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CD9$^+$ Regulatory B Cells Induce T Cell Apoptosis via IL-10 and Are Reduced in Severe Asthmatic Patients

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CD9 was recently identified as a marker of murine IL-10-competent regulatory B cells. Functional impairments or defects in CD9$^+$ IL-10-secreting regulatory B cells are associated with enhanced asthma-like inflammation and airway hyperresponsiveness. In mouse models, all asthma-related features can be abrogated by CD9$^+$ B cell adoptive transfer. We aimed herein to decipher the profiles, features, and molecular mechanisms of the regulatory properties of CD9$^+$ B cells in human and mouse. The profile of CD9$^+$ B cells was analyzed using blood from severe asthmatic patients and normal and asthmatic mice by flow cytometry. The regulatory effects of mouse CD9$^+$ B cells on effector T cell death, cell cycle arrest, apoptosis, and mitochondrial depolarization were determined using yellow dye, propidium iodide, Annexin V, and JC-1 staining. MAPK phosphorylation was analyzed by western blotting. Patients with severe asthma and asthmatic mice both harbored less CD19$^+$CD9$^+$ B cells, although these cells displayed no defect in their capacity to induce T cell apoptosis. Molecular mechanisms of regulation of CD9$^+$ B cells characterized in mouse showed that they induced effector T cell cycle arrest in sub G0/G1, leading to apoptosis in an IL-10-dependent manner. This process occurred through MAPK phosphorylation and activation of both the intrinsic and extrinsic pathways. This study characterizes the molecular mechanisms underlying the regulation of CD9$^+$ B cells to induce effector T cell apoptosis in mice and humans via IL-10 secretion. Defects in CD9$^+$ B cells in blood from patients with severe asthma reveal new insights into the lack of regulation of inflammation in these patients.

Keywords: regulatory B cells, CD9$^+$ B cells, severe asthma, apoptosis, effector T cells

INTRODUCTION

Beyond their capacity to secrete antibodies, B cells are also able to produce cytokines (1), express major histocompatibility complex and co-stimulatory molecules (2), present antigens (3), and regulate T-cell-mediated immune responses (4). Depending on the specific subset, B cells may thus promote, enhance, and/or regulate inflammation depending on the pro- and anti-inflammatory cytokines secreted, the balance of which influences the immune response (4). It is now well-established that specific B cell subtypes are involved in the maintenance of homeostasis of the immune system and regulate inflammation in pathological situations (5, 6). Also called regulatory B cells (Bregs), these specific B cells have immunosuppressive properties; their functional impairment is associated with exacerbated and/or persistent autoimmune processes (7–11), whereas their presence correlates with a state of tolerance in transplantation (12–17).

Although the presence and role of Bregs are thus clearly evidenced in different models and pathologies, their full molecular characterization remains elusive, primarily because no specific markers or transcription factors have been identified in rodents and humans (18). To date, the most commonly used marker is IL-10 secretion (7, 18). Interestingly, allergic patients display a lower frequency of IL-10-secreting Bregs with altered function than healthy volunteers (HV) or allergen-tolerant patients (19, 20). Patients with allergic asthma harbor a defective expansion of such IL-10–producing B cells in response to lipopolysaccharide (LPS) stimulation and a weaker IL-10 response to house dust mite (HDM) allergen–activated T cells (21–23). We have previously demonstrated that murine IL-10$^+$ Bregs are enriched in a CD9$^+$ B cell subset (24). Induction of allergic asthma in mice alters the homeostasis of IL-10$^+$ Bregs, and adoptive transfer of CD9$^+$ B cells alone is sufficient to abrogate asthma in an IL-10-dependent manner (24). Finally, we have shown that CD9 expression in humans is dramatically increased at the surface of CD24^{hi}CD38^{hi} immature B cells, thus defining an important IL-10 Breg subset (24). This finding has been confirmed by others (25–27), and CD9 thus appears to be a reliable marker for defining both mouse and human Bregs.

We show herein, for the first time, that mouse and human CD9$^+$ B cells elicit regulatory properties through IL-10 secretion, and transitional CD24^{hi}CD38^{hi}B cells expressing CD9 are decreased in the blood of severe asthmatic patients. We report that CD9$^+$ B cells induce effector T cell apoptosis via the secretion of IL-10. In mouse, T cell proliferation is blocked at the sub G0/G1 cell cycle phase, leading to activation of the intrinsic and extrinsic apoptotic pathways via a MAPK-dependent mechanism. These data reveal new insights on the lack of regulation of inflammation in severe asthmatic patients and help pave the way to the discovery of potential novel therapies.

MATERIALS AND METHODS

Asthmatic Patients

This study was performed in accordance with the recommendations of the University Hospital Ethical Committee of Nantes and the Committee for the Protection of Patients from Biologic Risks with written informed consent from all subjects. All subjects provided written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University Hospital Ethical Committee of Nantes and the Committee for the Protection of Patients from Biologic Risks. Blood samples were collected from patients included in the EXPRESA clinical study (NCT00721097), which is a prospective cohort of severe asthmatic patients (28). Pulmonary function tests, clinical data, and blood samples were collected each month for 1 year. Nine severe asthmatic patients were selected within EXPRESA cohort based absence of asthma exacerbation in the previous month, absence of systemic corticosteroids (short and long course) and frozen PBMC samples availability. Ten age- and sex-matched Healthy Volunteers (HV), who were free from atopy, asthma (whatever phenotype), allergic rhinitis, atopic dermatitis any other inflammatory diseases, and drugs (whatever route) were used as controls. All severe asthmatic patients had high doses of inhaled corticosteroids (>1,200 microg of beclometasone or equivalent). In HV, % of predicted FEV1 and its coefficient of variation was assumed to 100% (70–130) and age-matched according to reference lung values consensus (29, 30). ACQ7 score was assumed to be at 0 in HV (**Supplementary Data Table 1**).

Immunophenotyping of Asthmatic Patient Samples

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare, Marolles-en-Hurepoix, France) gradient centrifugation and frozen. Immunophenotyping of PBMCs from 9 severe asthmatic patients was performed using flow cytometry. PBMCs from 10 HV were analyzed as controls. PBMCs were rapidly thawed by placing cryovials at 37°C, washed and stained according to standard protocols using the following mAbs: CD19-BUV395, CD27-BUV737, CD38-BV605, CD24-PerCP-Cy5.5, and CD9-BV510 (BD Biosciences, Le Pont de Claix, France). These markers were used to distinguish CD19$^+$ B lymphocytes, CD19$^+$CD27$^+$ memory cells, CD19$^+$CD27$^-$ naïve cells, CD19$^+$CD24^{hi}CD38^{hi} transitional cells, CD19$^+$CD24$^-$CD38$^+$ plasma cells, and CD19$^+$CD9$^+$ Bregs. For all experiments, dead cells were excluded using the Zombie NIR™ Fixable Viability kit (BioLegend, London, UK). Human anti–IL-10 (BD Biosciences) was used to inhibit the IL-10 pathway. Samples were assessed on a BD LSRFORTESSA X-20 (BD Biosciences, Le Pont de Claix, France), and the data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Asthmatic Mouse Model

Six week-old wild-type BALB/c mice were purchased from Charles River Laboratories (Ecully, France). Allergic inflammation was induced using a total House Dust Mite (HDM) extract (Dermatophagoïdes farinae) provided by Stallergenes (Antony, France), as previously described (31). This study was performed in accordance with the recommendations of the Regional Ethical Committee for Animal Experiments of Pays de la Loire (ceea.2012.77) under accreditation number 3455.

The protocol was approved by the Regional Ethical Committee for Animal Experiments of Pays de la Loire.

Cell Sorting

Six week-old wild-type BALB/c mice were purchased from Charles River Laboratories (Ecully, France). Splenic cells were isolated and stained with the following antibodies for cell sorting by flow cytometry: CD19-APC-H7 (1D3), CD9-BV421 (KMC8), CD4-FITC (GK1.5) (BD Biosciences, Le Pont-de-Claix, France); and CD3-APC (145-2C11), and CD25-PE (PC61.5) (eBioscience, Paris, France). These markers were used to distinguish $CD19^+CD9^-$ non-regulatory B cells, $CD19^+CD9^+$ Breg cells and $CD3^+CD4^+CD25^-$ effector T cells. Cells were sorted on a BD FACSARIA III (BD Biosciences, Le Pont-de-Claix, France).

Cell Activation, Co-culture, and Treatments

Mouse cells (1 million/mL) were cultured for 48 h in RPMI-1640 medium with 10% fetal calf serum and 2 mM glutamine. $CD19^+CD9^-$ non-regulatory B cells or $CD19^+CD9^+$ Breg cells were activated with 2 μg/mL anti-CD40 (HM40-3) (BD Biosciences, Le Pont-de-Claix, France) for 48 h and 10 μg/mL LPS for 5 h. $CD3^+CD4^+CD25^-$ effector T cells were activated with 100 U/mL interleukin-2 (IL-2) for 48 h. Effector T cells were then co-cultured for 48 h with non-regulatory or Breg cells at a ratio of 1:1 and at a concentration of 1 million/ml on plates coated with anti-CD3 (145-2C11) (BD Biosciences, Le Pont-de-Claix, France). T cells alone were cultured as controls. For human co-culture experiments, the same protocol was used except that B cells were activated with 50 ng/mL recombinant human soluble CD40L (R&D Systems Europe, Lille, France) plus 2.5 mg/mL CpG oligodeoxynucleotide 2006 (InvivoGen, San Diego, CA, USA), and T cells were activated with 50 U/mL recombinant IL-2 (SARL Pharmaxie, Aigueperse, France). During co-culture, the cells were treated with 50 nM Z-VAD (R&D Systems Europe, Lille, France) to inhibit apoptosis. To inhibit IL-10 and the transforming growth factor-beta (TGF-β) and Fas pathways, the cells were treated during co-culture with 10 μg/mL anti–IL-10 (BD Biosciences, Le Pont-de-Claix, France), 10 μg/mL anti–TGF-β1 (Abcam, Inc., Cambridge, UK), or 20 μg/mL anti-Fas ligand (R&D Systems Europe, Lille, France), respectively. To block CD9 function, the cells were treated during co-culture with 0.2 μg/μL KMC8 monoclonal antibody (BD Biosciences, Le Pont-de-Claix, France). To activate the IL-10 pathway, the cells were treated with 10 ng/mL recombinant IL-10 (R&D Systems Europe, Lille, France).

Viability, Apoptosis, and Cell Cycle Assays

To determine the percentage of dead $CD3^+CD4^+CD25^-$ effector T cells, the cells were stained using a LIVE/DEAD Fixable Yellow Dead Cell Stain Kit according to the manufacturer's recommendation (Invitrogen, Waltham, US). B cells were removed from the analysis using a gating strategy based on CD19-APC-H7 (1D3) staining. T cell cycle stages were assessed using propidium iodide (PI) staining (Beckman Coulter, Roissy CDG, France) and the percentage of apoptotic T cells by Annexin V-FITC staining (BD Biosciences, Le Pont-de-Claix, France). Samples were run on a BD LSRFORTESSA X-20 (BD Biosciences, Le Pont de Claix, France), and the data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Western Blotting

The analysis of $CD3^+CD4^+CD25^-$ effector T cells protein expression was performed by western blotting after negative selection with MACS columns (Miltenyi Biotec. Paris, France). The following primary antibodies were used: anti-Bid, anti-actin, and anti-cleaved and total caspase 8, 9, and 12 (Cell Signaling Technology, St Quentin en Yvelines, France).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using the potential-sensitive fluorescent probe tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Life Technologies, Saint-Aubin, France). Cells were incubated in Hank's Balanced Salt Solution (Gibco Life Technologies, Saint-Aubin, France) with JC-1 at 5 mg/mL for 30 min at 37°C. CD19-APC-H7 (1D3) antibody staining allowed the removal of B cells from the analysis. Samples were assessed on a BD LSRFORTESSA X-20 (BD Biosciences, Le Pont de Claix, France), and the data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Measurement of Mitogen-Activated Protein Kinase (MAPK) Phosphorylation

After 48 h of co-culture, cells were stained with the following Phosflow antibodies: anti-phospho-p38-PeCy-7 and anti-phospho-JNK-PE (BD Biosciences, Le Pont de Claix, France); anti-phospho-ERK1/2-PE (eBioscience, Paris, France); and CD19-APC-H7 (1D3) antibody to remove B cells from the analysis. Samples were assessed on a BD LSRFORTESSA X-20 (BD Biosciences, Le Pont de Claix, France), and the data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Statistical Analyses

Comparisons of experimental values between the two groups were analyzed using the Mann–Whitney *U*-test. The non-parametric Kruskal–Wallis test with Dunn's posttest were used for comparisons between more than two groups. All statistical analyses were performed using GraphPad Prism v7 (La Jolla, CA, USA).

RESULTS

Severe Asthmatic Patients and Asthmatic Mice Harbor a Defect in $CD19^+CD9^+$ B Cell Frequency

The frequencies of $CD19^+$ B lymphocytes, $CD19^+CD27^+$ memory cells, $CD19^+CD27^-$ naïve cells, $CD19^+CD24^{hi}CD38^{hi}$ transitional cells, $CD19^+CD24^-CD38^+$ plasma cells, and $CD19^+CD9^+$ B cells were assessed by flow cytometry using PBMCs from severe asthmatic patients and HV as controls (gating strategy shown in **Figure 1A**). The total $CD19^+$ B cell

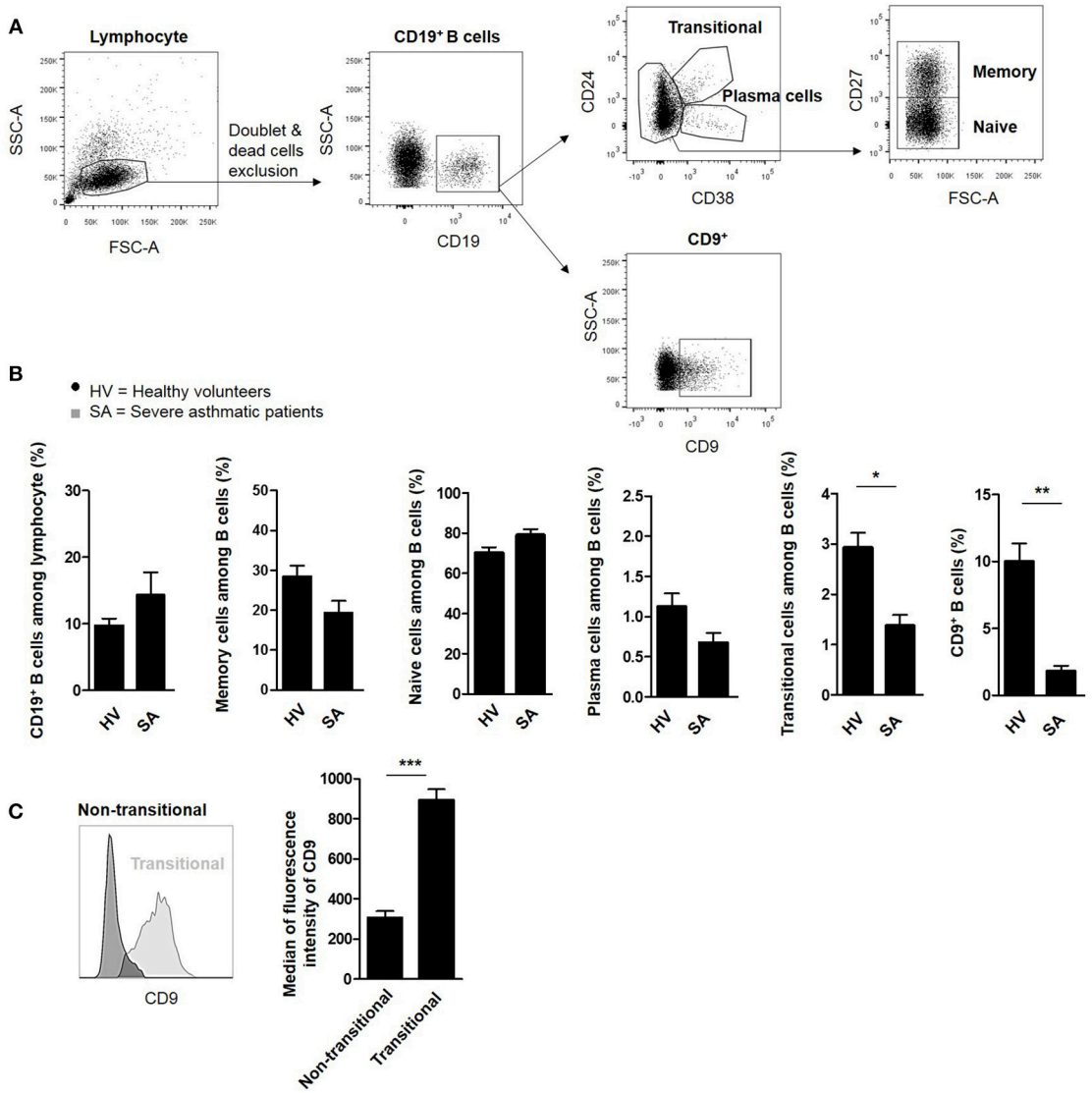

FIGURE 1 | B lymphocyte subpopulations in the blood of severe asthmatic patients. **(A)** Gating strategy used after immunostaining to determine all B cell subsets. **(B)** Assessment of $CD19^+$ B lymphocytes, $CD19^+CD27^+$ memory cells, $CD19^+CD27^-$ naive cells, $CD19^+CD24^-CD38^+$ plasma cells, $CD19^+CD24^{hi}CD38^{hi}$ transitional cells, and $CD9^+$ B cells in 10 healthy volunteers (HV) and 9 severe asthmatic patients (SA) (*$p < 0.05$, **$p < 0.01$). **(C)** Expression of the mean fluorescence intensity of CD9 in transitional and non-transitional B cell subsets (***$p < 0.001$).

frequency was not significantly different between HV and severe asthmatic patients (**Figure 1B**), suggesting the absence of a systemic effect of the treatment. No significant differences were observed for naive, memory and, plasma cell subtypes between the two groups. The frequencies of $CD19^+CD24^{hi}CD38^{hi}$ transitional and $CD19^+CD9^+$ B cells, however, were significantly lower in asthmatic patients than in HV (2.9% ± 0.3 and 9.9% ± 1.3 vs. 1.3% ± 0.2 and 1.8% ± 0.3 for HV and asthmatic patients, respectively, $p < 0.05$ and $p < 0.01$). Interestingly, all $CD19^+CD24^{hi}CD38^{hi}$ transitional B cells expressed CD9 (median fluorescence intensity of CD9 306% ± 34 vs. 894% ± 52 in non-transitional and transitional cells, respectively, $p < 0.001$) (**Figure 1C**), showing that $CD19^+CD24^{hi}CD38^{hi}$ transitional cells were included in the $CD9^+$ B cell subset.

We have previously demonstrated that murine IL-10^+ Bregs are enriched in a $CD9^+$ B cell subset and that adoptive transfer of $CD9^+$ B cells alone is sufficient to abrogate asthma in an IL-10-dependent manner (24). To decipher the regulatory potential of $CD19^+CD9^+$ B cells under inflammatory conditions, allergic asthma was induced in a mouse model using HDM as previously described (31) and summarized in **Figure 2A**. The percentage of $CD19^+CD9^+$ B cells was estimated in the spleen and lung of control and asthmatic mice using flow cytometry (**Figure 2B**). Asthmatic mice had significantly fewer $CD19^+CD9^+$ B cells in the spleen and lung than control mice (4.5% ± 0.3 and 3.1% ± 0.2

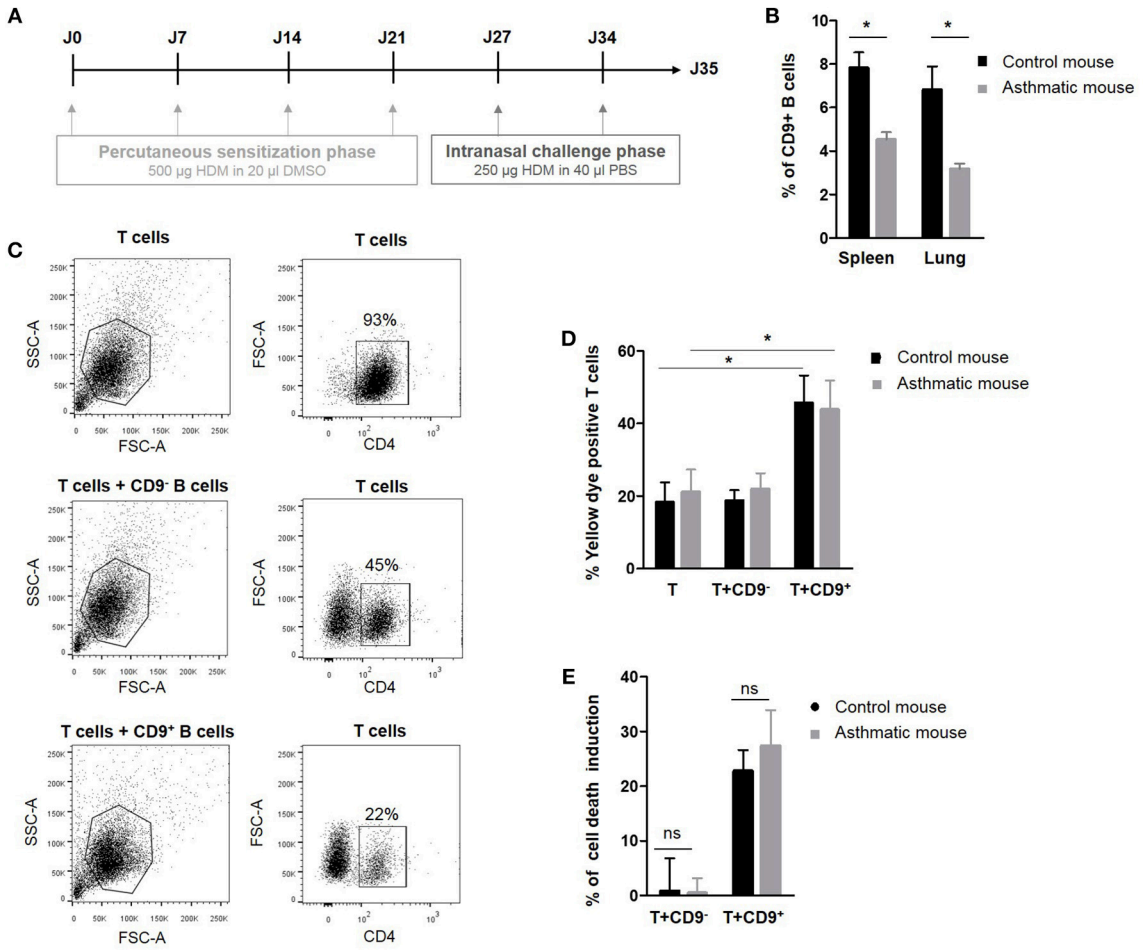

FIGURE 2 | Percentage and regulatory properties of CD9+ B cells in asthmatic mice. **(A)** Induction protocol in asthma mice: House dust mite model. **(B)** Percentage of CD9+ B cells among CD19+ cells in the spleen and lung of control and asthmatic mice ($n = 4$, $^*p < 0.05$). **(C)** Gating strategy used to remove B cells from the analysis by CD4 FITC staining. **(D)** After 48 h of activation, splenic CD3+CD4+CD25− effector T cells from asthmatic and naive Balb-c mice were co-cultured for 48 h with CD19+CD9+ or CD19+CD9− B cells or alone as controls. Cells were stained with yellow dye to measure T cell death induced by CD9+ or CD9− B cells. Percentage of Annexin V-positive T cell staining ($n = 6$, $^*p < 0.05$). **(E)** Percentage of T cell death induction by CD19+CD9+ or CD19+CD9− B cells (ns, non-significant).

vs. 7.8% ± 0.7 and 6.8% ± 1 in the spleen and lung of asthmatic and control mice, respectively, $p < 0.05$). These data validate the mouse as a relevant model for asthma in humans. All together, we report that patients with severe asthma and asthmatic mice both harbor a defect in number of CD19+CD9+ B cells.

CD19+CD9+ B Cells From Asthmatic Mice Harbor no Suppressive Property Defects

The next step was to analyze the regulatory function of CD19+CD9+ B cells in normal and pathologic situations. Thus, we analyzed the effects of CD19+CD9+ B cells from asthmatic and wild type control mice on CD3+CD4+CD25− effector T cell death in co-cultures. To achieve this goal, splenic CD19+CD9− or CD19+CD9+ B cells were activated for 48 h with anti-CD40/LPS. CD3+CD4+CD25− effector T cells were activated for 48 h with IL-2. CD19+CD9− or CD19+CD9+ B cells were then co-cultured for 48 h with CD3+CD4+CD25− effector T cells at a 1:1 ratio, and cell death was measured using yellow dye staining (**Figure 2C**). CD19+CD9+ B cells from asthmatic mice or controls both induced CD3+CD4+CD25− effector T cell death (18.2% ± 5.5 vs. 45.6% ± 7.6 in T cells alone or co-cultured with CD19+CD9+ B cells, respectively, in control mice, $p < 0.01$; 21% ± 6.2 vs. 43.8% ± 8 in T cells alone or co-cultured with CD19+CD9+ B cells, respectively, in asthmatic mice, $p < 0.01$) (**Figure 2D**). Moreover, the percentages of CD3+CD4+CD25− effector T cell death induced by CD19+CD9+ B cells from asthmatic mice or controls were the same (22.7% ± 3.8 vs. 27.3% ± 6.5 in control and asthmatic mice, respectively, non-significant–ns) (**Figure 2E**). Finally, CD19+CD9− B cells from asthmatic mice or controls did not induce CD3+CD4+CD25− effector T cell death (0.8% ± 5.9 vs. 0.4% ± 2.6 in control and

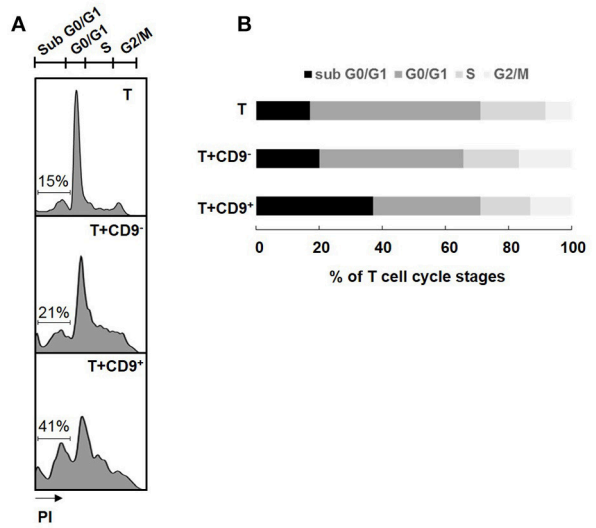

FIGURE 3 | Effects of CD9$^{+/-}$ B cells on T cell proliferation and death. After 48 h of activation, splenic CD3$^+$CD4$^+$CD25$^-$ effector T cells from Balb-c mice were co-cultured for 48 h with CD19$^+$CD9$^+$ or CD19$^+$CD9$^-$ B cells at a 1:1 ratio or alone as controls. Cells were stained with CD4 FITC antibody to remove B cells from the analysis. **(A)** Cells were stained with propidium iodide to measure T cell cycle stages. Representative staining of the different cell cycle stages. **(B)** Percentage of T cells in the different cell cycle stages ($n = 6$).

asthmatic mice, respectively, ns). These data show that although asthmatic mice have reduced number of CD19$^+$CD9$^+$ B cells, these cells display no defects in their capacity to induce T cell apoptosis. Although CD9 has been identified as a marker of B cells that are secreting IL-10, their full regulatory mechanisms remain unknown. Because there was no difference in function between CD19$^+$CD9$^+$ from naïve and asthmatic mouse, we further investigated the regulatory properties of these cells to decipher the molecular pathway leading to T cell death in naïve mice.

CD19$^+$CD9$^+$ B Cells Induce CD3$^+$CD4$^+$CD25$^-$ Effector T Cell Cycle Arrest in subG0/G1 and Cell Death

Determination of the cell cycle stage at which a cell is stopped is a good indicator of the type of cell death induced (32). CD19$^+$CD9$^+$ B cells, but not CD19$^+$CD9$^-$ B cells, induced significant CD3$^+$CD4$^+$CD25$^-$ effector T cell cycle arrest in sub G0/G1, as shown by PI staining (37.8% ± 4.28 and 20.2% ± 3.29 for T cells co-cultured with CD19$^+$CD9$^+$ B cells and T cells co-cultured with CD19$^+$CD9$^-$ B cells, respectively; $p < 0.01$) (**Figures 3A,B**). No difference was observed when T cells were co-cultured with or without CD19$^+$CD9$^-$ B cells. CD3$^+$CD4$^+$CD25$^-$ effector T cell S-phase progression did not appear to be significantly altered under any of the conditions, showing that B cells had no effect on T cell proliferation *per se*. All together, these data show that CD19$^+$CD9$^+$ B cells, but not CD19$^+$CD9$^-$ B cells, induce CD3$^+$CD4$^+$CD25$^-$ effector T cell cycle arrest in sub G0/G1, resulting in cell death.

CD19$^+$CD9$^+$ B Cells Induce CD3$^+$CD4$^+$CD25$^-$ Effector T Cell Apoptosis via an IL10-Dependent Mechanism

Cells in sub G0/G1 display sub-diploid content, which is an indicator of the DNA fragmentation characteristic of apoptotic cells (33). Activated CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured with CD19$^+$CD9$^+$ or CD19$^+$CD9$^-$ B cells for 48 h and then stained with Annexin V (**Figure 4A**). The percentage of Annexin V-positive CD3$^+$CD4$^+$CD25$^-$ effector T cells was significantly higher following co-culture with CD19$^+$CD9$^+$ B cells than that of T cells alone (42.1% ± 5.5 vs. 18.6% ± 3.3, respectively; $p < 0.001$), whereas no difference was observed for T cells co-cultured with CD19$^+$CD9$^-$ B cells (21% ± 6.5; ns) (**Figure 4B**). Treatment with an anti-CD9 agonist had no effect on the T cell death induced by CD19$^+$CD9$^+$ B cells, showing that this effect was not mediated by CD9 (50.3% ± 4.6; ns compared with T cells co-cultured with CD19$^+$CD9$^+$ B cells). Treatment with Z-VAD, a specific inhibitor of apoptotic cell death, blocked the T cell apoptosis induced by CD19$^+$CD9$^+$ B cells (18.8% ± 5.6; $p < 0.01$). Taken together, these data show that CD19$^+$CD9$^+$ B cells induce CD3$^+$CD4$^+$CD25$^-$ effector T cell cycle arrest in sub G0/G1, leading to apoptosis.

To determine whether direct B cell-T cell contact was necessary for CD19$^+$CD9$^+$ B cells to induce T cell apoptosis, CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured with CD19$^+$CD9$^+$ B cells in classical or trans-well plate assays (**Figure 4C**). CD19$^+$CD9$^+$ B cells induced T cell death in both situations (44% ± 4.1 vs. 40% ± 2, respectively, ns). Moreover, the T cell death induced by CD19$^+$CD9$^+$ B cells was not affected when the co-culture was performed in the presence of anti-TGF-β (40% ± 2 vs. 33.8% ± 3.6 with or without anti-TGF-β, respectively, ns) nor anti-Fas ligand (45% ± 5.1, ns compared with T cells co-cultured with CD19$^+$CD9$^+$ B cells). In contrast, anti-IL-10 prevented T cell death and fully restored T cell viability (17.2% ± 1.9 vs. 17.6% ± 4.1 for T cells alone and T cells co-cultured with CD19$^+$CD9$^+$ B cells/anti-IL-10, respectively, ns). Interestingly, CD3$^+$CD4$^+$CD25$^-$ effector T cells treated with IL-10 underwent apoptosis (35% ± 3.4 $p < 0.05$ compared with T cells alone), confirming its involvement in T cell apoptosis induced by CD19$^+$CD9$^+$ B cells. In summary, these data show that T cell-B cell contact is not necessary for T cell apoptosis induction by CD19$^+$CD9$^+$ B cells and that apoptosis induction is dependent on IL-10.

To determine whether the regulatory molecular pathways of CD19$^+$CD9$^+$ B cells is similar in humans, CD19$^+$CD9$^-$ B cells, CD19$^+$CD9$^+$ B cells, and CD3$^+$CD4$^+$CD25$^-$ effector T cells were sorted from the PBMCs of HV. Cells were activated for 48 h (anti-CD40 + CpG for CD19$^+$CD9$^-$ and CD19$^+$CD9$^+$B cells and IL-2 for CD3$^+$CD4$^+$CD25$^-$ effector T cells). CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured for 48 h with CD19$^+$CD9$^-$ B cells or CD19$^+$CD9$^+$ B cells at ratios of 1:1 or alone as controls. The percentage of apoptotic T cells was analyzed by Annexin V staining (**Figure 4D**). As for the mice, only human CD19$^+$CD9$^+$ B cells, but not CD19$^+$CD9$^-$ B cells, induced apoptosis of T cells (2.8% ± 0.8 for T cells alone,

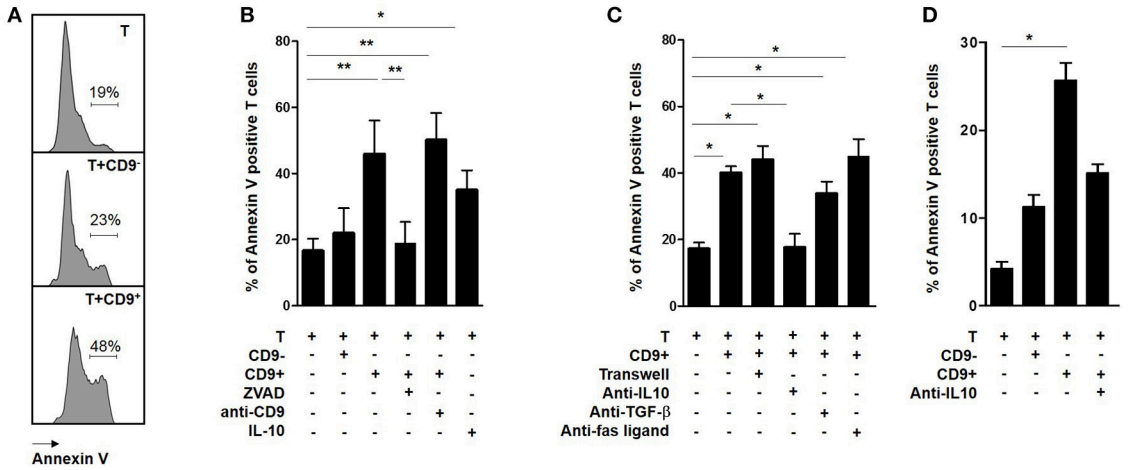

FIGURE 4 | Effects of CD9$^{+/-}$ B cells on T cell apoptosis. **(A–C)** After 48 h of activation, splenic CD3$^+$CD4$^+$CD25$^-$ effector T cells from Balb-c mice were co-cultured for 48 h with CD19$^+$CD9$^+$ or CD19$^+$CD9$^-$ B cells or alone as controls. During co-culture, the cells were also treated with 2 μg/μL KMC8 anti-CD9 antibody, 50 nM Z-VAD, 10 ng/mL IL-10, 10 μg/mL anti–IL-10, 10 μg/mL anti–TGF-β1, or 20 μg/mL anti-Fas ligand. Cells were also co-cultured in trans-wells. The cells were stained with CD4 antibody to remove B cells from the analysis and with Annexin V to measure T cell apoptosis. **(A)** Representative results of Annexin V staining. **(B,C)** Percentage of Annexin V-positive T cells in each co-culture condition ($n = 6$, $^*p < 0.05$, $^{**}p < 0.01$). **(D)** After 48 h of activation, CD3$^+$CD4$^+$CD25$^-$ effector T cells from 9 healthy volunteers were co-cultured for 48 h with CD19$^+$CD9$^+$ or CD19$^+$CD9$^-$ B cells or alone as controls. Cells were also treated with 20 μg/mL anti–IL-10 and stained with CD4 antibody to remove B cells from the analysis and stained with Annexin V to measure T cell apoptosis. Percentage of Annexin V-positive T cells in the different co-culture conditions ($n = 9$; $^*p < 0.05$).

14.3% ± 1.7 for T cells co-cultured with CD19$^+$CD9$^-$ B cells, and 21.4% ± 2.7 for T cells co-cultured with CD19$^+$CD9$^+$ B cells; T cells alone vs. T cells co-cultured with CD19$^+$CD9$^+$ B cells, $p < 0.001$). Treatment with anti-IL-10 reduced apoptosis induced by human T cells (15.7% ± 1.1; T cells alone vs. T cells co-cultured with CD19$^+$CD9$^+$ B cells/anti-IL-10, ns). Taken together, these data show that, as in mice, human CD19$^+$CD9$^+$ B cells induce CD3$^+$CD4$^+$CD25$^-$ effector T cell apoptosis via IL-10.

CD19$^+$CD9$^+$ B Cells Induce Apoptosis of CD3$^+$CD4$^+$CD25$^-$ Effector T Cells Through Extrinsic and Intrinsic Apoptosis Pathways via a MAPK-Dependent Mechanism

To determine the molecular pathways that were activated by CD19$^+$CD9$^+$ B cells, we used flow cytometry to analyze the phosphorylation levels of MAPK p38, extracellular signal–regulated kinases (ERK1/2) and c-Jun N-terminal kinases (JNK), transcription factors that are known to be major activators of apoptosis (34, 35), in CD3$^+$CD4$^+$CD25$^-$ effector T cells after co-culture with or without CD19$^+$CD9$^+$ or CD19$^+$CD9$^-$ B cells. Only CD19$^+$CD9$^+$ B cells, but not CD19$^+$CD9$^-$ B cells, induced phosphorylation of p38, JNK, and ERK1/2 in CD3$^+$CD4$^+$CD25$^-$ effector T cells undergoing apoptosis (**Figure 5A**). Cleavage of caspases 8 and 9 is characteristic of the activation of the extrinsic and intrinsic pathways of apoptosis, respectively. Caspase 12 cleavage is characteristic of endoplasmic reticulum stress. CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured with CD19$^+$CD9$^-$ or CD19$^+$CD9$^+$ B cells and negatively selected by MACS columns to perform western blotting only on T cells (purity, 94% ± 2). CD3$^+$CD4$^+$CD25$^-$ effector T cells cultured alone were used as controls. Both caspase 8 and 9 were cleaved when CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured with CD19$^+$CD9$^+$ B cells (**Figure 5B**) (ratio of cleaved/pro-caspases was 1.5 and 1.4, respectively). Caspases 8 and 9 were also cleaved when CD3$^+$CD4$^+$CD25$^-$ effector T cells were co-cultured with CD19$^+$CD9$^-$ B cells, but to a reduced extent, probably due to the small percentage of Bregs not expressing CD9 (ratio of cleaved/pro-caspases was 0.7 and 0.4, respectively). Caspase 12 was not cleaved under any condition. This finding correlated with the cleavage and activation of the pro-apoptotic protein Bid, linking the intrinsic and extrinsic pathways (**Figure 5C**). Finally, the significantly higher percentage of monomeric JC-1 in CD3$^+$CD4$^+$CD25$^-$ effector T cells co-cultured with CD19$^+$CD9$^+$ B cells confirmed the mitochondrial depolarization of T cells and activation of the intrinsic pathway of apoptosis (48.6% ± 5.7 vs. 28.6% ± 2.5, respectively; $p < 0.05$) (**Figure 5D**). Taken together, these data show that CD19$^+$CD9$^+$ B cells induce CD3$^+$CD4$^+$CD25$^-$ effector T cell apoptosis via both extrinsic and intrinsic pathways through MAPK activation.

DISCUSSION

The prevalence of allergic asthma has dramatically increased worldwide during the last decade. Asthma is a chronic inflammatory disease of the airways associated with airway hyper-responsiveness to inhaled allergens and deregulation of type 2 immunity (36); however, its pathogenesis is still not fully understood. Asthma is characterized by an expansion of CD4$^+$ helper T lymphocytes (Th2, Th17), increased production

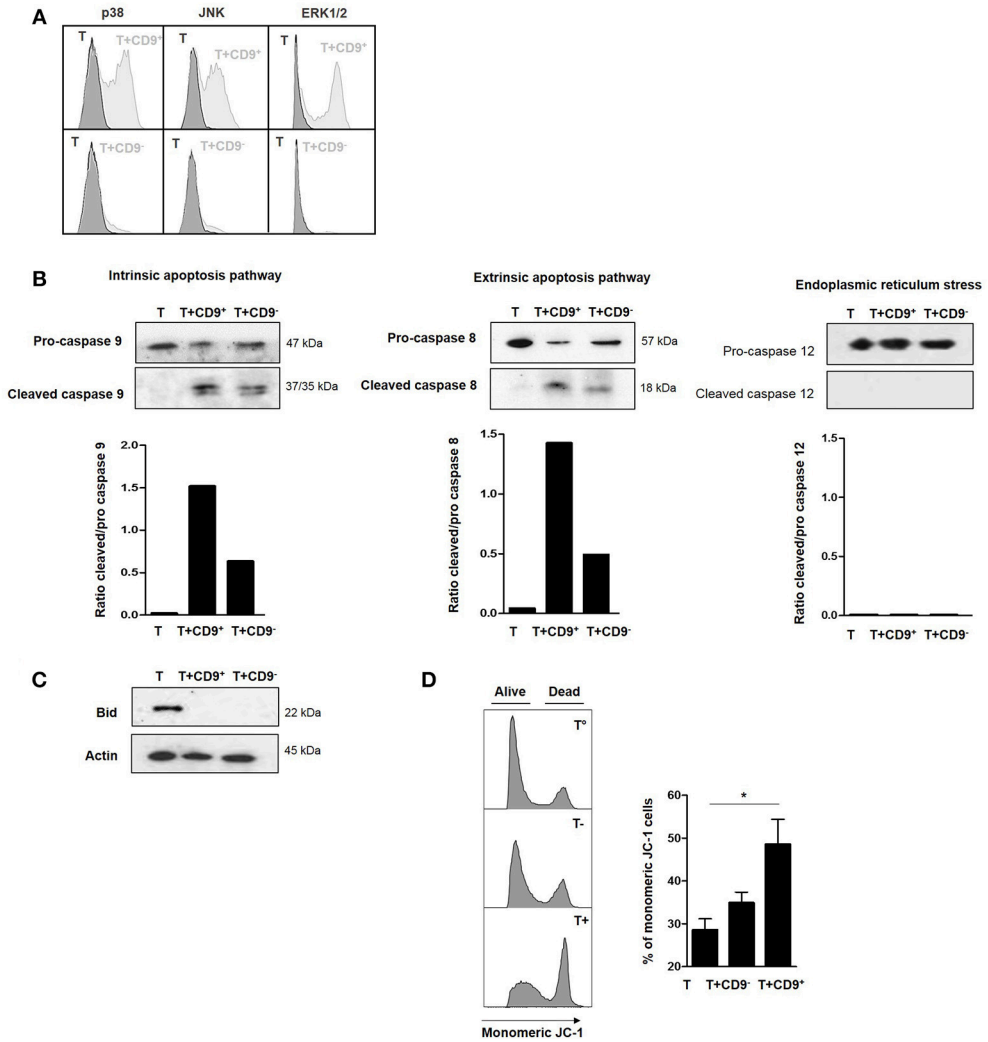

FIGURE 5 | Effects of CD9+ B cells on MAPK phosphorylation, Bid and caspase cleavage and mitochondrial depolarization. After 48 h of activation, splenic CD3+CD4+CD25− effector T cells from Balb-c mice were co-cultured for 48 h with CD19+CD9+, CD19+CD9− B cells or alone as controls. **(A)** B cells were removed from the analysis by CD4 staining. Phosphorylation of MAPK p38, JNK, and ERK1/2 in CD3+CD4+CD25− effector T cells was assessed by flow cytometry. Representative results of MAPK staining. **(B)** CD3+CD4+CD25− effector T cells were negatively selected using MACS columns. Cleavage of caspase 8, 9, and 12 was assessed by western blotting, and the ratios of cleaved/pro-caspases were calculated. **(C)** CD3+CD4+CD25− effector T cells were negatively selected by MACS columns. Bid cleavage was assessed by western blotting. Actin was used as the loading control. **(D)** Mitochondrial depolarization was assessed by JC-1 staining. Representative results of JC-1 staining and percentage of monomeric JC-1 cells in all culture conditions ($n = 5$, $^*p < 0.05$).

of the Th2 cytokines IL-4, IL-5, and IL-13, an increased level of allergen-specific immunoglobulin E (IgE), eosinophilia and inflammation of the airways (37). The roles of other T cells, including natural killer T cells, γδ T cells, and CD8 T cells, has also been reported in asthma, although the relative importance of these different cell populations remains to be confirmed (38). The role of "conventional" B cells, as an actor in immunity, has been clearly demonstrated in asthma (22, 39), and several studies in mice and humans that have also evidenced a role for "specific subsets" of B cells with regulatory properties have reported that these "Breg cells" are involved in the suppression and control of airway inflammation (6, 10). Their numbers are a crucial determinant that clearly contributes to the regulation of the immune system, and their deficiency in asthma is now well-demonstrated (21, 22, 24).

The most commonly used marker of Bregs is their ability to produce and have a suppressive effect via IL-10, but other cytokines or molecules, such as TGF-β, IL-35, and granzyme B, have been shown to mediate regulatory properties on different immune cell targets (1, 12, 14, 23), and no exclusive cell surface marker has yet been evidenced for this Breg population (5, 18). We have previously reported that asthmatic mice are characterized by a lack of IL-10-secreting Bregs enriched in a CD9+ B cell subset. The adoptive transfer of these CD9+ B cells alone is thus sufficient to abrogate asthma in an IL-10-dependent manner (24). The ability of Bregs to normalize lung function and

airway inflammation and the notable alterations of this Breg pool in asthmatic mice clearly point to these cells as an interesting target in allergic diseases.

We demonstrated that these cells regulate effector T cell proliferation, but the mechanisms responsible for such an effect remain unknown. In this study, we demonstrated that CD9$^+$ B cells harbored strong suppressive properties. These cells induced the death of effector T cells via the intrinsic and extrinsic pathways of apoptosis in an IL-10-dependent manner and through the MAPK cascade. We also confirmed these findings, for the first time, in humans and reported that severe asthmatic patients have reduced number of CD9$^+$ B cells with regulatory properties.

CD9 is a tetraspanin molecule that is involved in different mechanisms, including adhesion, migration, cell fusion and membrane signaling (40, 41). Interestingly, CD9 is also involved in the enhancement and maintenance of IL-10 secretion in murine and human antigen-presenting cells (40, 41). We have demonstrated that an IL10$^+$ B cell population is enriched in a CD9$^+$ B cell subset that is decreased in asthmatic mice (24). Similar data have been published by Sun and Matsushita, who identified CD9 as a marker of murine IL-10-competent Bregs (26, 27). It has been reported that the PI3K-Akt pathway is essential for their development and is involved in their regulatory effects in a model of contact hypersensitivity (27). These data confirmed a study conducted by Ostrowski et al. in a model of foot-and-mouth disease virus-infected mice, in which CD9$^+$ B cells were the main source of splenic IL-10 (25). Thus, these data not only clearly support CD9 as a potential biomarker of Bregs but also suggest a possible role in asthma physiopathology, differing from the classical role of B cells as IgE producers. However, no mechanism of action has yet been reported for CD9$^+$ B cells.

The unique link between CD9 and asthma is related to the observation that certain tetraspanins interact with tetrameric high-affinity IgE receptors (FcεRI) on effector cells (42, 43). This interaction is associated with the negative regulation of FcεRI-mediated degranulation of mast cells, which may at least in part explain their possible role in asthma. We suggest herein another or complementary potential mechanism for CD9 Breg cells in this pathology. We report that CD9$^+$ B cells induce T cell cycle arrest in sub G0/G1, leading to the cell death of effector T cells by apoptosis via IL-10 secretion and activation of the MAPK cascade independently from CD9 itself. Apoptosis is a physiologically programmed cell death program that is also involved in defense mechanisms against damaged or stressed cells and cells stimulated by any agent to prevent the accumulation of non-functional cells in tissues (44). Apoptosis may be activated via two different pathways: the intrinsic and extrinsic pathways (45, 46). Following the activation of different signaling cascades, both pathways converge at the final caspase 3 activation step and commonly lead to the cleavage of different proteins leading to cell death. The link between IL-10 and apoptosis is clear, and our data are concordant with those of O'Farrell's data showing that IL-10 increases the expression of p19 to inhibit macrophage proliferation through cell cycle arrest in G1 and implicating the MAPK phosphorylation cascade (47). In our experiments, IL10 alone is able to induce the apoptosis of T cells as CD9$^+$ B cells. IL-10 inhibits the proliferation of other cells with various other effects, such as the induction of endoplasmic reticulum stress (48). These cells did not induce cleavage of caspase 12, which is characteristic of endoplasmic reticulum stress, nor necroptosis, as treatment with necrostatin-1 did not abrogate T cell death (data not shown), confirming the involvement of apoptosis.

The intrinsic apoptosis pathway is initiated by the activation of pro-apoptotic mediators leading to mitochondrial depolarization, the release of cytochrome c and cleavage of caspase 9 (49). The extrinsic pathway is activated when death receptors are ligated, resulting in caspase 8 activation (50). Caspase 8 can directly induce apoptosis or activate the intrinsic pathway by cleavage of Bid. Here, we report that CD9$^+$ B cells induced T cell apoptosis through both the extrinsic and intrinsic pathways, involving caspases 8 and 9 and cleavage of the molecule Bid required for apoptosis activation. Activation of the intrinsic pathway in T cells by CD9$^+$ Bregs was confirmed by mitochondrial depolarization. These data corroborate Bailey's results showing that IL-10 induces apoptosis in mast cells and macrophages via the intrinsic cascade through mechanisms involving caspase 3 cleavage and mitochondrial depolarization (51). Surprisingly, when T cells are co-cultured with non-regulatory CD9- B cells, low levels of caspase 8 and 9, and Bid are cleaved. These phenomena could explain the small but substantial number of apoptotic cells compared with T cells alone. This low level of T cell death is more likely a consequence of CD9 as a known marker of enrichment of IL-10-secreting B cells (88% of total IL-10 secreting B cells) (26) and the presence of a few Breg cells in the CD9$^-$ subset.

We have also confirmed that CD9$^+$ B cells also exist in humans. CD9$^+$ B cells from HV induced apoptosis of autologous effector T cells in an IL-10-dependent manner, as observed for mouse CD9$^+$ B cells. Finally, we report for the first time that CD9$^+$ B cells, which are also associated with a CD19$^+$CD24^{hi}CD38^{hi} transitional B cell phenotype, were reduced in number in the blood of severe asthmatic patients. There is evidence in different models that enhanced activity of Bregs producing IL-10 strongly suppresses inflammatory processes (4–7, 20), and conversely, Van Der Vlugt reported that patients with allergic asthma displayed B cells with impaired regulatory activity (21). Here, we report that the number of CD9$^+$ B cells was reduced in the blood of severe asthmatic patients, as demonstrated in asthmatic mice lacking CD9$^+$ B cells but displaying no functional defect. Furthermore, these cells were able to induce effector T cell apoptosis in the same manner as CD9$^+$ B cells from wild type mice.

Interestingly, we also report herein that severe asthmatic patients had fewer CD9$^+$ B cells not only at basal levels between exacerbations but also following acute inflammation events (data not shown), suggesting that even under a strong inflammatory environment, patients are not able to upregulate CD9$^+$ Breg production. These data suggest that the restoration of a normal number of CD9$^+$ B cells may "correct" a potential defect and suggest that increasing Breg cells in allergic/asthmatic patients may be envisioned as a new therapeutic strategy. In mice, the adoptive transfer of CD9$^+$ B cells reverses asthma

(24), and the adoptive transfer of CD1d^{hi} Bregs suppresses airway inflammation by inducing natural forkhead box protein 3-positive CD4+ regulatory T cell recruitment into the lungs (10). Interestingly, parasites and infectious agents drive Breg expansion and have been proposed as a potential mechanism for reducing host immune responses (19, 52–54). The potential for use of helminth molecules as a new-anti-inflammatory therapy has been postulated using the worm's molecules to generate Breg cells (54, 55). Modulation of anti-inflammatory cytokines (56), the possibility to expand Breg cells *in vitro* (9, 57) or strategies aimed at controlling signaling pathways in favor of *in vivo* Breg expansion (58) have been seriously considered. Compared with allergen-specific immunotherapy or non-specific treatments, such strategies may offer the possibility for a reduced treatment time and superior effects resulting from self-sustaining or the induction of regulatory mechanisms since targeting Breg cells may also embrace other regulatory systems simultaneously, such as IL-10-producing regulatory T cells (19).

In conclusion, our data are the first to characterize the molecular mechanisms of the regulation of CD9$^+$ B cells to induce effector T cell apoptosis in mice and humans. The lack of CD9$^+$ Breg cells in severe asthmatic patients suggests Bregs as a potential target for future therapeutic strategies. A better understanding of the immunological mechanisms underlying allergic diseases is essential for the development of new preventive and therapeutic Breg interventions.

AUTHOR CONTRIBUTIONS

CB, MD, LuC, GB, SB, and AM: study conception and design. CB, MD, and ED: acquisition of data. CB, MD, LuC, GB, SB, and AM: analysis and interpretation of data. All authors critical revision for important intellectual content. CB and MD: statistical analysis. CB, MD, AM, and SB: obtained funding. ED, AF, M-AC, LuC, and MK: administrative, technical, or material support. SB and AM: carried out study supervision. All authors approved the final version of the manuscript prior to submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03034/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1: Demographic and clinical data.

Definition of abbreviations: HV, healthy volunteers; SA, severe asthmatic patients, FEV1, Forced Expiratory Volume in the first second; ACQ, asthma control questionnaire, NA, not applicable.

	HV	SA	p-value
Number of patients	**10**	**9**	NA
Median Age [range]	52 [30-66]	63 [28-70]	0.06
Sex, n (%)			0.06
Male	6 (60)	4 (44)	
Female	4 (40)	5 (56)	
Median % of predicted FEV1 [range]	100 [70-130]	71 [37-109]	0.38
Median ACQ [range]	0 [0-0]	9 [4-21]	NA
Inhaled Corticosteroids, n (%)	0 (0)	9 (100)	NA
Inhaled Salbutamol on demand, n (%)	0 (0)	9 (100)	NA