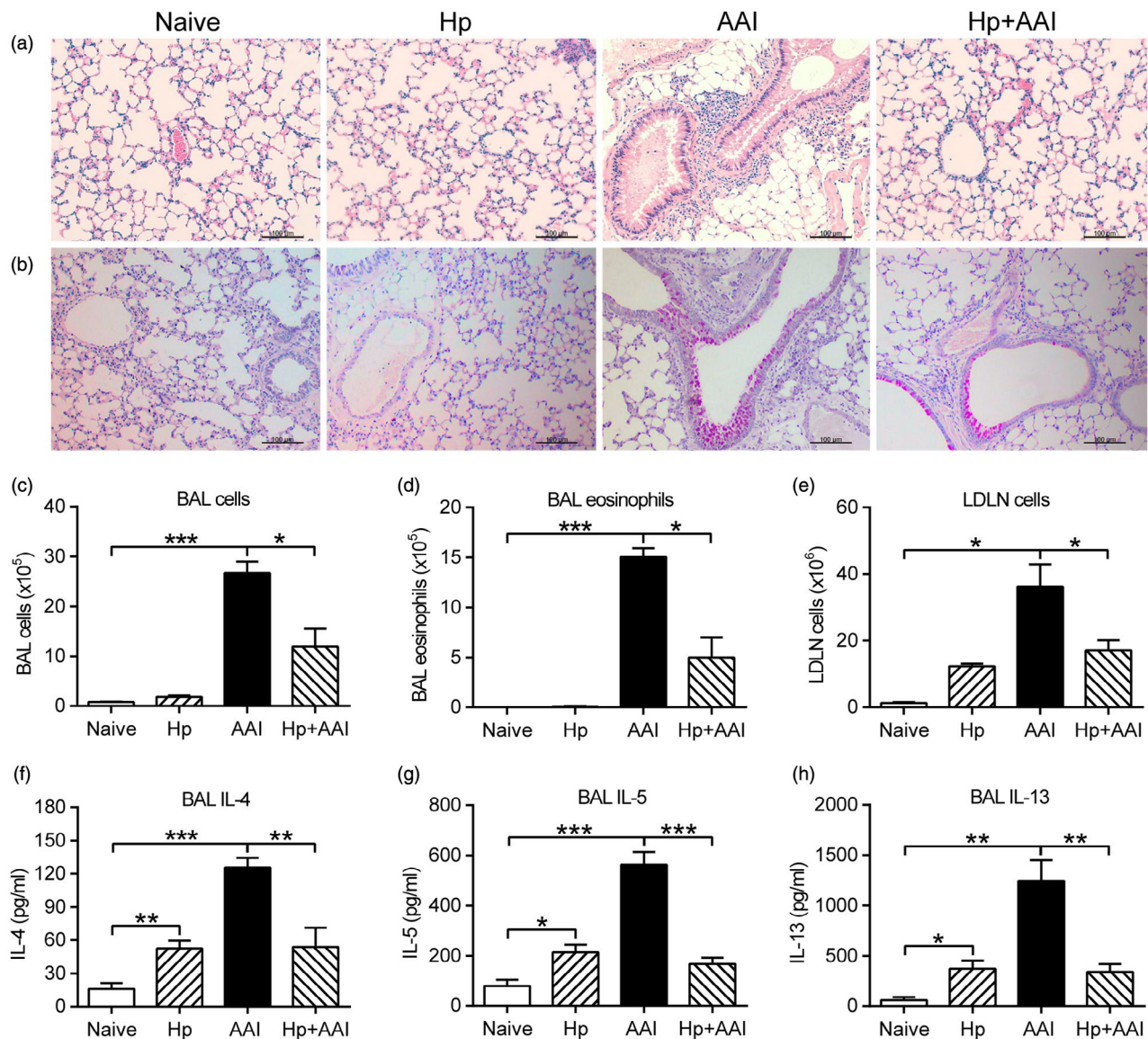


Fig. 1. Suppression of allergic airway inflammation (AAI) and T helper type 2 (Th2) cytokine responses by *Heligmosomoides polygyrus*. Four groups of C57BL/6 mice were treated following the experimental protocol as described in Supporting information, Fig. S1 and euthanized at day 18. (a) Photomicrographs of lung sections stained with hematoxylin and eosin (H&E). (b) Photomicrographs of lung sections stained with Alcian blue periodic acid-Schiff (AB-PAS) showing mucus-containing goblet cells. Scale bar = 100 μ m. (c) Total number of bronchoalveolar lavage (BAL) cells. (d) Total number of eosinophils in BAL determined by percentage of CD11c⁺Siglec-F⁺ cells in total BAL cells. (e) Total number of lung-draining lymph nodes cells. (f–h) Interleukin (IL)-4, IL-5 and IL-13 levels in BAL fluid determined by enzyme-linked immunosorbent assay (ELISA). Results presented are from one of three experiments. Data are means $\pm$ standard error of the mean (s.e.m.) of three to four mice per group. Statistical significances are indicated by * P < 0.05, ** P < 0.01, *** P < 0.001.

of H&E-stained lung sections showed that parasite infection did not result in apparent histological changes in lung compared to that of naive animals. Lung sections from AAI mice showed considerable cellular infiltrates. As observed in the previously reported studies [10–12], AAI mice pre-infected with *H. polygyrus* showed significantly reduced lung histopathology compared to the AAI animals (Fig. 1a). AB-PAS staining of lung sections revealed

a severe hyperplasia of goblet cells and mucus production in bronchi of AAI mice. However, these histopathological changes were suppressed in *H. polygyrus*-infected AAI mice (Fig. 1b).

Analysis of BAL cells also revealed substantial cellular infiltrates into lung tissue of AAI mice. In parasite-infected AAI mice, however, the cellular influx was significantly reduced compared with that of AAI mice (P < 0.05)

(Fig. 1c). Compared with the naive mice, the frequency and number of eosinophils were significantly increased in AAI mice, and *H. polygyrus* infection suppressed eosinophil response (Fig. 1d, Supporting information, Fig. S2). Lung-draining lymph nodes showed increased cellularity in AAI mice, and this response was suppressed in *H. polygyrus*-infected AAI mice (Fig. 1e). We also observed that *H. polygyrus* infection given 3 or 4 weeks before OVA sensitization was able to suppress the AAI immunopathologies (data not shown).

BAL fluids were collected and analyzed by ELISA to determine the cytokine levels. BAL fluid from *H. polygyrus*-infected mice showed increased levels of IL-4 ($P < 0.01$), IL-5 and IL-13 compared to the samples from naive mice (both $P < 0.05$). BAL fluids from AAI mice contained high levels of these Th2 cytokines. BAL samples from *H. polygyrus*-infected AAI mice showed significantly reduced IL-4, IL-5 and IL-13 levels ($P < 0.01$ and $P < 0.001$) (Fig. 1f–h). Cytokine production *in vitro* by spleen cells showed different patterns from that of BAL fluids. Spleen cells from parasite-infected mice and parasite-infected AAI mice produced significantly higher levels of IL-4 and IL-5 than the cells from AAI mice (Supporting information, Fig. S3). IL-13 was undetectable in the supernatants of spleen cell culture.

Responses of IL-10 cytokine and IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells to *H. polygyrus* infection

The observation that Th2-inducing *H. polygyrus* infection suppressed Th2-driven AAI suggests that other immunoregulatory factors may be involved in modulation of the AAI by the parasite. We then examined IL-10 production in sera of *H. polygyrus*-infected and found that IL-10 levels in serum increased significantly 1 and 2 weeks following infection, and declined to lower levels 3 weeks after infection (Fig. 2a). In addition, BAL fluids and cell culture supernatants of spleen cells from parasite-infected AAI mice stimulated *in vitro* with OVA and parasite antigen showed higher levels of IL-10 than that from AAI mice ($P < 0.01$ and 0.05) (Fig. 2b,c).

To determine the cellular sources of IL-10, we analyzed the frequency of CD19⁺IL-10⁺ B_{reg} cells in MLN and spleen of the mice following *H. polygyrus* infection. The frequency of IL-10⁺CD19⁺ B_{reg} cells was increased significantly in MLN of the infected mice and peak level occurred 1 week following infection ($P < 0.001$ versus day 0) (Fig. 2d). Parasite infection also induced increased frequency of IL-10⁺CD4⁺T_{reg} cells that peaked 1 week after *H. polygyrus* infection ($P < 0.001$ versus day 0) (Fig. 2g). We also examined the absolute numbers of IL-10⁺ B_{reg} and IL-10⁺T_{reg} cells in MLN during the course of *H. polygyrus* infection and observed that peak numbers of IL-10⁺ B_{reg} cells occurred 2 weeks after

infection, but the number of IL-10⁺ T_{reg} cells peaked 3 weeks post-infection ($P < 0.001$ versus day 0) (Fig. 2e,h). The frequencies of IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells in spleen also increased and peak levels were detected 2 weeks after parasite infection ($P < 0.001$ versus day 0) (Fig. 2f,i).

Suppression of AAI by transferred IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells

The increase in frequencies and numbers of IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells in *H. polygyrus*-infected mice suggests that these immunoregulatory cells may play roles in modulation of OVA-induced AAI. To confirm the functional association between the B_{reg} and IL-10⁺ T_{reg} cells and the protection against allergic inflammation in the lung, IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells, as well as their IL-10⁻ counterparts, were purified from spleens of *H. polygyrus*-infected IL-10-GFP mice and transferred to mice 1 day before OVA sensitization. In comparison with the AAI control group, mice receiving IL-10⁺ B_{reg} cells showed reduced cell infiltration in the lung, significantly less numbers of total BAL cells ($P < 0.05$) and eosinophils ($P < 0.05$) and reduced serum levels of IgE ($P < 0.01$). The mice receiving IL-10⁻ B cells showed lung cellular infiltrates, the numbers of BAL cells and eosinophil and IgE production that were comparable to those of AAI animals (Fig. 3a–c, Supporting information, Fig. S4a). Adoptive transfer of IL-10⁺ T_{reg} cells also suppressed lung immunopathology, as shown by lung histology sections, BAL total cell and eosinophil numbers and serum IgE levels (all $P < 0.01$), but this protective effect was not seen in transfer of IL-10⁻ T cells (Fig. 3d–f, Supporting information, Fig. S4b). Transfer of IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells from uninfected mice to AAI mice were also able to suppress the AAI immunopathology in recipient mice (Supporting information, Fig. S5a–d).

Interaction between IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells

Both IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells showed protective roles against OVA-induced AAI in the adoptive transfer experiments, but the relationship between the two types of immunoregulatory cell is not known. To address this question, *in-vitro* cell co-culture experiments were performed to analyze their interaction. Effector IL-10⁺ B_{reg} and IL-10⁻ B cells were isolated from IL-10-GFP mice and co-cultured with target CD4⁺ T cells. CD4⁺ T cells co-cultured with IL-10⁺ B_{reg} showed significantly increased frequency of IL-10⁺ T_{reg} cells and the increase of IL-10⁺ T_{reg} cell frequency showed a dose-response to the increasing effector : target ratios. CD4⁺ T cells co-cultured with IL-10⁻ B cells also showed an increase in frequency of IL-10⁺ T_{reg} cells compared with CD4⁺ T cells cultured alone ($P < 0.05$), but the response was lower than the CD4⁺T cells co-cultured with IL-10⁺

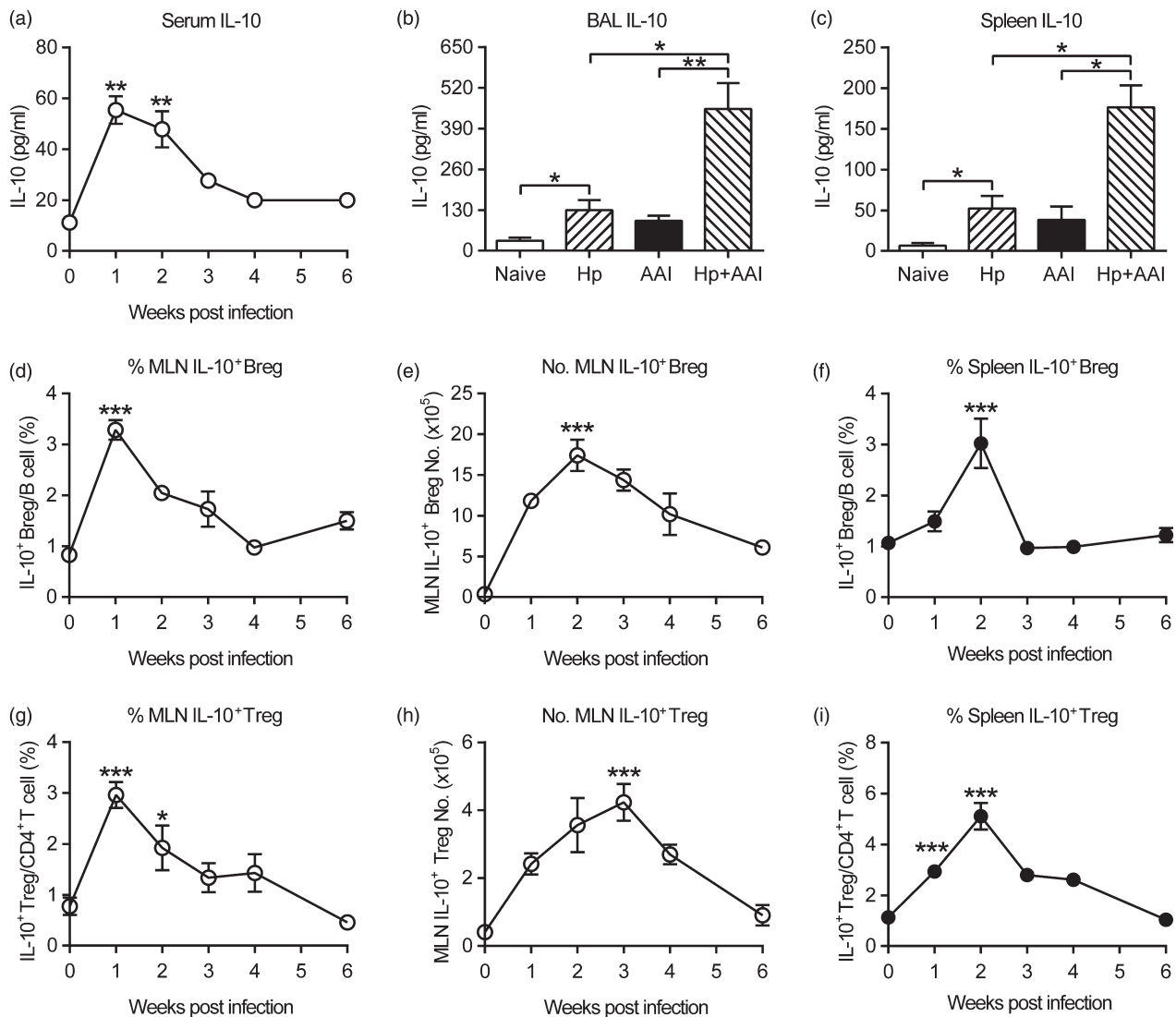


Fig. 2. Responses of interleukin (IL)-10 cytokine in serum and IL-10⁺ regulatory B cells (B_{reg}) and IL-10⁺ regulatory T cells (T_{reg}) in mesenteric lymph node (MLN) and spleen following *Heligmosomoides polygyrus* infection. (a). Serum levels of IL-10. (b). IL-10 levels in bronchoalveolar lavage (BAL) fluids of the four groups of mice. (c). Levels of IL-10 produced by spleen cells *in vitro*. Spleen cells from four groups of mice were cultured in medium (naive group), in the presence of *H. polygyrus* antigen (HpAAI) (20 µg/ml, Hp group), ovalbumin (OVA) [20 µg/ml, allergic airway inflammation (AAI) group] or Hp antigen and OVA (Hp+AAI group). Cell culture supernatants were harvested and IL-10 level was determined by enzyme-linked immunosorbent assay (ELISA). (d). Percentage of IL-10⁺ B_{reg} cells in CD19⁺ B cells in mesenteric lymph nodes (MLN). (e) Number of IL-10⁺ B_{reg} cells in MLN. (f) Percentage of IL-10⁺ B_{reg} cells in CD19⁺ B cells in spleen. (g). Percentage of IL-10⁺ T_{reg} cells in CD4⁺ T cells in MLN. (h). Number of IL-10⁺ T_{reg} cells in MLN. (i). Percentage of IL-10⁺ T_{reg} cells in CD4⁺ T cells in spleen. Results from one of two experiments are shown. Data are means ± standard error of the mean (s.e.m.) of three to five mice per group. Statistical significances are indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001. In (a,d–g), statistical significance of the differences *versus* day 0 are indicated.

B_{regs} (Fig. 4a). When this co-culture assay was performed in Transwell culture plates, promotion of IL-10⁺ T_{reg} cell proliferation by IL-10⁺ B_{reg} cells was abrogated (Fig. 4b). We then determined whether IL-10⁺ T_{reg} cells were able to promote IL-10⁺ B_{reg} cell differentiation, and observed that B cells co-cultured with IL-10⁺ T_{reg} cells or IL-10⁺ CD4⁺ T cells showed no significant change in proportion of IL-10⁺

B_{reg} cells (Fig. 4c). Further IL-10⁺ B_{reg} cell transfer experiment was performed to determine whether the B_{reg} cells were able to promote IL-10⁺ T_{reg} cell expansion *in vivo*. CD4⁺ T cells from spleen of mice receiving IL-10⁺ B_{reg} cells showed significantly increased frequency of IL-10⁺ T_{reg} cells in comparison with the mice that received no cells or IL-10⁺ B cells (Fig. 4d).

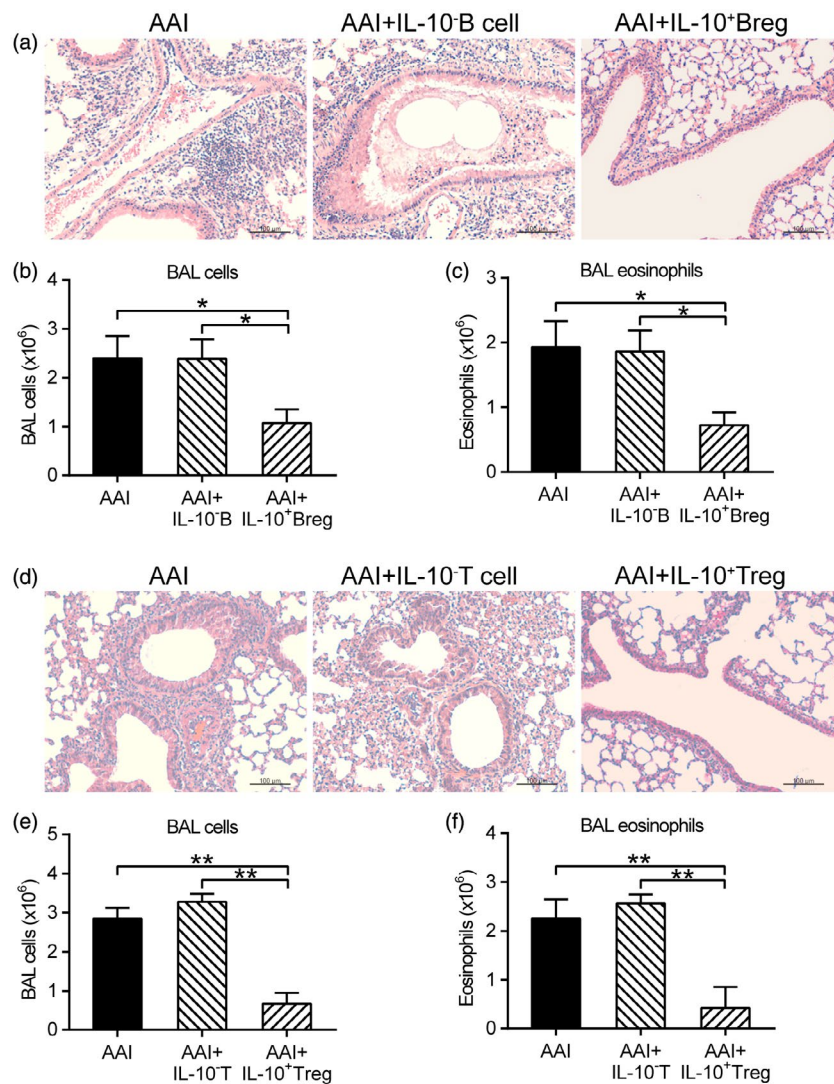


Fig. 3. Suppression of lung immunopathology by adoptively transferred interleukin (IL)-10⁺ regulatory B cells (B_{reg}) and interleukin (IL)-10⁺ regulatory T cells (T_{reg}). (a). Photomicrographs of hematoxylin and eosin (H&E)-stained lung sections from the three groups of allergic airway inflammation (AAI) mice receiving phosphate-buffered saline (PBS), IL-10⁻ B cells or IL-10⁺ B_{reg} cells. (b,c) Total number of bronchoalveolar lavage (BAL) cells (b) and eosinophils (c). (d) Photomicrographs of H&E-stained lung sections from the three groups of AAI mice receiving PBS, IL-10⁻ CD4⁺ T cells or IL-10⁺ T_{reg} cells. (e,f) Total number of BAL cells (e) and eosinophils (f). Results from one of two experiments are presented. Scale bar = 100 μ m. (b,c,e,f) Means $\pm$ standard error of the mean (s.e.m.) of three to four mice per group. Statistical significance is indicated by *P < 0.05, **P < 0.01.

Role of FoxP3⁺ T_{reg} cells in mediation of parasite-induced suppression of AAI

FoxP3⁺ T_{reg} cells are known to play an important role in counteracting inflammation and maintenance of immune homeostasis, but its role in prevention from lung immunopathology is not fully understood. Analysis of the change in frequency of CD4⁺FoxP3⁺ T_{reg} cells revealed that the frequency of FoxP3⁺ T_{reg} cells in total CD4⁺ T cells were increased in MLN and spleen and peaked 2 weeks

following *H. polygyrus* infection ($P < 0.05$ – 0.001 versus day 0) (Fig. 5a,b). In the *in-vitro* cell co-culture assay, we found that CD4⁺ T cells co-cultured with IL-10⁺ B_{reg} cells showed a higher frequency of FoxP3⁺ T_{reg} cells than the CD4⁺ T cells cultured alone or co-cultured with IL-10⁻ B cells (Fig. 5c). However, the FoxP3⁺ T_{reg} cells did not show the ability to induce IL-10⁺ B_{reg} cell proliferation (Fig. 5d). Passive transfer of IL-10⁺ B_{reg} cells did not increase the frequency of FoxP3⁺ T_{reg} cells in spleen CD4⁺ T cells *in vivo* (data not shown).

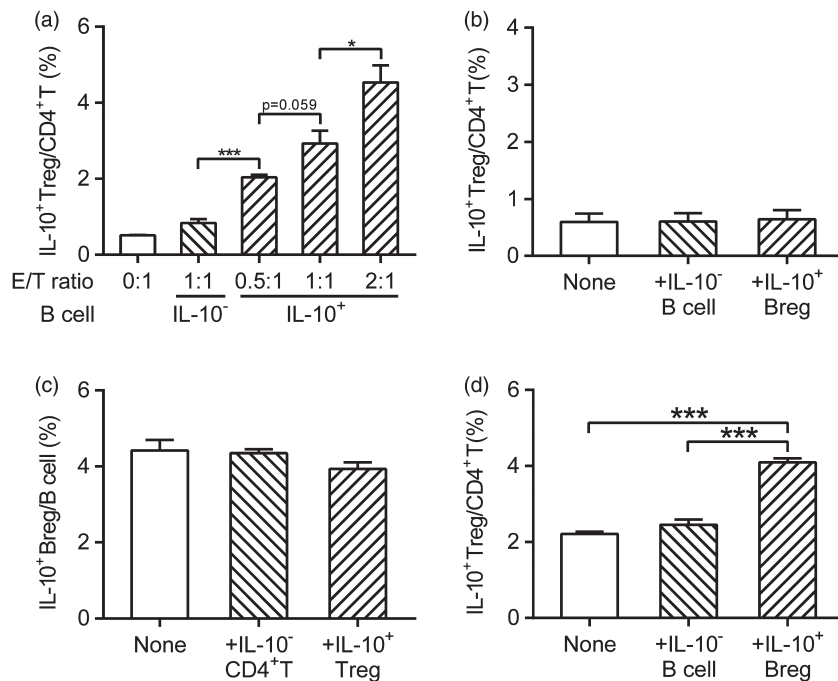


Fig. 4. Analysis of interaction between interleukin (IL)-10⁺ regulatory B cells (B_{reg}) cells and IL-10⁺ regulatory T cells (T_{reg}) cells. (a). CD4⁺ T cells were co-cultured with IL-10⁻ B cells (E : T ratio 1 : 1) or IL-10⁺ B_{reg} cells (E : T ratio 0.5 : 1, 1 : 1, 2 : 1) and percentage of IL-10⁺CD4⁺ T_{reg} cells in CD4⁺ T cells were examined. (b) CD4⁺ T cells were co-cultured with IL-10⁻ B cells or IL-10⁺ B_{reg} cells in Transwell cell culture plates and percentage of IL-10⁺ T_{reg} cells in CD4⁺ T cells were examined. (c). CD19⁺ B cells were co-cultured with IL-10⁻CD4⁺ T cells or IL-10⁺CD4⁺ T_{reg} cells and percentage of IL-10⁺ B_{reg} cells in total CD19⁺ B cells were examined. (d). IL-10⁻CD19⁺ B cells and IL-10⁺CD19⁺ B_{reg} cells were isolated and transferred to naive IL-10 green fluorescent protein (GFP) mice. Frequency of IL-10⁺ T_{reg} cells in total CD4⁺ T cells in spleen was determined 48 h after cell transfer. Results presented are from one of two experiments. Data shown are means ± standard error of the mean (s.e.m.) of four cell culture samples. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01.

The *in-vivo* depletion approach was then used in DEREK mice to determine the role of FoxP3⁺ T_{reg} cells in mediation of suppression of AAI by parasite. In the preliminary experiments, we found that DT treatment of DEREK mice depleted 60–75% FoxP3⁺ T_{reg} cells in spleen (Supporting information, Fig. S6a). Treatment of wild-type naive and AAI C57BL/6 mice with DT had no effect on the BAL cell numbers (Supporting information, Fig. S6b).

Groups of DEREK mice that were either untreated (naive), sensitized and challenged with OVA for induction of AAI (AAI) or infected with *H. polygyrus* prior to induction of AAI (Hp+AAI) were given four treatments of DT before and during OVA sensitization (Supporting information, Fig. S6c). Three corresponding control groups were not treated with DT. As observed in wild-type C57BL/6 mice (Fig. 1), AAI DEREK mice that were not treated with DT developed severe lung cellular infiltrates and showed increased numbers of BAL total cells and eosinophils in comparison with the naive DEREK mice (*P* < 0.01), and Hp+AAI DEREK mice without DT treatment showed alleviated lung histopathology and reduced numbers of total cells and eosinophils in BAL compared with the

DEREG AAI mice (Fig. 5e–g). The AAI DEREK mice that were depleted of the FoxP3⁺ T_{reg} cells by DT treatment showed exacerbated lung histopathology and significantly increased numbers of BAL total cells and eosinophils (*P* < 0.05–0.01) compared with the AAI DEREK mice that were not treated with DT. The Hp+AAI DEREK mice treated with DT showed some lung cellular infiltrates and higher numbers of BAL cells compared with the Hp+AAI DEREK mice that were not treated with DT (*P* < 0.05) (Fig. 5f,g). These results demonstrated that absence of FoxP3⁺ T_{reg} cells resulted in more severe allergic inflammation in both parasite-free and parasite-infected AAI mice.

Discussion

Epidemiological studies have revealed an inverse association between the increased frequency of allergic asthma and reduced prevalence of parasitic infections during the past few decades in the populations of industrialized countries. Laboratory studies with animal models provide firm evidence for the causal relationship between the parasite infection and protection against allergic asthma

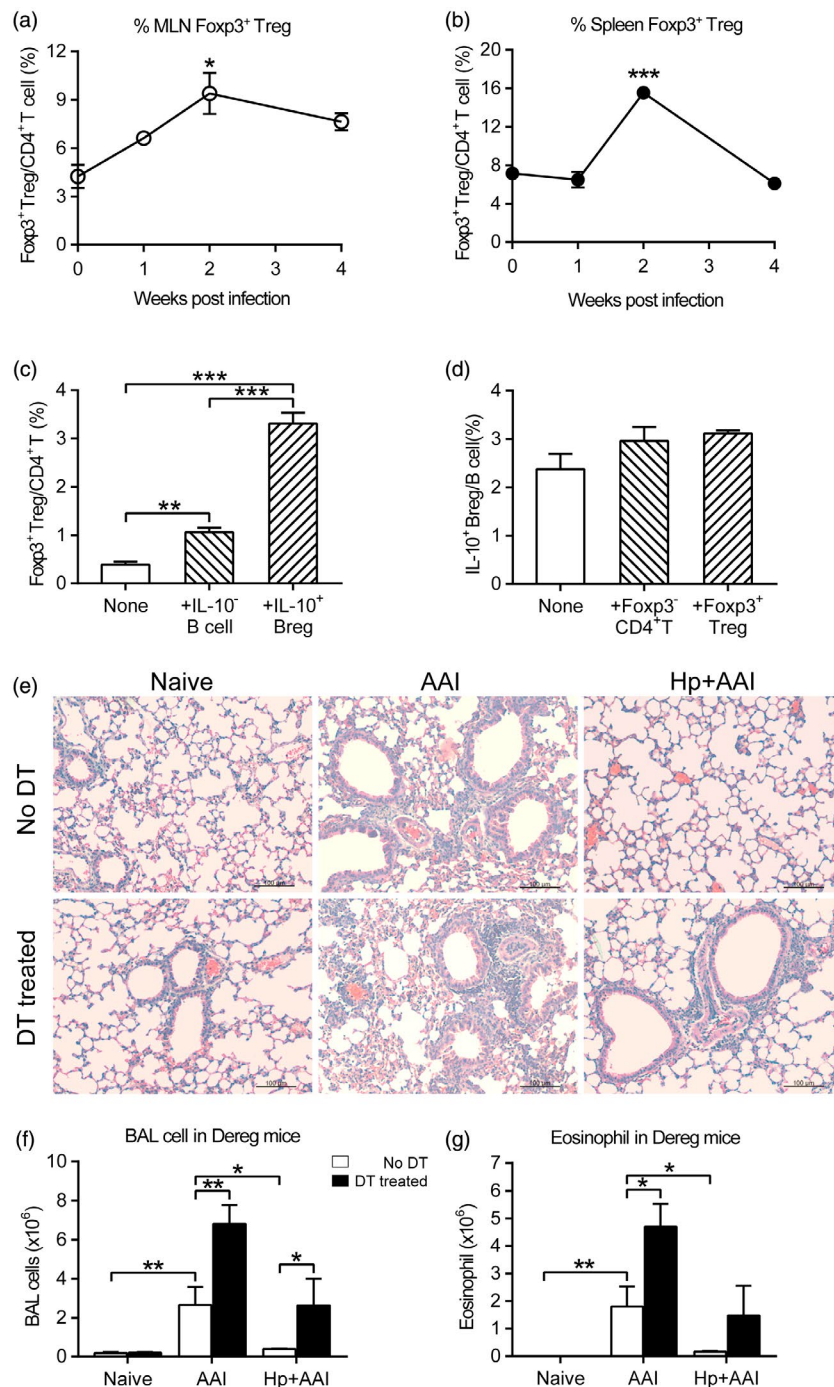


Fig. 5. Roles of forkhead box protein 3 (FoxP3)⁺ regulatory T cells (T_{reg}) cells in mediation of parasite-associated immunosuppression of ovalbumin (OVA)-induced lung immunopathology. (a,b) Response of FoxP3⁺ T_{reg} cells in mesenteric lymph nodes (MLN) (a) and spleen (b) during the course of *Heligmosomoides polygyrus* infection. (c) Stimulation of FoxP3⁺ T_{reg} cell proliferation by interleukin (IL)-10⁻ B cells and IL-10⁺ regulatory B cells (B_{reg}) cells *in vitro*. (d) Stimulation of IL-10⁺ B_{reg} cell proliferation by FoxP3⁺CD4⁺ T cells and FoxP3⁺CD4⁺ T_{reg} cells *in vitro*. (e) Photomicrographs of hematoxylin and eosin (H&E)-stained lung sections of naive, allergic airway inflammation (AAI) or *H. polygyrus* antigen (HpAAI) DERE mice that were either untreated or treated with diphtheria toxin (DT). Scale bar = 100 µm. (f,g) Total number of bronchoalveolar lavage (BAL) cells (f) and eosinophils (g) of naive, AAI or Hp+AAI DERE mice that were either untreated or treated with DT. Results presented are from one of two experiments. Data are means ± standard error of the mean (s.e.m.) of four cell culture samples. Statistical significances are indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (a,b) Statistical significance of the differences *versus* day 0 are indicated.

[24–26]. In this study, we used an OVA-AAI asthma model and *H. polygyrus*, a nematode parasite that dwells in the host's intestine during its entire life cycle, to investigate the immune mechanisms underlying the protection against allergic asthma by concurrent parasite infection. In agreement with findings by others [10,11,15], we observed that *H. polygyrus* infection established before induction of AAI significantly suppressed the lung immunopathology evaluated by pulmonary histopathological changes and infiltration of inflammatory cells (Fig. 1a–d). AAI mice that harbor Th2-inducing *H. polygyrus* infection showed increased levels of immunosuppressive cytokine IL-10 and reduced levels of IL-4, IL-5 and IL-13 in local lung air-space fluid than the parasite-free AAI mice (Figs. 1f–h, Fig. 2b), indicating that immunomodulatory pathways other than Th1/Th2 antagonism may operate to modulate the immunopathology in the local inflammatory site by parasite. Further experiments revealed that *H. polygyrus* infection induced increased level of IL-10 in the periphery, increased frequencies of IL-10-producing B_{reg} cells, IL-10-producing T_{reg} and FoxP3⁺ T_{reg} cells in MLN and spleen (Figs. 2, 5a,b). Passive transfer of the IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells protected the AAI mice from developing severe immunopathology, mimicking the effects of live parasite infection (Fig. 3). Furthermore, depletion of FoxP3⁺ Treg cells *in vivo* in *H. polygyrus*-infected AAI mice partially abrogated the immune protection against AAI by parasite (Fig. 5). These results demonstrate that these three types of immunoregulatory cells are involved in the mediation of suppression of allergic inflammation in lung by concurrent parasite infection.

FoxP3⁺ T_{reg} and IL-10⁺ T_{reg} cells are known to play critical roles in control of excessive immune response to maintain the immunohomeostasis and immune tolerance [27,28]. IL-10⁺ B_{reg} cells are also recognized in recent years as important immunosuppressive cells and critical in suppression of immunopathology [29,30]. The roles of these immunoregulatory cells in modulation of lung allergic response by parasite have been studied, but inconsistent results were obtained. It was reported that CD4⁺ T cells or CD19⁺ B cells from *H. polygyrus*-infected IL-10-deficient mice were able to transfer protection from the Der p 1 allergen-induced allergic response, and depletion of CD25⁺ cells with mAb *in vivo* partially blocked the suppression of lung allergic response by parasite [11,15]. However, study by Kitagaki *et al.* showed that suppression of airway allergic response by *H. polygyrus* infection was absent in IL-10-deficient mice [10]. With passive transfer of purified IL-10⁺ B_{reg} or IL-10⁺ T_{reg} cells and *in-vivo* depletion of FoxP3⁺ T_{reg} approaches, we demonstrated that IL-10⁺ B_{reg} cells and FoxP3⁺ T_{reg} cells play important roles in modulation of allergic response in lung by parasite. Our study further demonstrates for the first time, to our knowledge, that IL-10⁺ T_{reg} cells are an important modulator involved

in the suppression of allergic response by parasite. The observation that IL-10⁺ B_{reg} and IL-10⁺ T_{reg} cells from uninfected and parasite-infected mice showed similar potency to suppress the AAI response in the recipient mice (Fig. 3, Supporting information, Fig. S5) indicated that these immunoregulatory cells exert their suppressive effect in an antigen-unspecific manner.

Although IL-10⁺ B_{regs}, IL-10⁺ T_{regs} and FoxP3⁺ T_{regs} have been shown to mediate the immunosuppression of allergic diseases by parasites, the relationship and interaction between these immune regulatory cells are not fully known. IL-10⁺ T_{reg} and FoxP3⁺ T_{reg} are two distinct types of immune regulatory T cells. Analysis of the functional marker expression revealed that a minor portion of these two types of T_{regs} co-express IL-10 and FoxP3 (Supporting information, Fig. S7). In arthritis and tumor animal model studies, it was observed that B cells or IL-10⁺ B_{reg} cells are required for maintenance of normal levels of IL-10⁺ T_{regs} and FoxP3⁺ T_{regs} and sufficient immunosuppression [31,32]. We observed that *H. polygyrus* infection induced increased frequencies and numbers of IL-10⁺ B_{regs} and IL-10⁺ T_{regs} (Fig. 2). *In-vitro* cell co-culture assay revealed that IL-10⁺ B_{reg} cells were able to promote CD4⁺ cells to differentiate into IL-10⁺ T_{reg} cells, but IL-10⁺ T_{reg} cells were unable to induce IL-10⁺ B_{reg} cell expansion. Furthermore, transfer of IL-10⁺ B_{reg} cells induced proliferation of IL-10⁺ T_{reg} cells (Fig. 4). These results, taken together, suggest a possible series of events, i.e. parasite infection activates and induces IL-10⁺ B_{reg} cell response that promotes IL-10⁺ T_{reg} cell expansion, and the expanded IL-10⁺ T_{reg} cell population exerts the immunosuppression function to prevent the excessive inflammation.

Carter *et al.* reported that induction of IL-10⁺ T_{reg} by B_{reg} requires cell–cell contact [33]. A study by Mann *et al.* demonstrated that B_{reg} cells regulate T_{reg} cells by cell interaction through a CD80/CD86–CD28-dependent mechanism [34]. Mizoguchi *et al.* also observed that B cell transfer to suppress colitis requires the presence of CD40 and CD86 [35]. It is clear that IL-10 is not sufficient to induce T_{regs} directly, but requires a cognate interaction mediated by co-stimulatory molecules on the surface of B cells and CD4⁺ T cells. Our observation that IL-10⁺ B_{reg} cells, when separated from CD4⁺ T cells in the Transwell cell culture system, were not able to induce IL-10⁺ T_{reg} cell proliferation (Fig. 4b), supports the notion that co-stimulatory molecules are involved in the cell–cell contact for induction of T_{regs} by IL-10⁺ B_{reg} cells [33–35].

The frequency of FoxP3⁺ T_{reg} cells was increased in both MLN and spleen following *H. polygyrus* infection. To determine whether this FoxP3⁺ T_{reg} response requires IL-10⁺ B_{reg} cells, a cell co-culture assay was performed. The CD4⁺ T cells cultured alone for 48 h showed reduced the frequency of FoxP3⁺ T_{regs} (Fig. 5c) compared with the frequency of FoxP3⁺ T_{regs} in *ex-vivo* cell samples (Fig. 5b,

day 0), and the CD4⁺ T cells co-cultured with IL-10⁺ B_{reg}s showed elevated levels of FoxP3⁺ T_{reg}s. Passive transfer of IL-10⁺ B_{reg}s failed to induce FoxP3⁺ T_{reg} expansion *in vivo* (data not shown). These results suggest that IL-10⁺ B_{reg} cells are required for maintenance, but not for induction, of the FoxP3⁺ T_{reg} cell population [31]. Our previous study showed that *H. polygyrus* infection induced expansion of a subset of dendritic cells with CD11c^{low}CD45RB^{mid} phenotypes that were able to promote proliferation of FoxP3⁺ T_{reg} cells [36].

Depletion of FoxP3⁺ T_{reg} cells *in vivo* in AAI DERE mice enhanced the allergic airway inflammation, indicating that FoxP3⁺ T_{reg} cells are essential for control of severity of AAI in parasite-free conditions (Fig. 5e–g). Removal of FoxP3⁺ T_{reg}s in Hp+AAI mice also resulted in more severe allergic inflammation compared with the Hp+AAI mice that were not depleted of FoxP3⁺ T_{reg} cells. This increment in severity of allergic inflammation in Hp+AAI mice may result from the loss of FoxP3⁺ T_{reg} cells that intrinsically occur in mice to control the basic severity of AAI. However, infection of *H. polygyrus* induced substantial increase of FoxP3⁺ T_{reg} cells in MLN and spleen. The increased severity of allergic inflammation in DT-treated Hp+AAI mice may also be attributable to the loss of parasite-induced FoxP3⁺ T_{reg} cells.

Helminth parasites may modulate the response of host immune system directly or via alteration of gut microbiota. A recent study by Zaiss *et al.* showed that *H. polygyrus* infection altered the intestinal microbiota community and transfer of the modified microbiota conferred protection against allergic asthma [37]. We found that mouse spleen and MLN B cells stimulated with bacterial lipopolysaccharide (LPS) and cytosine–phosphate–guanosine (CpG) *in vitro* showed increased frequency of IL-10⁺ B_{reg} cells [38] (and unpublished data). It is possible that the nematode parasite-modified intestinal microbiota preferentially promotes IL-10⁺ B_{reg} response, a pathway that warrants further study.

In conclusion, this study demonstrates the protective effects of intestinal parasite infection on the OVA-induced allergic airway inflammation. This immune protection is mediated by B_{reg}, IL-10⁺ T_{reg} and FoxP3⁺ T_{reg} cells. Our results also demonstrate that IL-10⁺ B_{reg} cells is one of the key upstream immunoregulatory cells that promote expansion of IL-10⁺ T_{reg} and maintenance of FoxP3⁺ T_{reg} cells. These findings not only provide new insight into the complex immunological relationship between helminth parasite infection and allergic immunopathology, but also reveals the potential targets for development of immune therapy against allergic asthma.

Acknowledgements

This study was supported by the grants from the Science and Technology Specific Program of Guangzhou Science

and Innovation Commission (201607020046) and the Major Research Plan of NSFC-Guangdong Joint Project (U1801286) to J. L. and Z. S. We thank Dr Marilyn Scott (McGill University, Canada) for kindly providing the nematode parasite.

Disclosures

The authors declare no conflicts of interest.

Author contributions

Z. S., J. L. and X. G. conceived and designed the study. X. G. and X. R. performed the experiments. Q. W., Y. L. and Z. Y. contributed to experimental design and data analysis. X. G. and Z. S. prepared the manuscript.

References

- Nieto A, Wahn U, Bufe A *et al.* Allergy and asthma prevention 2014. *Pediatr Allergy Immunol* 2014; **25**:516–33.
- Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; **347**:911–20.
- Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med* 2006; **355**:2226–35.
- Bach JF. The hygiene hypothesis in autoimmunity: the role of pathogens and commensals. *Nat Rev Immunol* 2018; **18**:105–20.
- Maizels RM, McSorley HJ. Regulation of the host immune system by helminth parasites. *J Allergy Clin Immunol* 2016; **138**:666–75.
- Medeiros M Jr, Figueiredo JP, Almeida MC *et al.* *Schistosoma mansoni* infection is associated with a reduced course of asthma. *J Allergy Clin Immunol* 2003; **111**:947–51.
- Ponte EV, Rasella D, Souza-Machado C, Stelmach R, Barreto ML, Cruz AA. Reduced asthma morbidity in endemic areas for helminth infections: a longitudinal ecological study in Brazil. *J Asthma* 2014; **51**:1022–7.
- Leonardi-Bee J, Pritchard D, Britton J. Collaboration PiA. Asthma and current intestinal parasite infection: systematic review and meta-analysis. *Am J Respir Crit Care Med* 2006; **174**:514–23.
- Aranzamendi C, de Bruin A, Kuiper R *et al.* Protection against allergic airway inflammation during the chronic and acute phases of *Trichinella spiralis* infection. *Clin Exp Allergy* 2013; **43**:103–15.
- Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN. Intestinal helminths protect in a murine model of asthma. *J Immunol* 2006; **177**:1628–35.
- Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 2005; **202**:1199–212.
- Mangan NE, van Rooijen N, McKenzie AN, Fallon PG. Helminth-modified pulmonary immune response protects mice from allergen-induced airway hyperresponsiveness. *J Immunol* 2006; **176**:138–47.

- 13 Barnes PJ. Targeting cytokines to treat asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2018; **18**:454–66.
- 14 Kang SA, Park MK, Cho MK *et al.* Yu HS Parasitic nematode-induced CD4⁺Foxp3⁺ T cells can ameliorate allergic airway inflammation. *PLOS Negl Trop Dis* 2014; **8**:e3410.
- 15 Wilson MS, Taylor MD, O’Gorman MT *et al.* Helminth-induced CD19⁺CD23^{hi} B cells modulate experimental allergic and autoimmune inflammation. *Eur J Immunol* 2010; **40**:1682–96.
- 16 Madan R, Demircik F, Surianarayanan S *et al.* Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *J Immunol* 2009; **183**:2312–20.
- 17 Lahl K, Loddenkemper C, Drouin C *et al.* Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007; **204**:57–63.
- 18 Su Z, Segura M, Morgan K, Loredó-Osti JC, Stevenson MM. Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. *Infect Immunity* 2005; **73**:3531–9.
- 19 Bates JH, Rincon M, Irvin CG. Animal models of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009; **297**:L401–L410.
- 20 Pichavant M, Goya S, Hamelmann E, Gelfand EW, Umetsu DT. Animal models of airway sensitization. *Current protocols in immunology* 2007; Chapter 15: Unit 15.8–Unit. 8.
- 21 Rayamajhi M, Redente EF, Condon TV, Gonzalez-Juarrero M, Riches DW, Lenz LL. Non-surgical intratracheal instillation of mice with analysis of lungs and lung draining lymph nodes by flow cytometry. *J Vis Exp* 2011; **51**:e2702.
- 22 Bochner BS. Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors. *Clin Exp Allergy* 2009; **39**:317–24.
- 23 McSorley HJ, O’Gorman MT, Blair N, Sutherland TE, Filbey KJ, Maizels RM. Suppression of type 2 immunity and allergic airway inflammation by secreted products of the helminth *Heligmosomoides polygyrus*. *Eur J Immunol* 2012; **42**:2667–82.
- 24 Okada H, Kuhn C, Feillet H, Bach JF. The ‘hygiene hypothesis’ for autoimmune and allergic diseases: an update. *Clin Exp Immunol* 2010; **160**:1–9.
- 25 Liu AH. Revisiting the hygiene hypothesis for allergy and asthma. *J Allergy Clin Immunol* 2015; **136**:860–5.
- 26 Cruz AA, Cooper PJ, Figueiredo CA, Alcantara-Neves NM, Rodrigues LC, Barreto ML. Global issues in allergy and immunology: Parasitic infections and allergy. *J Allergy Clin Immunol* 2017; **140**:1217–28.
- 27 Palomares O, Martín-Fontecha M, Lauener R *et al.* Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF- β . *Genes Immun* 2014; **15**:511–20.
- 28 Chien CH, Chiang BL. Regulatory T cells induced by B cells: a novel subpopulation of regulatory T cells. *J Biomed Sci* 2017; **24**:86.
- 29 Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* 2012; **30**:221–41.
- 30 Rosser EC, Blair PA, Mauri C. Cellular targets of regulatory B cell-mediated suppression. *Mol Immunol* 2014; **62**:296–304.
- 31 Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. *Arthritis Res Ther* 2012; **4**:R32.
- 32 Tadmor T, Zhang Y, Cho H-M, Podack ER, Rosenblatt JD. The absence of B lymphocytes reduces the number and function of T-regulatory cells and enhances the anti-tumor response in a murine tumor model. *Cancer Immunol Immunother* 2011; **60**:609–19.
- 33 Carter NA, Vasconcellos R, Rosser EC *et al.* Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol* 2011; **186**:5569–79.
- 34 Mann MK, Maresz K, Shriver LP, Tan Y, Dittel BN. B cell regulation of CD4⁺CD25⁺ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* 2007; **178**:3447–56.
- 35 Mizoguchi E, Mizoguchi A, Pfeffer FI, Bhan AK. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. *Int Immunol* 2000; **12**:597–605.
- 36 Li Z, Liu G, Chen Y, Liu Y, Liu B, Su Z. The phenotype and function of naturally existing regulatory dendritic cells in nematode-infected mice. *Int J Parasitol* 2011; **41**:1129–37.
- 37 Zaiss MM, Rapin A, Lebon L *et al.* The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. *Immunity* 2015; **43**:998–1010.
- 38 Liu Y, Chen Y, Li Z *et al.* Role of IL-10-producing regulatory B cells in control of cerebral malaria in *Plasmodium berghei* infected mice. *Eur J Immunol* 2013; **43**:2907–18.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1. Schematic of experimental design. Two groups of C57BL/6 mice were infected with 300 *H. polygyrus* third-stage larvae, and one of them were left untreated (Hp group) and another group were sensitized by i.p. injection of OVA in PBS 14 days (as day 0 of the experiment) after parasite infection. After second OVA sensitization at day 7, mice were challenged with three aerosolized OVA treatment at day 14–16 (Hp+AAI group). Mice that were untreated (Naïve group) or OVA-sensitized and challenged as described above (AAI group) were included. All mice were sacrificed at day 18.

Fig. S2. Representative flow cytometric profiles of bronchoalveolar cells from the four groups of mice stained with fluorescent anti-CD11c and anti-Siglec-F mAbs. CD11c^{high}Siglec-F⁺ population is defined as alveolar macrophages and CD11c^{high}Siglec-F⁺ population is defined as eosinophils.

Fig. S3. Cytokines production *in vitro* by splenic cells from the four groups of mice. Spleens were collected from the

four groups mice, single cell suspensions were prepared and cultured in the presence of parasite antigen (Hp group cells), OVA (AAI group cells) or parasite antigen and OVA (Hp+AAI group cells). Supernatants were collected and levels of IL-4 (a) and IL-5 (b) were determined by ELISA. Results shown are from one of two experiments. ***, $P < 0.001$.

Fig. S4. Serum levels of total IgE in mice. Groups of mice were sensitized and challenged with OVA to induce AAI and transferred of IL-10⁺Breg cells (a) or IL-10⁺Treg cells (b). AAI mice receiving PBS, IL-10⁺CD19⁺ B cells (a) or IL-10⁺CD4⁺ T cells (b) were included as control groups. Mice were sacrificed and serum levels of total IgE were quantified by ELISA. The data shown are mean $\pm$ SEM of 3–4 mice per group. ***, $P < 0.01$.

Fig. S5. Adoptive transfer of IL-10⁺Breg and IL-10⁺Treg cells collected from normal uninfected mice. IL-10⁺CD19⁺Breg and IL-10⁺CD19⁺ B cells (a, b) and IL-10⁺CD4⁺Treg and IL-10⁺CD4⁺ cells (c, d) were isolated from spleens of normal

uninfected mice by magnetic beads negative selection and cell sorting. Cells were adoptively transferred into AAI mice. Total number of BAL cells (a, c) and eosinophils (b, d) were determined in recipient mice at sacrifice. Data shown are mean $\pm$ SEM of 4–6 mice per group. **, $P < 0.01$.

Fig. S6. Diphtheria toxin (DT) treatment to delete Foxp3⁺ Treg cells *in vivo*. (a) Efficiency of Foxp3⁺ Treg cell depletion in Dereg mice by DT treatment. (b) Effect of DT treatment on bronchoalveolar cells of wild-type C57BL/6 naive and AAI mice. (c) Schematic of DT treatment experiment. DT treatment were given four times at day -1, 3, 8 and 12 as highlighted in red.

Fig. S7. Co-expression of IL-10 and Foxp3 in IL-10⁺Treg and Foxp3⁺Treg cell populations. Spleen cells were collected from IL-10-GFP mice and stained with anti-CD4-PE and intracellularly stained with anti-Foxp3-APC. GFP⁺ (IL-10⁺) and Foxp3⁺ populations in gated CD4⁺ cells were analyzed by flow cytometry.

Supporting information

Critical roles of regulatory B and T cells in helminth parasite-induced protection against allergic airway inflammation

Xiang Gao, Xia Ren, Qiong Wang, Zhaowei Yang, Yinxiong Li, Zhong Su, Jing Li

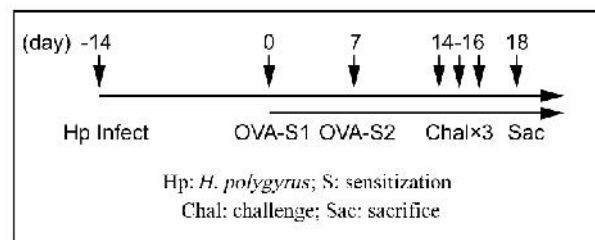


Figure S1. Schematic of experimental design. Two groups of C57BL/6 mice were infected with 300 *H. polygyrus* third-stage larvae, and one of them were left untreated (Hp group) and another group were sensitized by i.p. injection of OVA in PBS 14 days (as day 0 of the experiment) after parasite infection. After second OVA sensitization at day 7, mice were challenged with three aerosolized OVA treatment at day 14-16 (Hp+AAI group). Mice that were untreated (Naïve group) or OVA-sensitized and challenged as described above (AAI group) were included. All mice were sacrificed at day 18.

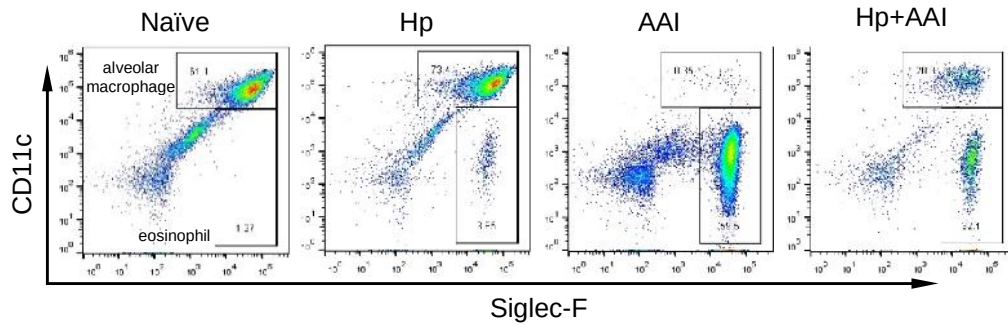


Figure S2. Representative flow cytometric profiles of bronchoalveolar cells from the four groups of mice stained with fluorescent anti-CD11c and anti-Siglec-F mAbs. CD11c^{high}Siglec-F⁺ population is defined as alveolar macrophages and CD11c^{low}Siglec-F⁺ population is defined as eosinophils.

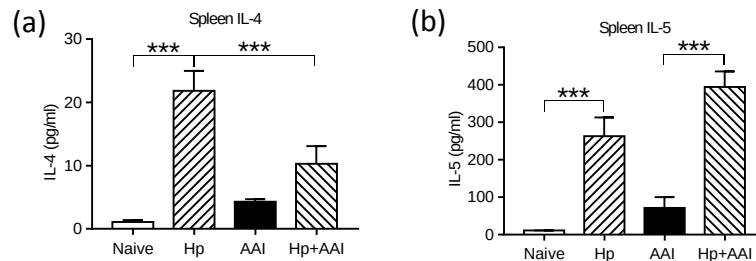


Figure S3. Cytokines production *in vitro* by splenic cells from the four groups of mice. Spleens were collected from the four groups mice, single cell suspensions were prepared and cultured in the presence of parasite antigen (Hp group cells), OVA (AAI group cells) or parasite antigen and OVA (Hp+AAI group cells). Supernatants were collected and levels of IL-4 (a) and IL-5 (b) were determined by ELISA. Results shown are from one of two experiments. ***, $p < 0.001$.

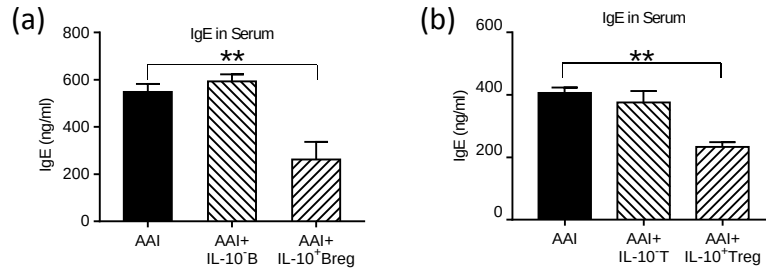


Figure S4. Serum levels of total IgE in mice. Groups of mice were sensitized and challenged with OVA to induce AAI and transferred of IL-10⁺Breg cells (a) or IL-10⁺Treg cells (b). AAI mice receiving PBS, IL-10⁺CD19⁺ B cells (a) or IL-10⁺CD4⁺ T cells (b) were included as control groups. Mice were sacrificed and serum levels of total IgE were quantified by ELISA. The data shown are mean $\pm$ SEM of 3-4 mice per group. ***, $p < 0.01$.

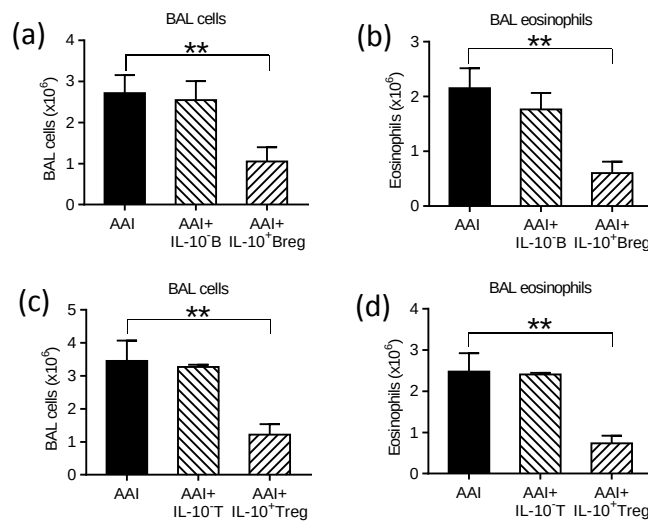


Figure S5. Adoptive transfer of IL-10⁺Breg and IL-10⁺Treg cells collected from normal uninfected mice. IL-10⁺CD19⁺Breg and IL-10⁺CD19⁺ B cells (a, b) and

IL-10⁺CD4⁺Treg and IL-10⁻CD4⁺ cells (c, d) were isolated from spleens of normal uninfected mice by magnetic beads negative selection and cell sorting. Cells were adoptively transferred into AAI mice. Total number of BAL cells (a, c) and eosinophils (b, d) were determined in recipient mice at sacrifice. Data shown are mean $\pm$ SEM of 4-6 mice per group. **, $p < 0.01$.

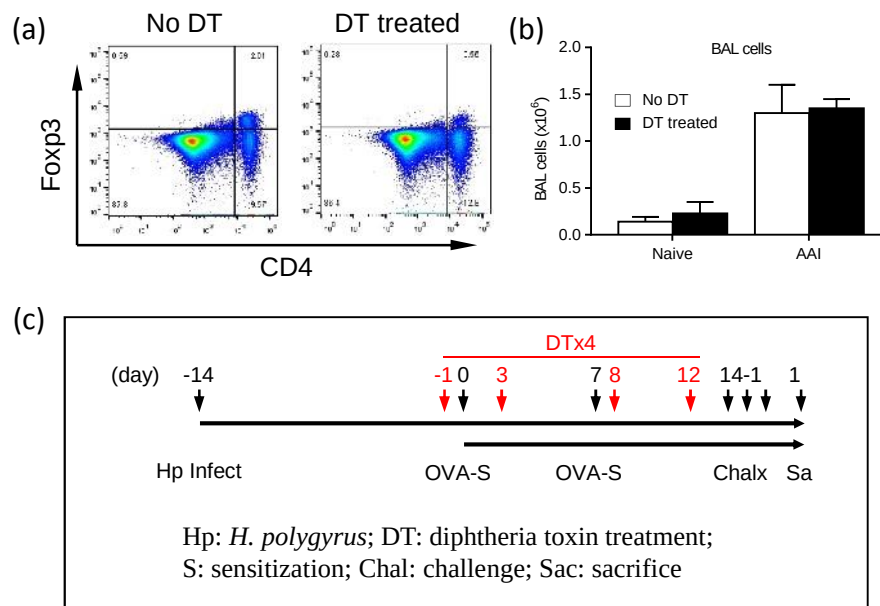


Figure S6. Diphtheria toxin (DT) treatment to delete Foxp3⁺Treg cells in vivo. (a) Efficiency of Foxp3⁺ Treg cell depletion in Dereg mice by DT treatment. (b) Effect of DT treatment on bronchoalveolar cells of wild-type C57BL/6 naive and AAI mice. (c) Schematic of DT treatment experiment. DT treatment were given four times at day -1, 3, 8 and 12 as highlighted in red.

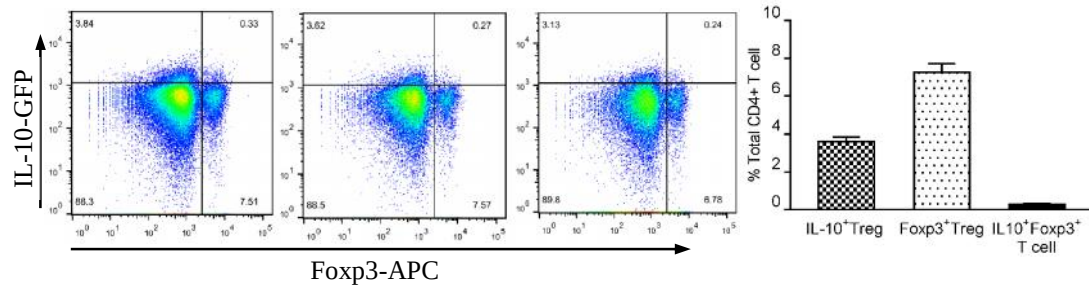


Figure S7. Co-expression of IL-10 and Foxp3 in IL-10⁺Treg and Foxp3⁺Treg cell populations. Spleen cells were collected from IL-10-GFP mice and stained with anti-CD4-PE and intracellularly stained with anti-Foxp3-APC. GFP⁺ (IL-10⁺) and Foxp3⁺ populations in gated CD4⁺ cells were analyzed by flow cytometry.