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A regulatory CD9$^+$ B-cell subset inhibits HDM-induced allergic airway inflammation

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Abstract

Background: Exposure to respiratory allergens triggers airway hyperresponsiveness and inflammation characterized by the expansion of T_H2 cells and the production of allergen specific IgE. Allergic asthma is characterized by an alteration in immune regulatory mechanisms leading to an imbalance between pro- and anti-inflammatory components of the immune system.
Aims: Recently B cells have been described as central regulators of exacerbated inflammation, notably in the case of autoimmunity. However, to what extent these cells can regulate airway inflammation and asthma remains to be elucidated.
Materials & Methods: We took advantage of a allergic asthma model in mice induced by percutaneous sensitization and respiratory challenge with an extract of house dust mite.
Results: In this study, we showed that the induction of allergic asthma alters the homeostasis of IL-10$^+$ Bregs and favors the production of inflammatory cytokines by B cells. Deeper transcriptomic and phenotypic analysis of Bregs revealed that they were enriched in a CD9$^+$ B cell subset. In asthmatic mice the adoptive transfer of CD9$^+$ B cells normalized airway inflammation and lung function by inhibiting T_H2- and T_H17-driven inflammation in an IL-10-dependent manner, restoring a favorable immunological balance in lung tissues. Indeed we further showed that injection of CD9$^+$ Bregs controls the expansion of lung effector T cells allowing the establishment of a favorable regulatory T cells/effector T cells ratio in lungs.
Conclusion: This finding strengthens the potential for Breg-targeted therapies in allergic asthma.

Allergic asthma is a chronic inflammatory disease characterized by airway hyper-responsiveness and deregulated inflammation in response to allergens. This pathology is controlled by CD4$^+$ T helper (T_H) lymphocytes, which cause cellular infiltration in the lungs, overproduction of mucus, and airway constriction. In general, B cells have been described in asthma as deleterious cells secreting allergen-specific IgE under pro-T_H2 inflammatory conditions. IgE interacts with Fcε receptor I at the surface of basophils and mast cells, promoting the release of pro-inflammatory mediators such as chemokines, prostaglandins, and leukotrienes, which leads to airway inflammation and airway narrowing (1).

Beyond their antibody secretion capacities, B cells can also present antigens, produce cytokines, and regulate T-cell-mediated immune responses (2). Indeed, B cells can secrete both pro-inflammatory and inhibitory cytokines, the balance of which influences the immune response (2). Recently, a sub-

Abbreviations
BAL, bronchoalveolar lavage; Bregs, regulatory B cells; HDM, house dust mite; LPS, lipopolysaccharide; MZ, marginal zone; PIM, PMA + ionomycin + monensin; PMA, phorbol 12-myristate 13-acetate.

population of regulatory B cells (Bregs) that produces large amounts of IL-10 was identified in both mice and humans (3, 4). These cells are able to suppress inflammation by constraining T_H1 and T_H17 responses and inducing regulatory T cells (Tregs) (3, 4). Accordingly, functional impairment of the IL-10-producing B-cell subset is associated with exacerbated persistent autoimmunity and dermal allergic inflammation (5–7). In allergic asthma models, infection with parasites protects mice from the development of lung inflammation and airway hyper-responsiveness through the generation of IL-10-producing B cells (8, 9). Notably, elevated levels of allergen-specific, IL-10-producing Bregs have been found after immunotherapies for food and bee venom allergies, suggesting that increased proportions of Bregs are a specific feature of induced tolerance toward allergens (10, 11). However, to what extent the development of allergic asthma influences the homeostasis of Bregs has not yet been clearly addressed. To investigate this, we took advantage of an acute model of house dust mite (HDM)-induced allergic airway inflammation.

Herein, we show that B cells from allergic mice preferentially secrete a pro-inflammatory cytokine profile that counters the effects of the anti-inflammatory cytokine IL-10. Consistently, the frequency of IL-10^+ Bregs was decreased in the spleen and lungs of asthmatic mice. Microarray and cytometry analysis further demonstrated that Bregs were enriched in a $CD9^+$ B-cell subset that was decreased in asthmatic mice, and adoptive transfer of these $CD9^+$ B cells abrogated asthma in an IL-10-dependent manner.

Materials and methods

Mice

Six- to eight-week-old wild-type and IL-$10^{-/-}$ BALB/c mice were purchased from Charles River Laboratories (Ecully, France). Allergic inflammation was induced in mice using a total HDM extract *(Dermatophagoïdes farinae)* provided by Stallergenes (Antony, France). Induction of allergic inflammation was performed as previously described (12) (see Appendix S1 for more details).

Flow cytometry – The following antibodies were used for flow cytometric analyses

Ly6G-PercP.Cy5.5 (1A8), CD8-APC-H7 (53–6.7) (BD Biosciences, Le Pont-de-Claix, France), CD3-APC (145.2C11), CD19-PeCy7 (1D3), F4/80-FITC (BM8) (eBioscience, Paris, France), CCR3-PE (83101; R&D systems, Lille, France). CD3-APC (145-2C11), CD4-FITC (OKT4), CD25-PE (PC61), Foxp3-PECy7 (FJK-16s), CD19-PeCy7 (1D3), IgM-APC or PE (II/41), IgD-eFluor®450 or APC (11-26c), GL7-A488 (GL-7), CD9-FITC (KMC8), CD70-PerCP-eFluor®710 (FR70), Ctla4-APC (UC10-4B9), CD73-eFluor®450 (TY/11.8), CD5-APC (53–7.3), CD1d A488 (1B1), CD23-FITC (B3B4), CD21-eFluor®450 (4E3), IL-4-APC (11B11) and IL-13-FITC (eBio13A) (all from eBioscience), CD95-Bv421® (Jo2), PD-1-Bv421® (J43) and IL-17A (TC11-18H10) (BD Biosciences). For all experiments, dead cells were excluded using DAPI or Fixable Viability Dye 450 (BD Biosciences). Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) before the intracellular staining (see Appendix S1 for more details).

Analysis of IL-10 production

We analyzed IL-10-producing B cells as previously described (13). In some experiments, splenic B-cell subsets were purified using the Pan B Cell Isolation Kit (Miltenyi Biotec, Paris, France) or the FACS ARIA III (BD Biosciences) and then incubated for 2 days with a CD40 agonist (BD Biosciences, 2 μg/ml), Tim-1 (BioXcell, 10 μg/ml), IL-21 (eBiosciences, 100 ng/ml), or BAFF (R&D system, 20 ng/ml) and activated with LPS + PIM (PMA, Ionomycin and monensin) for the last 5 h (see Appendix S1 for more details).

Adoptive transfer

Spleen cells from asthmatic WT or IL-$10^{-/-}$ mice were stained for CD19 and CD9. Fluorescence minus one was used as a negative control for CD9 expression. $CD9^+$ and $CD9^-$ B cells were sorted using a FACSAria III (BD Bioscience). Purity was routinely superior of 95%. HDM-treated mice received 5×10^5 sorted cells intravenously (i.v.) 1 day before the second challenge. To follow the migration of B cells into the lungs, we stained them with PKH26 before the sorting.

Statistical analysis

Comparisons of experimental values between two groups were analyzed using the Mann–Whitney *U*-test. The nonparametric Kruskal–Wallis test with Dunn's posttest was used for comparisons between more than two groups. Enhanced pause (Penh) and lung resistance results were analyzed using a two-way analysis of variance (ANOVA). All statistic analyses were performed in GraphPad Prism v6, La jolla, USA.

Results

Exposure to allergen elicits attraction of B cells to the airways

As previously published by our team (12, 14), exposure to total *Dermatophagoides farinae* (Der f) extract induced airway hyper-responsiveness (Fig. S1A,B) associated with severe inflammation, as characterized by the presence of mucus-secreting cells, eosinophilic-neutrophilic influx, production of systemic Der f1-specific IgE, and T_H2-T_H17 cytokine secretion (Fig. S1C–E).

We found an increased expression of the B-cell chemoattractant factor, CXCL13, in the lungs and a higher level of CXCL13 in the BAL of asthmatic mice 24 h after the last challenge (Fig. S2A). This was associated with a significant increase of B cells in the BAL and lungs of house dust mite-treated mice (HDM mice) (Fig. 1A–B). Notably, we detected perivascular and peribronchial B-cell infiltrate in the lung tis-

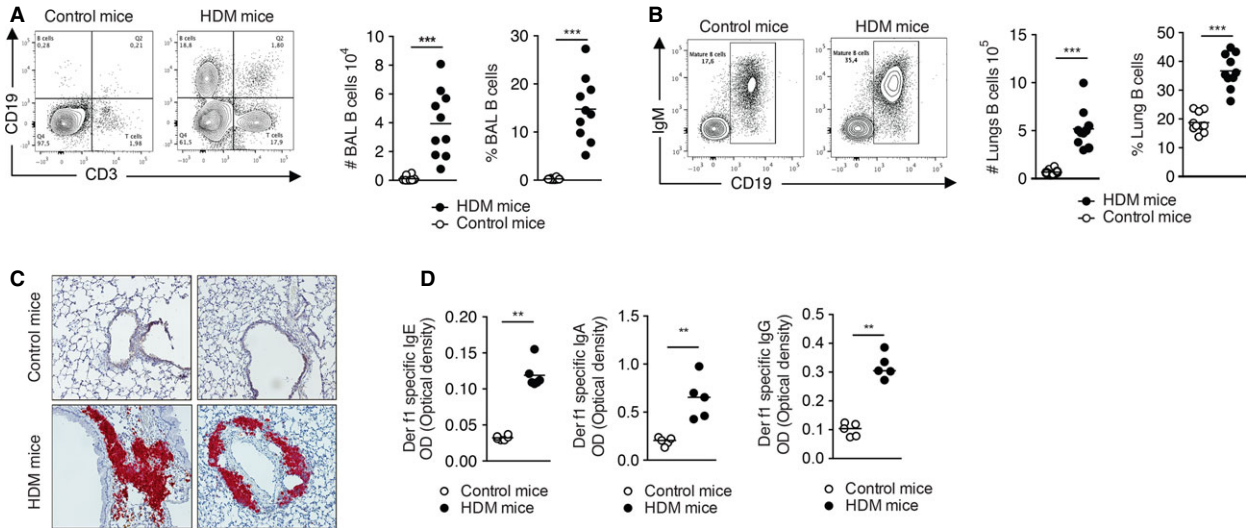

Figure 1 HDM promotes the infiltration and maturation of B cells. (A) Quantification of B cells in the BAL. (B) Quantification of total B cells in lungs of mice. (C) Representative B-cell staining in the lung tissue (Red staining = B220$^+$ B cells). (D) Levels of Der f1-specific IgE, IgG, and IgA in BAL. $^{**}P < 0.01$, $^{***}P < 0.001$.

sues from mice exposed to the allergen (Fig. 1C). Our cytometry analysis highlighted in lung of HDM mice an increase of germinal center B cells (CD19$^+$ Fas$^+$ GL7$^+$) and total switched memory B cells (CD19$^+$ IgM$^-$ IgD$^-$) (Fig. S2B,C), which was associated with the production of Der f1-specific IgE, IgG, and IgA in BAL fluid, supporting the establishment of a local B-cell response toward the allergen (Fig. 1D).

Allergic inflammation influences the cytokine profile of B cells and importantly alters the homeostasis of IL-10$^+$ regulatory B cells

Various cytokine-producing B cells able to regulate the immune response have been recently described (2). To more precisely define the cytokine profile of B cells in asthma, we assessed the mRNA levels of a panel of cytokines including IFN-γ, IL-2, IL-4, IL-6, IL-10, and TGF-β, in purified *ex vivo* lung B cells obtained from control and HDM-treated mice (Fig. 2A). We observed a higher mRNA expression of IL-4 and IL-6 cytokines in B cells from lungs of asthmatic mice (Fig. 2A), whereas the expression of IL-10 was significantly decreased in the lung B cells from HDM mice (Fig. 2A).

The decreased expression of IL-10 in lung B cells of asthmatic mice prompted us to examine whether allergic inflammation affects the homeostasis of Bregs. Lung cells were isolated and stimulated during 5 h with LPS and PIM, followed by staining for cytoplasmic IL-10 expression (13). IL-10-secreting B cells represented 3.14% (±0.4) of total B cells in the lungs of control mice, whereas only 2.1% (±0.2, $P < 0.01$) was observed in the lungs of asthmatic mice (Fig. 2B). Consistently LPS-stimulated lung B cells from allergic mice produce lower levels of IL-10 than LPS-stimulated lung B cells from control mice (Fig. 2C). Finally, a lower frequency of IL-10-secreting B cells was found in the lung of allergic mice after restimulation of total lung cells with HDM extract (Fig. 2D). Altogether, these results indicate an altered homeostasis of IL-10-secreting B cells in the context of allergy.

Transcriptomic analysis highlights new specific surface markers for IL-10-secreting B cells

Stimulation with CD40 agonist optimally induces IL-10 in purified spleen B cells (Fig. S3A–C). CD40-stimulated B cells were labeled with anti-IL-10 antibody conjugated to anti-CD45 antibody and incubated to allow secretion and capture of IL-10 to the cell surface for the purification of live IL-10$^+$ B cells (Fig. S3D,E). To better define Bregs, we analyzed the transcriptomic profile of sorted IL-10$^+$ B cells and IL-10$^-$ B cells of asthmatic and control mice using whole-genome microarray expression analysis (Fig. 3A and Table S1). We identified 3420 and 5374 differentially expressed probes in IL-10$^+$ and IL-10$^-$ B cells in control and HDM mice, respectively (Fold >0.3; $P < 0.01$, Table S1). A total of 954 common probes were differentially expressed in both control and HDM mice (Table S1).

To investigate the Breg phenotype, we focused on the expression of surface markers that were differentially expressed in IL-10$^-$ and IL-10$^+$ cells in both control and HDM mice. Interestingly, classical markers described for Bregs, including CD5, CD1d, CD23, CD21, CD43, IgM, and TIM-1, were not differentially expressed between IL-10$^-$ and IL-10$^+$ B cells in our microarray experiments (Table S1). Instead, surface proteins with higher expression in IL-10$^+$ B cells were CD80, CD70, Nt5e (coding for CD73), CD9, CTLA4, and Pdcd1 (coding for PD-1). Surface markers with significantly lower expression in IL-10$^+$ B cells included

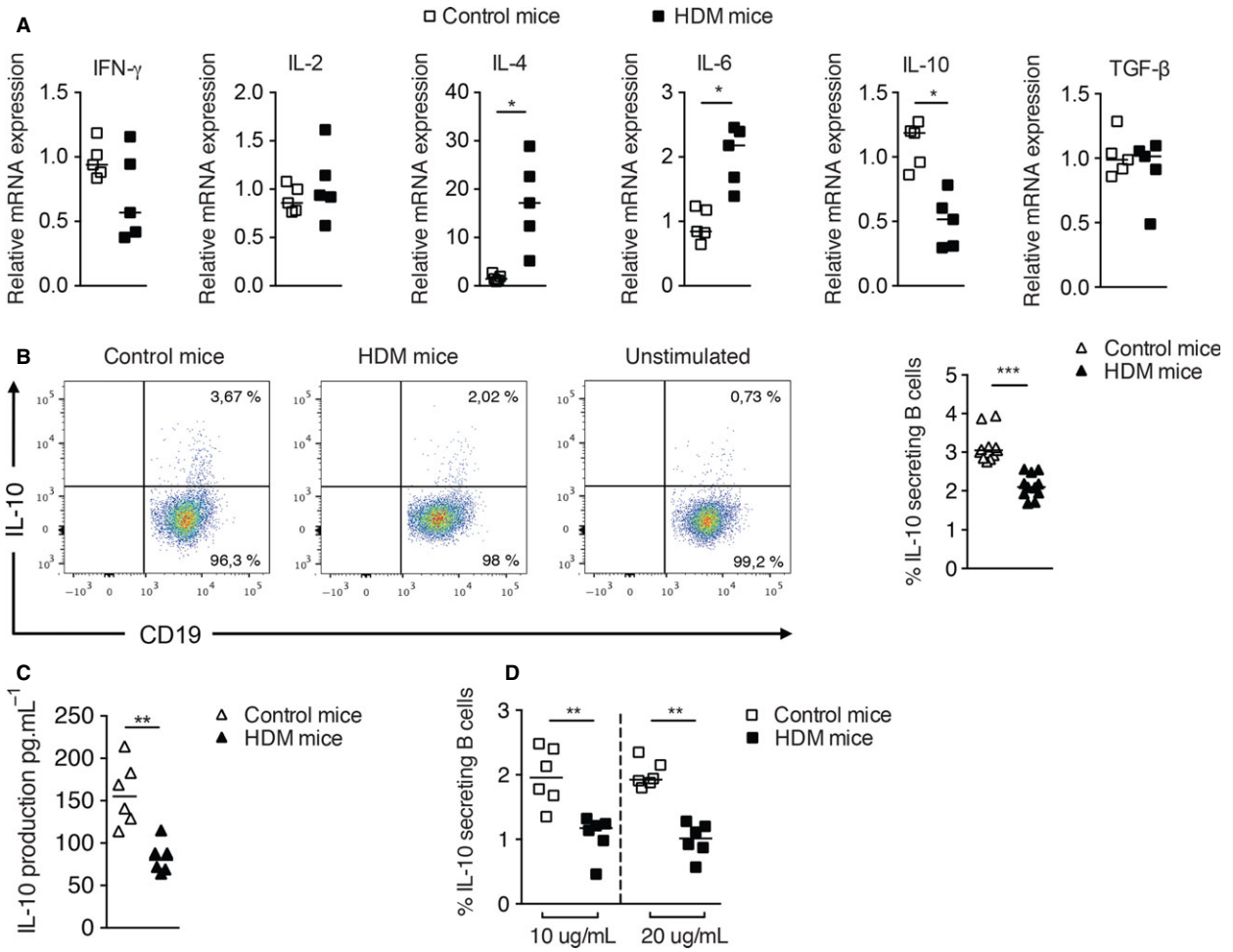

Figure 2 HDM-induced asthma alters the homeostasis of Bregs. (A) The mRNA levels of cytokines were analyzed using RT-PCR in purified B cells from the lungs of control and allergic mice. (B) IL-10$^+$ B-cell frequencies in the lungs of mice. Lungs were cultured with LPS + PIM for 5 h, stained with CD19 mAb, permeabilized, and stained with IL-10 mAb for flow cytometric analysis. Representative results demonstrate the frequency of IL-10-producing cells within the indicated gates among total CD19$^+$ B cells in lungs. (C) IL-10 protein levels in the supernatant of purified lung B cells activated with LPS + PIM for 5 h. (D) Lung cells from control and HDM-treated mice were stimulated for 3 days with a total extract of HDM. At the end of the culture period, the cells were restimulated with PMA, and the IL-10$^+$ B-cell frequency was analyzed by flow cytometry. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

Pdpn (coding for podoplanin), Itga10, and Itgb3 (Fig. 3A). Differential expression of surface CD9, CD70, CD73, CD80 was confirmed by flow cytometric analysis (Fig. 3B), further supporting the identification of new markers to phenotype IL-10$^+$ Bregs in mice.

CD9 is a specific marker for Bregs in mice and humans

To phenotype Bregs, we focused on the expression of CD9, CD73, and CD70 (Fig. 3C). Other B-cell markers that have been linked to Breg cells in previous reports (15, 16) were analyzed as well (Fig. 3C), and we found an equivalent enrichment in IL-10$^+$ B cells in the CD5$^+$ CD1d^{hi} and T2-MZP populations (Fig. 3C–D). However, a higher enrichment in IL-10 production was found in splenic CD9-expressing B-cell subsets (Fig. 3C–D).

Because the differential expression of CD9, CD70, and CD73 could be induced by our protocol of activation, we stimulated spleen purified B cells with LPS-PIM for 5 h to identify spontaneous Bregs (Fig. 4A). In this setting, only the expression of CD9 remains differential between IL-10$^+$ and IL-10$^-$ B cells (Fig. 4A). Additionally, around 16.3% (±1.8) of these CD9$^+$ B cells express IL-10, whereas less than 1% of CD9 express IL-10 (Fig. S4A). Consistently sorted CD9$^+$ splenic B cells produce the highest levels of IL-10 after *in vitro* activation (Fig. 4B) further confirming the reliability of this marker to identify Bregs.

In spleen, the CD9 expression pattern is heterogeneous and is not restricted to one B-cell subset. Indeed, overexpres-

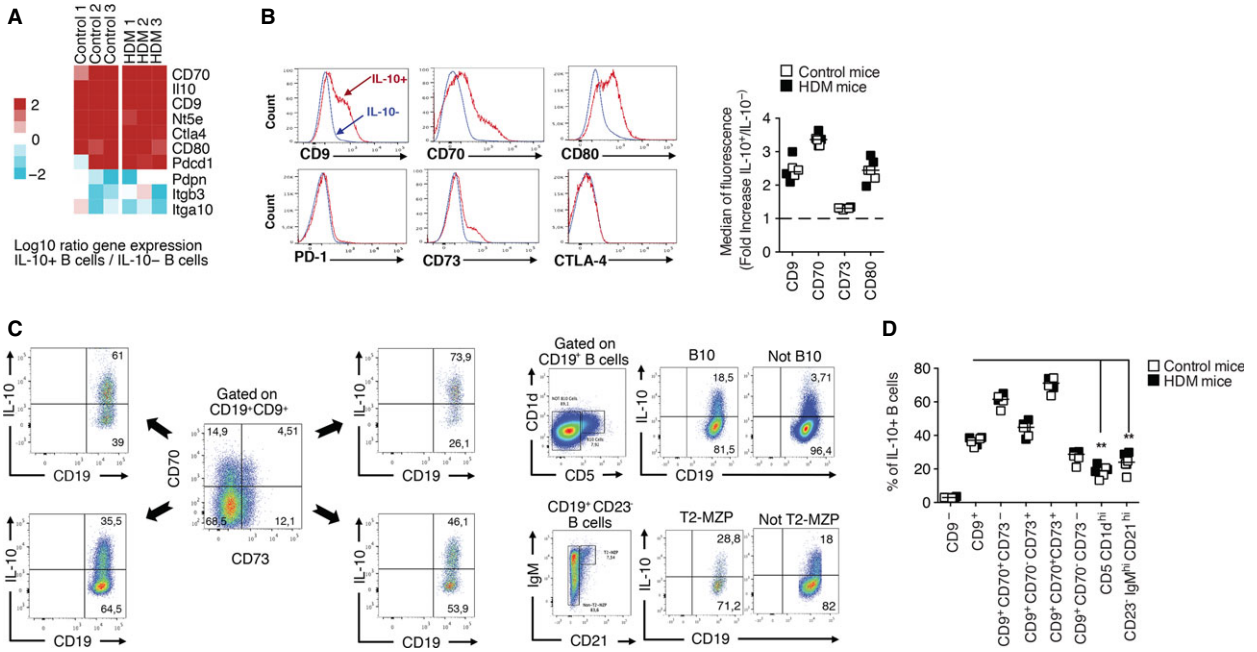

Figure 3 Characterization of IL-10$^+$ and IL-10$^-$ B cells. (A) Heat map showing upregulated and downregulated genes coding for surface proteins between IL-10$^+$ and IL-10$^-$ B cells. (B) Representative staining of CD9, CD70, CD73, CD80, CTLA4, and PD-1 at the surface of IL-10$^+$ and IL-10$^-$ B cells and differential protein expression of CD9, CD70, CD73, and CD80 at the surface of IL-10$^+$ B cells of control and HDM mice. (C) Purified B cells from the spleens of control and HDM mice were activated for 2 days with CD40 mAb agonist and LPS + PIM during the last 5 h. Then, IL-10-secreting B cells were quantified in the CD5$^+$ CD1d$^+$ (B10), CD23$^-$ CD21^{hi} IgM^{hi} (T2-MZP) and newly identified CD9$^+$ B-cell subsets. (D) Quantification of IL-10$^+$ B cells in CD9-expressing B-cell subsets, CD5$^+$ CD1d$^+$ (B10), and in CD23$^-$ CD21^{hi} IgM^{hi} (T2-MZP) $^{**}P < 0.01$.

sion of CD9 is found in B1a B cells (CD5$^+$), MZ B cells, and T2-MZP B cells (Fig. 4C; Gating strategy is available in Fig. S5). These three populations have been described for their role in the regulation of inflammation in many experimental mouse models (4, 17).

In lungs, the phenotype of IL-10 Bregs was similar, with higher expression of CD9, CD73, and CD70 at the surface of IL-10-secreting B cells (Fig. 4D). Also, cytometry analysis revealed that 6.6% (±0.3) of lung CD9$^+$ B cells express IL-10 in control mice (Fig. S4B). Interestingly, frequency of these cells was decreased in allergic mice confirming the defect in Bregs (Fig. S4C).

Finally, in humans, expression of CD9 was dramatically increased at the surface of CD24^{hi} CD38^{hi} immature B cells describing as an important IL-10 Breg subset able to control T-cell inflammation (Fig. 4E) (18–20). Altogether, our findings suggest that CD9 can be a reliable marker to purify both mouse and human Bregs.

Injected CD9$^+$ B cells home to the lungs and reduce airway inflammation

In a last step, we explored the ability of CD9$^+$ B cells to regulate airway inflammation and asthma exacerbation *in vivo*. A total of 5×10^5 splenic CD9$^+$ or CD9$^-$ B cells were isolated by cell sorting and were intravenously injected in asthmatic mice 24 h before the second challenge (Fig. S6A). To follow the migration of injected B cells, we labeled them with a fluorescent tracer. Interestingly, a fraction of injected CD9$^+$ B cells were found in the lungs of mice 24 h after the second challenge (Fig. S6B-D). Lung function and allergic airway inflammation were then analyzed, and we found that the recipients of splenic CD9$^+$ B cells from HDM mice were protected from HDM-induced asthma, with lower bronchial hyper-reactivity (Fig. 5A), lower levels of systemic Der f1-specific IgE, and dampened cellular infiltration in BAL (Fig. 5B) including significantly lower levels of neutrophils and eosinophils (Fig. 5D,E). In contrast, CD9$^-$ B cells from the spleens of HDM mice did not influence the course of HDM-induced allergic airway inflammation, with comparable levels of airway hyper-responsiveness (Fig. 5A), Der f1-specific IgE (Fig. 5C), and cellular infiltration (Fig. 5B,D,E) than HDM mice treated with PBS (Fig. 5B–E). Similarly, transfer of CD9$^+$ B cells from IL-10$^{-/-}$ allergic animals was not protective, confirming the IL-10-dependent regulatory mechanism *in vivo* (Fig. 5A–E).

Next, we investigated to what extent CD9$^+$ B cells influenced T-cell responses in the airways. Figure 6A–C shows that injection of CD9$^+$ Bregs significantly reduced the production of IL-4, IL-13, and IL-17 by CD4$^+$ T cells in the lung tissues. Additionally, the levels of inflammatory cytokines were significantly reduced in the BAL after the second

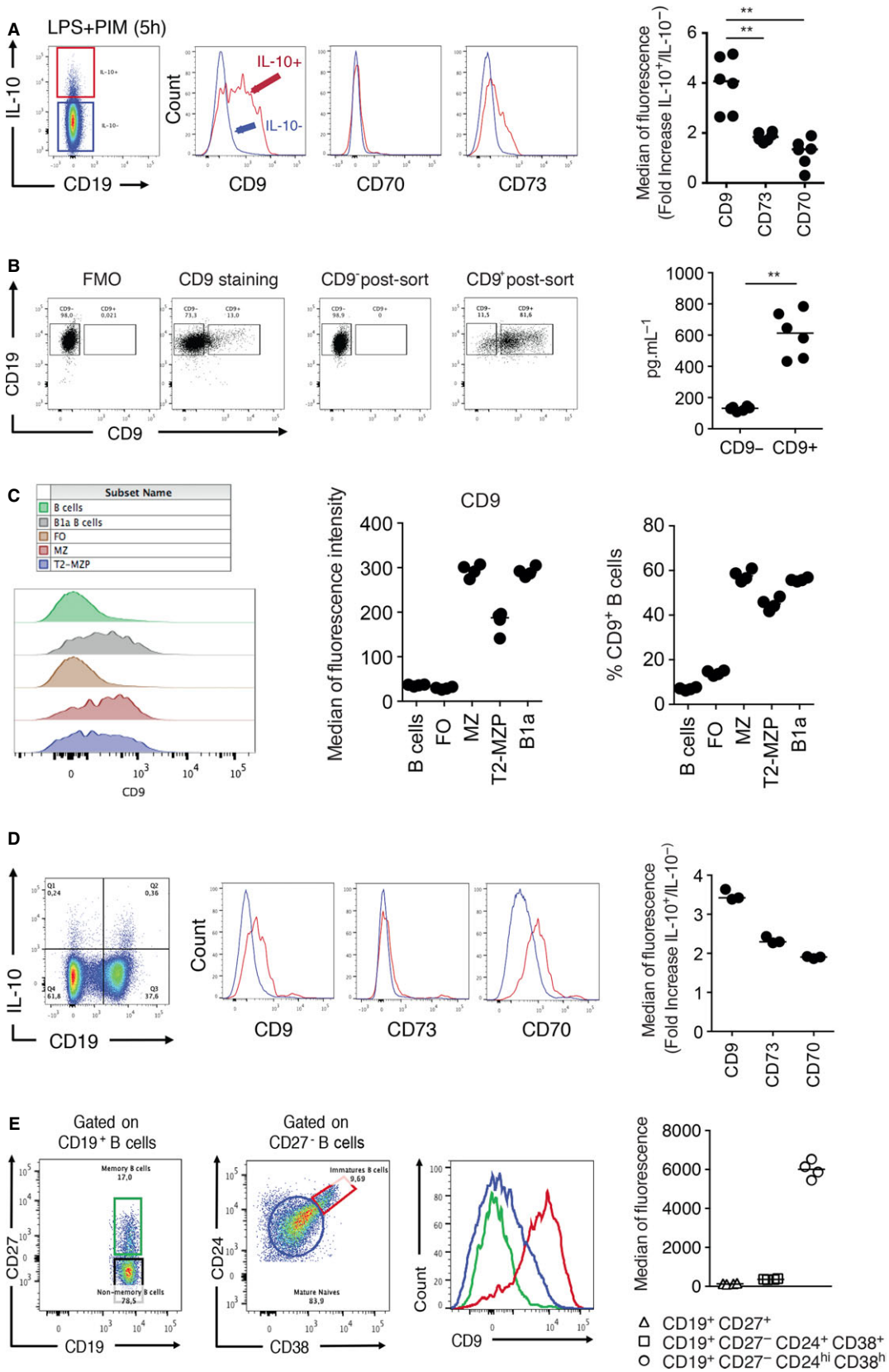

Figure 4 CD9 is a specific marker of Bregs. (A) Protein expression of CD9, CD73, and CD70 in spleen IL-10$^+$ B cells (relative to IL-10$^-$ cells) after 5 h of activation with LPS + PIM. (B) Sorted CD9$^+$ and CD9$^-$ B cells were stimulated for 48 h with CD40 agonist and with LPS + PIM for the last 5 h, and then, the IL-10 levels were measured in the supernatants by ELISA. (C) Expression of CD9 in spleen B-cell subsets. (D) Total lung cells were isolated from naïve BALB/c mice and stimulated for 5 h with LPS + PIM, and then the expression of CD9, CD70, and CD73 was quantified at the surface of IL-10$^+$ B cells. (E) Phenotype of human CD9-expressing B cells. $^{**}P < 0.01$.

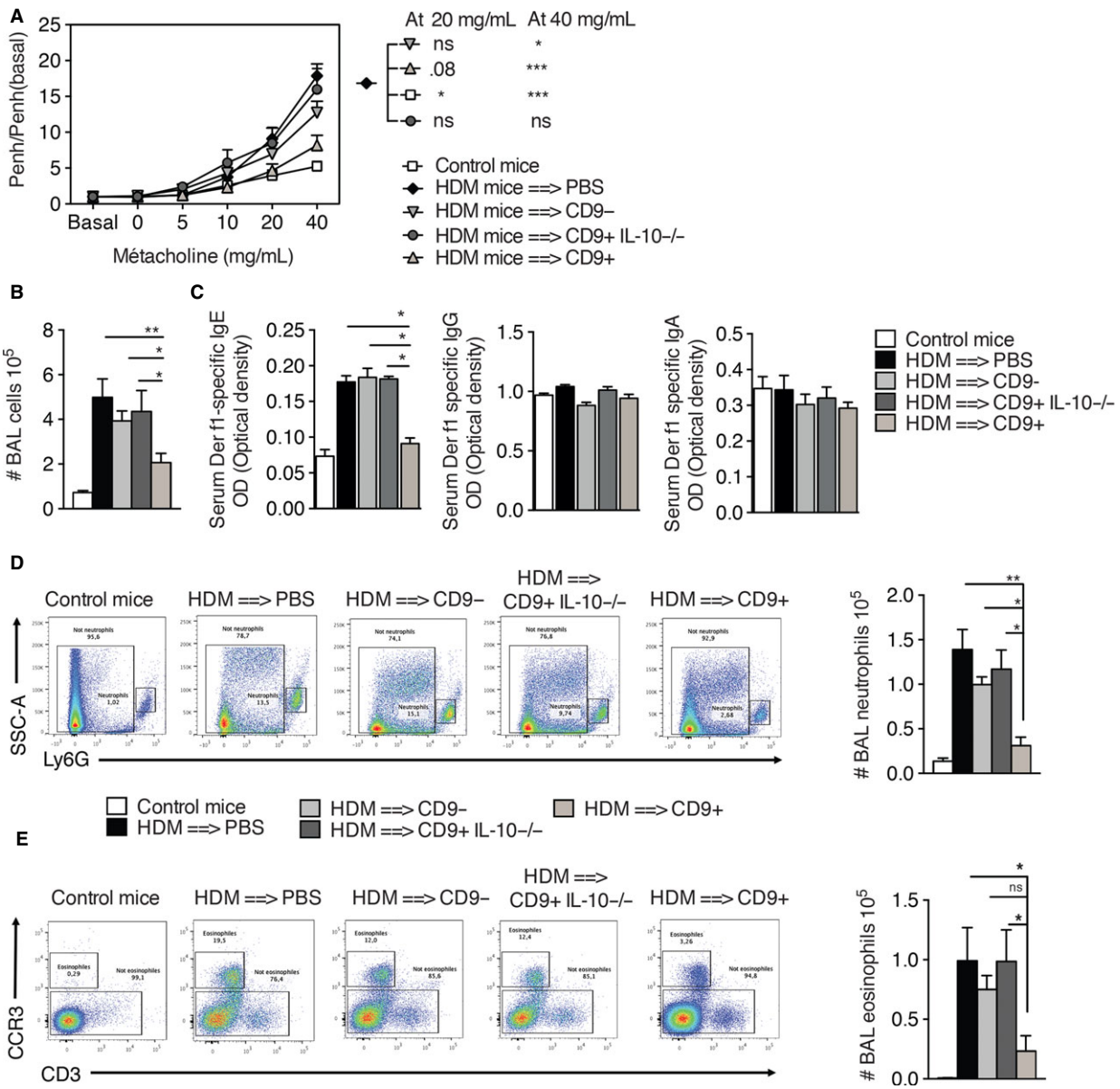

Figure 5 CD9$^+$ B cells control asthma exacerbation. (A) Penh values at day 35 after the adoptive transfer of B cells (n = 5–10 mice per group). (B) Total BAL cell counts after injection of CD9$^+$ regulatory B cells (n = 5–10 mice per group). (C) Levels of Der f1-specific IgG, IgA, and IgG in the sera of mice (n = 5 by groups). (D) Neutrophil, and (E) eosinophil BAL cell counts after injection of B-cell subsets (n = 5–10 mice per group) $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

challenge in CD9$^+$ recipient mice (Fig. 6D). Interestingly, level of IL-10 was increased in the BAL of CD9$^+$ recipient supporting a local release of this cytokine after Breg transfer (Fig. 6E).

Given the intimate link between Tregs and Bregs (5, 8) and the potent role of Tregs in asthma (21), we investigated whether injection of CD9$^+$ Bregs promoted the expansion of Tregs in the lung mucosa. As shown in

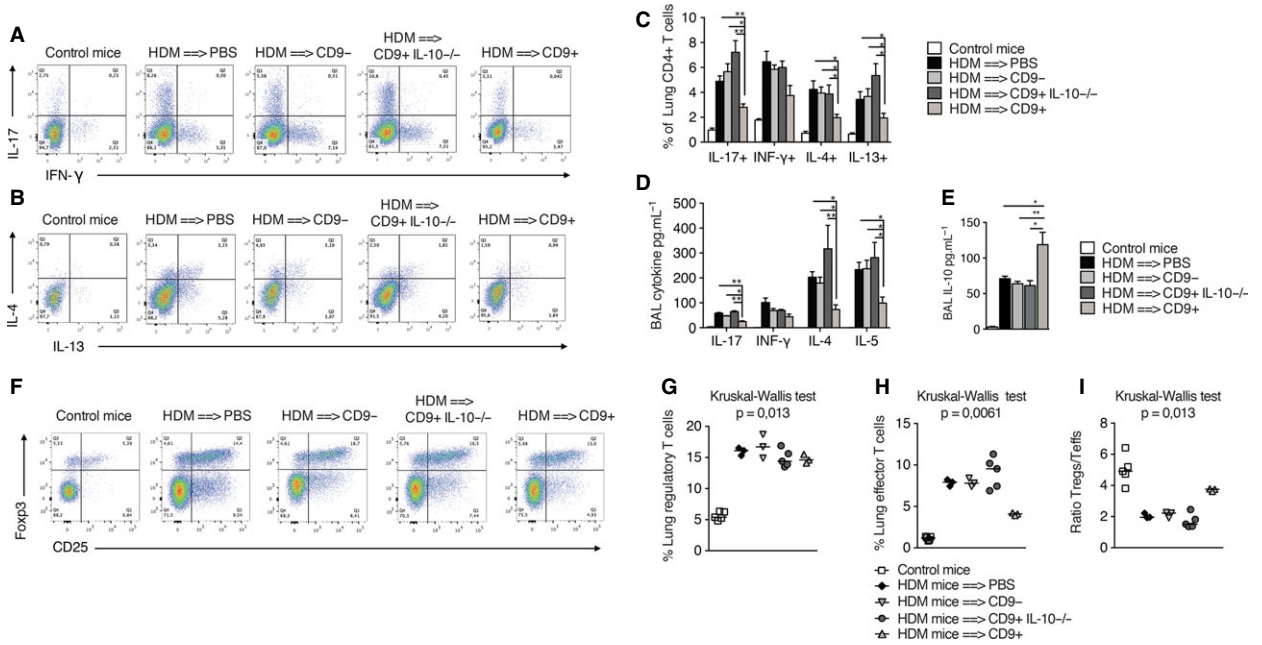

Figure 6 Adoptive transfer of CD9+ B cells influences airway inflammatory T-cell responses. (A–D) Quantification T_H2 and T_H17 responses after supplementation with CD9+ B cells by flow cytometry in lung and multiplex technologies in BAL (n = 5–10 mice per group). (E) Quantification of IL-10 in BAL of mice after supplementation with CD9+ B cells (n = 5–10 mice per group). (F–I) Frequency of regulatory T cells and effector T cells and the regulatory T-cell: effector T-cell ratio in the lungs of control mice and asthmatic mice injected with CD9+ B cells, CD9− B cells, or CD9+ IL-10−/− B cells. $^{**}P < 0.01$, $^{*}P < 0.05$; and ns, nonsignificant.

Fig. 6F,G, adoptive transfer of CD9+ Bregs did not affect the proportion of lung CD25+ Foxp3+ Tregs. Consistent with our findings, we found a decreased frequency of CD25+ Foxp3− effector T cells (Teff) after injection of CD9+ B cells, resulting in a higher Treg/Teff cell ratio (Fig. 6H,I). These data confirm that CD9+ Breg cells prevent the development of asthma by inhibiting allergic airway inflammation via IL-10-dependent mechanisms. Finally, by increasing the Treg/Teff ratio, injection of CD9+ Bregs is likely to contribute to immunological tolerance in allergic airway inflammation.

Discussion

The delicate balance between allergen-induced inflammation and tolerance is crucial for the management of exacerbated inflammatory responses in allergic diseases (22). Tregs have been extensively described as key protective cells against the development of these pathologies (22). However, a major role for IL-10-producing Bregs has also been recently reported in the control of exacerbated inflammation in different animal models as well as humans and uncontrolled inflammation can arise from a Breg deficit (3, 4). Notably, allergen immunotherapy using bee venom (11) or casein (10) was shown to promote the generation of IL-10+ Bregs, thus ensuring the establishment of allergen tolerance. Here, our results demonstrate that Bregs were less prevalent in lungs of mice after allergen exposure confirming that the development of asthma alters the homeostasis of IL-10+ regulatory B cells. Similar results have recently been reported in the context of allergy, in which patients displayed altered Breg functions and a lower frequency of IL-10-secreting B cells when compared to healthy volunteers or allergen-tolerant patients (9, 10). In contrast, we found that exposure to HDM preferentially induced the expression of inflammatory cytokines by B cells. In particular, we detected elevated IL-4 and IL-6 mRNA levels in the lung B cells of allergic mice. These findings fit perfectly with data obtained in experimental autoimmune encephalitis (EAE) and diabetes mouse models, demonstrating a key role for inflammatory B cells in the development of the pathology and maintenance of T-cell-mediated inflammation (23–26). The ability of B cells to preferentially produce inflammatory cytokines under pathogenic conditions suggests that pathogenic B cells and Breg responses might be generated with different kinetics and under specific conditions (23, 25, 26). Future investigations should assess why allergic inflammation alters the differentiation of Bregs and rather favors the emergence of inflammatory B cells. This would give very interesting findings concerning the biology of B cells in allergy.

Contrary to regulatory T cells (Tregs), a definitive characterization of Bregs still remains elusive (3, 4). No specific markers or transcriptional factors permit to characterize these cells, in rodents or in humans. In mice, IL-10+ Bregs have been associated with many phenotypes (15, 16, 27–29). Our microarray and flow cytometric analysis allowed us to identify new surface markers that were significantly and differentially expressed on IL-10+ B cells. In particular, we demonstrated that the expression of CD9 was sufficient to

identify the majority of IL-10$^+$ Bregs in both spleen and lungs of animals. Interestingly, the expression of this molecule was upregulated at the surface of human CD24^{hi} CD38^{hi} immature B cells, a Breg subset described for its capacity to inhibit T-cell-mediated inflammation and induce Tregs *in vitro* (18–20). This supports the idea that CD9 may be used to identify Bregs both in mice and humans.

CD9 is a tetraspanin molecule involved in the enhancement and maintenance of IL-10 secretion in murine and human antigen-presenting cells (30, 31), most likely by enhancing calcium signaling (32), an important pathway for Breg generation (33). CD9 knockout mice do not display abnormalities in their B-cell compartments (34). As previously described, we showed that this marker was essentially expressed at the surface of B1a, T2-MZP, and MZ B cells (35). All of these B-cell subsets have been described to be regulatory (4, 17). Consequently, the expression of CD9 is a good tool to purify B-cell subsets displaying potent regulatory functions in mice (4).

The existence of CD9$^+$ IL-10$^+$ B cells has already been demonstrated in mice (36). However, no data regarding their regulatory capacities have yet been reported. Herein, we establish for the first time a role for CD9$^+$ Bregs in the regulation of allergic asthma inflammation by demonstrating that injection of CD9$^+$ B cells altered the course of HDM-induced airway inflammation and bronchial hyper-reactivity. We showed that injected CD9$^+$ B cells home to the lungs and constrain T_H2- and T_H17-driven inflammation. These results are in accordance with previously published data in humans and mice demonstrating that IL-10-producing Bregs can inhibit T_H17- (20, 37) and T_H2-mediated inflammation (8). The ability of Bregs to inhibit IL-17-producing T cell is of particular interest given the suspected role of IL-17 in severe asthma (12, 38). Administration of splenic CD9$^+$ B cells from IL-10$^{-/-}$ mice did not regulate the asthmatic response mediated by HDM, demonstrating the IL-10-dependent mechanism of CD9$^+$ Bregs *in vivo*. As IL-10 is absolutely required to dampen allergic inflammation at mucosal surfaces such as the gut or lungs (21), our results support an important role for Bregs in allergic diseases.

IL-10-producing Bregs can mediate many immune regulatory mechanisms (3, 4). For example, several studies have demonstrated that IL-10-producing B cells are important for the generation or maintenance of Foxp3 Tregs (5, 8). In our study, the injection of Bregs did not influence Tregs in the lungs. Instead, the Bregs controlled T-cell activation, restoring a favorable immunological balance and most likely leading to less severe inflammation. Similar findings have also supported the idea that IL-10$^+$ Bregs are not necessary for the induction of Tregs (26, 39). However, we cannot exclude here the induction of type-1 regulatory T cells as we observed increased levels of IL-10 in the BAL of CD9$^+$ recipient mice. Additionally, Bregs can limit the maturation of antigen-presenting cells and influence the antigen presentation to T cells by inhibiting of MHC-II and costimulatory molecules (29, 40, 41). Moreover, IL-10-secreting B cells can inhibit the production of IL-6 and IL-23 by dendritic cells *in vitro*, which might explain how Bregs can reduce T_H17 responses *in vivo* (42). Nevertheless, how Bregs regulate T_H2 responses is less clear. Indeed, T_H2 cells might be induced independently of dendritic cells via basophil-dependent or innate lymphoid cell-dependent mechanisms (43). IL-10-producing B cells might therefore regulate T_H2 immunity by acting on other immune components besides dendritic cells.

In conclusion, our data show how the development of asthma modulates the profile of B cells, with a notable alteration of the Breg pool. We identify the CD9 protein as interesting marker to purify both mouse and human Bregs. The ability of Bregs to normalize lung function and airway inflammation points to these cells as an interesting target in allergic diseases. Also, our results emphasize the importance of B cells in asthma, not only as IgE producers but also as suppressive cells able to constrain the pathological process.

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Author contributions

F.B. and J.C., performed experiments, analyzed data, and wrote the study. M.D., C.B., S.D., G.M., and M-A.C. gave support to experiments and analyzed data. A.M. and S.B. designed the study and wrote the study.

Conflicts of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Exposure to HDM induces mixed inflammation in the lungs of mice

Figure S2. Accumulation of memory and germincal center B cells in lungs of asthmatic mice.

Figure S3. Expansion and sorting of IL-10 producing B cells.

Figure S4. Frequency of IL-10 secreting cells in CD9+ B cells.

Figure S5. Gating strategy to identify spleen B cell subsets.

Figure S6. Adoptive transfer of CD9$^+$ B cells.

Table S1. Log10 IL-10+/10- producing B cells from HDM mice

Appendix S1. Supplemental methods.

References

1. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. *J Allergy Clin Immunol* 2012;**129**:635–645.
2. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* 2010;**10**:236–247.
3. Candando KM, Lykken JM, Tedder TF. B10 cell regulation of health and disease. *Immunol Rev* 2014;**259**:259–272.
4. Braza F, Chesne J, Castagnet S, Magnan A, Brouard S. Regulatory functions of B cells in allergic diseases. *Allergy* 2014; **69**:1454–1463.
5. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M 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**:5569–5579.
6. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 2002;**3**:944–950.
7. Jin G, Hamaguchi Y, Matsushita T, Hasegawa M, Le Huu D, Ishiura N et al. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B cells. *J Allergy Clin Immunol* 2013;**131**:1674–1682.
8. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* 2010;**125**:1114–1124.
9. van der Vlugt LE, Labuda LA, Ozir-Fazalalikhan A, Lievers E, Gloudemans AK, Liu KY et al. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. *PLoS ONE* 2012;**7**:e30883.
10. Noh J, Lee JH, Noh G, Bang SY, Kim HS, Choi WS et al. Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br 1) in Cow Milk Allergy. *Cell Immunol* 2010;**264**:143–149.
11. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* 2013;**131**:1204–1212.
12. Chesne J, Braza F, Chadeuf G, Mahay G, Cheminant MA, Loy J et al. Prime role of IL-17A in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model. *J Allergy Clin Immunol* 2015;**35**:1643.
13. Matsushita T, Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. *Methods Mol Biol* 2011;**677**:99–111.
14. Bihouee T, Bouchaud G, Chesne J, Lair D, Rolland-Debord C, Braza F et al. Food allergy enhances allergic asthma in mice. *Respir Res* 2014;**15**:142.
15. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR et al. Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* 2007;**178**:7868–7878.
16. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* 2008;**28**:639–650.
17. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* 2012;**30**:221–241.
18. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 2010;**32**:129–140.
19. Cherukuri A, Rothstein DM, Clark B, Carter CR, Davison A, Hernandez-Fuentes M et al. Immunologic human renal allograft injury associates with an altered IL-10/TNF-alpha expression ratio in regulatory B cells. *J Am Soc Nephrol* 2014;**25**:1575–1585.
20. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* 2013;**5**:173ra23.
21. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity* 2009;**31**:438–449.
22. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;**133**:621–631.
23. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 2012;**209**:1001–1010.
24. Molnarfi N, Schulze-Topphoff U, Weber MS, Patarroyo JC, Prod'homme T, Varrin-Doyer M et al. MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* 2013;**210**:2921–2937.
25. DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR et al. B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. *Proc Natl Acad Sci USA* 2013;**110**:5133–5138.
26. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest* 2008;**118**:3420–3430.
27. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest* 2011;**121**:3645–3656.
28. Maseda D, Candando KM, Smith SH, Kalampokis I, Weaver CT, Plevy SE et al. Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-gamma+CD4+ T cell numbers during colitis development in mice. *J Immunol* 2013;**191**:2780–2795.
29. Matsumoto M, Baba A, Yokota T, Nishikawa H, Ohkawa Y, Kayama H et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity* 2014;**41**:1040–1051.
30. Ha CT, Waterhouse R, Wessells J, Wu JA, Dveksler GS. Binding of pregnancy-specific glycoprotein 17 to CD9 on macrophages induces secretion of IL-10, IL-6, PGE2, and TGF-beta1. *J Leukoc Biol* 2005;**77**:948–957.
31. Peng WM, Yu CF, Kolanus W, Mazzocca A, Bieber T, Kraft S et al. Tetraspanins CD9 and CD81 are molecular partners of trimeric FcvarepsilonRI on human antigen-presenting cells. *Allergy* 2011;**66**:605–611.
32. Halova I, Draberova L, Bambouskova M, Machyna M, Stegurova L, Smrz D et al. Cross-talk between tetraspanin CD9 and transmembrane adaptor protein non-T cell activation linker (NTAL) in mast cell activation and chemotaxis. *J Biol Chem* 2013;**288**:9801–9814.
33. Matsumoto M, Fujii Y, Baba A, Hikida M, Kurosaki T, Baba Y. The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity* 2011;**34**:703–714.
34. Cariappa A, Shoham T, Liu H, Levy S, Boucheix C, Pillai S. The CD9 tetraspanin is not required for the development of peripheral B cells or for humoral immunity. *J Immunol* 2005;**175**:2925–2930.
35. Won W-JJ, Kearney JF. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J Immunol* 2002;**168**:5605–5611.

36. Ostrowski M, Vermeulen M, Zabal O, Zamorano PI, Sadir AM, Geffner JR et al. The early protective thymus-independent antibody response to foot-and-mouth disease virus is mediated by splenic CD9+ B lymphocytes. *J Virol* 2007;**81**:9357–9367.
37. Yoshizaki A, Miyagaki T, DiLillo DJ, Matsushita T, Horikawa M, Kountikov EI et al. Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* 2012;**491**:264–268.
38. Chesne J, Braza F, Mahay G, Brouard S, Aronica M, Magnan A. IL-17 in severe asthma: where do we stand? *Am J Respir Crit Care Med* 2014;**190**:1094–1101.
39. Hoehlig K, Shen P, Lampropoulou V, Roch T, Malissen B, O'Connor R et al. Activation of CD4(+) Foxp3(+) regulatory T cells proceeds normally in the absence of B cells during EAE. *Eur J Immunol* 2012;**42**:1164–1173.
40. O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev* 2008;**223**:114–131.
41. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol* 2010;**185**:2240–2252.
42. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH et al. TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol* 2008;**180**:4763–4773.
43. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 2010;**10**:225–235.