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IL-10^{+} regulatory B cells mitigate atopic dermatitis by suppressing eosinophil activation

Dajeong Lee[1,5], Min Geun Jo[1,5], Keun Young Min[1], Min Yeong Choi[1], Young Mi kim[2], Hyuk Soon Kim[3]✉ & Wahn Soo Choi[1,4]✉

Atopic dermatitis (AD) presents significant therapeutic challenges due to its poorly understood etiology. Eosinophilia, a hallmark of allergic inflammation, is implicated in AD pathogenesis. Interleukin-10 (IL-10)-producing regulatory B (Breg) cells exhibit potent anti-inflammatory effects. However, their role in controlling AD-related eosinophilia is not well understood. To investigate the impact of eosinophils on AD, we employed IL-5Rα-deficient (*Il5ra*$^{-/-}$) mice, which lack functional eosinophils. Induction of AD in these mice resulted in attenuated disease symptoms, underscoring the critical role of eosinophils in AD development. Additionally, the adoptive transfer of purified Breg cells into mice with AD significantly alleviated disease severity. Mechanistic studies revealed that IL-10 produced by Breg cells directly inhibits eosinophil activation and infiltration into the skin. In vitro experiments further confirmed that Breg cells inhibited eosinophil peroxidase secretion in an IL-10-dependent manner. Our collective findings demonstrate that IL-10 from Breg cells alleviates AD by suppressing eosinophil activation and tissue infiltration. This study elucidates a novel regulatory mechanism of Breg cells, providing a foundation for future Breg-mediated therapeutic strategies for AD.

Keywords Allergic disease, Atopic dermatitis, Regulatory B cells, Eosinophil, Interleukin-10

Atopic dermatitis (AD) is a chronic inflammatory skin condition that significantly impacts quality of life[1]. It is characterized by itching, eczema, and dryness, with symptoms arising from a complex interplay of genetic predisposition and environmental factors[2,3]. The disease has a high prevalence in childhood, affecting approximately 15–30% of children and 2–10% of adults worldwide, thereby constituting a major public health concern[3,4]. Common allergens associated with AD onset include food antigens, house dust mites, and molds, among various other antigens encountered in everyday life[5].

Currently, the predominant treatment strategy for AD primarily focuses on allergen avoidance and nonspecific anti-inflammatory approaches, with corticosteroids being the most commonly used[2]. However, these treatments face challenges in achieving remission due to their limited efficacy in individual cases and potential side effects. Consequently, there is increasing interest in immunoregulatory therapies aimed at inducing peripheral tolerance to causative antigens and mitigating excessive immune responses[2,6]. Recent years have seen a surge in exploring immune regulatory mechanisms for allergic diseases like AD. Despite these advances, the development of effective and safe therapies remains an ongoing challenge[2,6].

In this context, regulatory immune cells offer a promising avenue for alleviating AD through their respective regulatory mechanisms. Regulatory T (Treg) cells, regulatory B (Breg) cells, and regulatory Natural Killer (NKreg) cells have demonstrated therapeutic potential in various immune disorders, including psoriasis and AD[7]. Among these, Treg cells have received considerable attention due to their ability to suppress symptoms in allergic and autoimmune diseases[7]. B cells, traditionally known for their antibody production and effector functions, can also differentiate into immunosuppressive subsets known as Breg cells[8]. Identified in the early 2000s[9–11], Breg cells are defined by their ability to suppress immune responses through secretion of cytokines like IL-10, IL-35, TGF-β, and IL-21[12–14]. Notably, IL-35 selectively inhibits the proliferation of T helper (T_H) 1 and T_H17 cells, leading to broader immune suppression. Additionally, anti-inflammatory cytokines secreted by regulatory immune cells,

[1]School of Medicine, Konkuk University, Chungju 27478, Korea. [2]College of Pharmacy, Duksung Women's University, Seoul 01369, Korea. [3]Department of Biomedical Sciences, College of Natural Science and Department of Health Sciences, The Graduate School of Dong-A University, Busan 49315, Korea. [4]Institute of Biomedical Sciences & Technology, Konkuk University, Seoul 05029, Korea. [5]These authors contributed equally: Dajeong Lee and Min Geun Jo. ✉email: hskimxo@dau.ac.kr; wahnchoi@kku.ac.kr

such as TGF-β and IL-10, can directly inhibit the activation of various immune cells[15]. Within the Breg cell population, a subset of mouse CD1d^{hi}CD5^{+}CD19^{+} B cells exhibit pronounced immune suppressive function mediated by IL-10 secretion[12,16]. Past studies have reported that Breg cells can regulate effector T cells, ILC2, and adipose tissue macrophages in various forms of inflammatory skin conditions[17–19]. However, most of these studies have focused on contact dermatitis or psoriasis, leaving limitations in understanding their role and mechanisms in the AD environment and their direct effects on other effector cells.

Eosinophils, multifunctional leukocytes, play a critical role in allergic diseases and helminth infections[20–22]. These bone marrow-derived cells constitute a small portion (1–5%) of peripheral blood but are abundant in the bone marrow and gastrointestinal tract[21,23]. Most allergic reactions are accompanied by peripheral blood eosinophilia, defined as having more than 450 eosinophils/μL[24]. These infiltrating eosinophils contribute to local inflammation by releasing granules containing various mediators, including cytokines, chemokines, lipid mediators, and cytotoxic proteins such as eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP)[22,23,25,26]. Eosinophilia is associated with a wide range of disorders, including T_H2 cell-mediated allergies, drug reactions, parasitic infections, malignancies, and eosinophilic gastrointestinal diseases[24,25]. Although limited data exist regarding the specific role of eosinophils in AD[26–28], their potential regulation of eosinophils by Breg cells remains virtually unexplored. Evidence suggests that IL-10, by inhibiting eosinophil activation and degranulation, alleviates chronic inflammation and the progression of allergic disease[29–31]. However, due to the short half-life of IL-10 in humans (approximately 2.6 h), alternative therapeutic strategies that explore the administration or promotion of endogenous IL-10-producing cells are needed rather than direct IL-10 administration[32].

This study investigated the potential of Breg cells in AD therapy by exploring the suppressive effects of IL-10-producing Breg cells on eosinophil activation and tissue infiltration within an MC903-induced AD animal model.

Materials and methods

Mice

Eight-week-old female C57BL/c mice were obtained from Orient Bio (Seongnam-si, Gyeonggi-do, Korea), and *Il10*$^{-/-}$ (Il10tm1Cgn) mice and CD19-deficient *Cd19Cre* (Cd19tm1(cre)Cgn) on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). The B6.129P2(C)-Cd19tm1(Cre) Cgn/J mice were crossbred with C57BL/6 mice to produce heterozygous mice. These heterozygous mice were then bred to generate homozygous CD19-deficient B6.129P2(C)-Cd19tm1(Cre) Cgn/J mice, while wild-type C57BL/6 littermates served as control mice for our study. *Il5ra*$^{-/-}$ mice, also on a C57BL/6 background, were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The mice were housed in a specific pathogen-free animal facility at Konkuk University (Seoul, Korea), and were provided with sterilized diet and autoclaved water for the following experiments. This study was conducted and reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Approval number: KU22156). The animal experiments were carried out in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Induction of AD mice by using MC903

Eight-week-old C57BL/6 female mice were treated with calcipotriol (MC903; TOCRIS, Bristol, UK). Based on previous reports by other researchers and our preliminary experiments[33], each mouse received 2 nmol of MC903 dissolved in 20 μl of ethanol (vehicle) applied to the outer and inner surfaces of both ears for 12 consecutive days to induce AD lesions. Ear thickness was measured 24 h post-application using digital vernier calipers by two independent blinded observers.

Preparation and transfer of B cell subset

Splenocytes were isolated from donor mice, from which B cells were derived by pre-sorting with CD19 mAb-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, CD19^{+}CD1d^{hi}CD5^{+} (Breg cells) or CD19^{+}CD1d^{-}CD5^{-} (non-Breg cells) B cells were sorted using FACSAria (Becton Dickinson, San Jose, CA) (Fig. S1). For adoptive transfer, 1.5×10^6 isolated Breg or non-Breg cells were intravenously injected into recipient mice one day prior to the induction of AD with MC903.

Flow cytometry analysis

Single-cell suspensions were isolated from the spleen (SP), cervical lymph nodes (cLN), and ear tissue. Prior to staining for cell surface markers, dead cells and aggregates were excluded by forward and side light scatter analysis, along. Along with staining using the Zombie NIR™ fixable viability kit (BioLegend, San Diego, CA). The following antibodies against mouse surface proteins were used: CD1d (1B1), CD19 (eBio1D3); CD5 (53–7.3) for the detection of Breg cells; CD3 (17A2) and CD4 (GK1.5) for the detection of T cells; c-Kit (2B8) and FcεRIα (MAR-1) for the detection of mast cells; and CD11b (M1/70) for the detection of eosinophils. Fixation and permeabilization were performed using a kit from eBioscience Inc. (San Diego, CA). Additional antibodies for the detection of eosinophils included Siglec-F (S17007L), CCR3 (J073E5), CD69 (H1.2F3), and CD49d (R1-2), sourced from BioLegend (San Diego, CA). Eosinophils analysis was conducted using various FACS strategies based on our laboratory's established gating strategy (Fig. S2). To detect IL-10^{+} B cells isolated from lymphoid tissues, the cells were stimulated with lipopolysaccharide (LPS; 10 μg/ml; Sigma-Aldrich), phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma), and monensin (2 μM; eBioscience) for 5 h. Subsequent cell analysis was performed using a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA) and FlowJo version 10 software (TreeStar, Ashland, OR).

Culture of bone marrow-derived eosinophil (BmEos)

The culture of bone marrow-derived eosinophils was prepared following the method described previously[33]. Briefly, bone marrow cells were cultured at density of 1×10^6 cells/ml in RPMI 1640 medium (Welgene, Korea) supplemented with 20% fetal bovine serum (FBS), 100 IU/ml penicillin, 2 mM L-glutamine, 10 µg/ml streptomycin, 25 mM HEPES, 1× nonessential amino acids, 1 mM sodium pyruvate, and 55 µM 2-ME. Additionally, the culture medium contained 100 ng/ml stem cell factor (SCF; Peprotech, NJ, USA) and 100 ng/ml FLT3 ligand (FLT3-L; Peprotech, NJ, USA) from days 0 to 4. On day 2, complete medium along with cytokines were added to the culture. On day 4, the medium containing SCF and FLT3-L was replaced with medium containing 20 ng/ml recombinant mouse (rm) IL-5 (Peprotech, NJ, USA). On day 8, the cells were transferred to new flasks and maintained in fresh medium supplemented with rmIL-5. From this point forward, one-half of the medium was replaced every other day with fresh medium containing rmIL-5, and the cell concentration was adjusted to 1×10^6 cells/ml during each medium replacement.

In vitro co-culture of regulatory B cells with eosinophils

Mouse B cells were isolated from splenocytes using CD19 mAb-microbeads (Miltenyi Biotec), and Breg cells or non-Breg cells were subsequently sorted by FACSAria. Regulatory B cells were identified as the live $CD19^+CD1d^{hi}CD5^+$ subset using flow cytometry, achieving the purity of over 95%. Bone marrow–derived eosinophils (BmEos) were cultured for 12 days as previously described. Isolated B cells and eosinophils were incubated with IL-5 (20 ng/ml) and lipopolysaccharide (LPS; 10 ng/ml) at 37 °C for 24 h. BmEos (5.0×10^5 cells) were co-cultured with Breg cells at ratios ranging from 1:1 to 1:5 at 37 °C for 24 h. The frequency of $CD69^+$ or $CCR3^+CD49d^+$ eosinophils was then analyzed by flow cytometry.

Histological analysis

The extracted ear tissue was fixed in 4% paraformaldehyde (PFA) and processed into paraffin blocks. The tissues were dehydrated through a series of ethanol solutions with increasing concentrations. Following dehydration, the tissues were washed three times in xylene for 2 min, and then embedded in paraffin. Five-micrometer-thick tissue sections were cut from paraffin blocks and stained with hematoxylin and eosin (H&E).

Eosinophil peroxidase (EPO) activity analysis

The processed culture of BmEos was prepared as described previously[34]. Briefly, after 12 days of culture, BmEos were collected using centrifugation and washed with incomplete media. Cells were then resuspended in phenol-red-free Roswell Park Memorial Institute (RPMI) medium at a concentration of 2×10^4 cells/100 µl per well in 96-well plates and starved for 5 min. Subsequently, 10 µM of platelet-activating factor (PAF C16; TOCRIS, Bristol, UK) was added to appropriate wells to achieve the indicated concentration. The cells were incubated at 37 °C with 5% CO_2 for 30 min and then kept on ice for 5 min to stop the reaction. After the incubation, the supernatant was collected and centrifuged at 1500 rpm for 10 min at 4℃. The eosinophil culture supernatant (100 µl) was incubated in a flat 96-well plate for 10–15 min at 37℃ with 5% CO_2, along with 100 µl of substrate buffer (5 mM o-phenylenediamine, 1 M Tris (pH 8.0), and 30% H_2O_2 in water). The reaction was stopped by adding 100 µl of 4 M H_2SO_4 to each well, and absorbance was determined at 492 nm using a spectrophotometer.

Statistical analysis

Data were expressed as the mean ± SEM. Three or more independent experiments were conducted for both in vitro and animal experiments, with each experiment involving three or more mice per group. Statistical analysis was performed using Student's *t* test or ANOVA with Bonferroni's correction for comparing the means of two numerical values. Data analyses were carried out using commercially available *GraphPad* Prism (GraphPad Software, San Diego, CA, USA). In all comparisons, statistical significance was determined with $^*p<0.05$ and $^{**}p<0.01$.

Results

Alterations in eosinophil population in mice with Atopic Dermatitis

Atopic dermatitis (AD) was induced in mice through the topical application of the vitamin D3 analogue MC903 (calcipotriol) to the ear tissue[35]. Compared to the ethanol-treated control group, it was observed that the thickness of the ears steadily increased with the duration of the application (Fig. 1A). The ear thickness was also discernible by visual inspection (Fig. 1B, right panel). Histological examination of ear tissue revealed the presence of hyperkeratosis and substantial immune cell infiltration in AD-induced mice (Fig. 1B, right panel). Upon induction of AD, a significant increase in the infiltration of $Siglec\text{-}F^+CD11b^+$ eosinophils in the ear tissue was observed compared to the control group (Fig. 1C). Additionally, the total number of eosinophils recruited to the ear tissue, as well as the number of activated eosinophils ($CD69^+Siglec\text{-}F^+CD11b^+$), significantly increased four days after AD induction (Fig. 1D). IL-5, a cytokine essential for the generation and maintenance of eosinophils[26], was investigated in relation to AD onset and eosinophils by inducing AD in *Il5ra*$^{-/-}$ mice, which have almost complete depletion of eosinophils (Fig. S3A). As a result, there was a significant decrease in the number of eosinophils infiltrating into the ear tissue in *Il5ra*$^{-/-}$ mice, and ear swelling was significantly reduced compared to AD-induced wild-type (WT) mice (Fig. S3B). These findings suggest a close association between eosinophil infiltration into the ear tissue in AD mice and the severity of the disease.

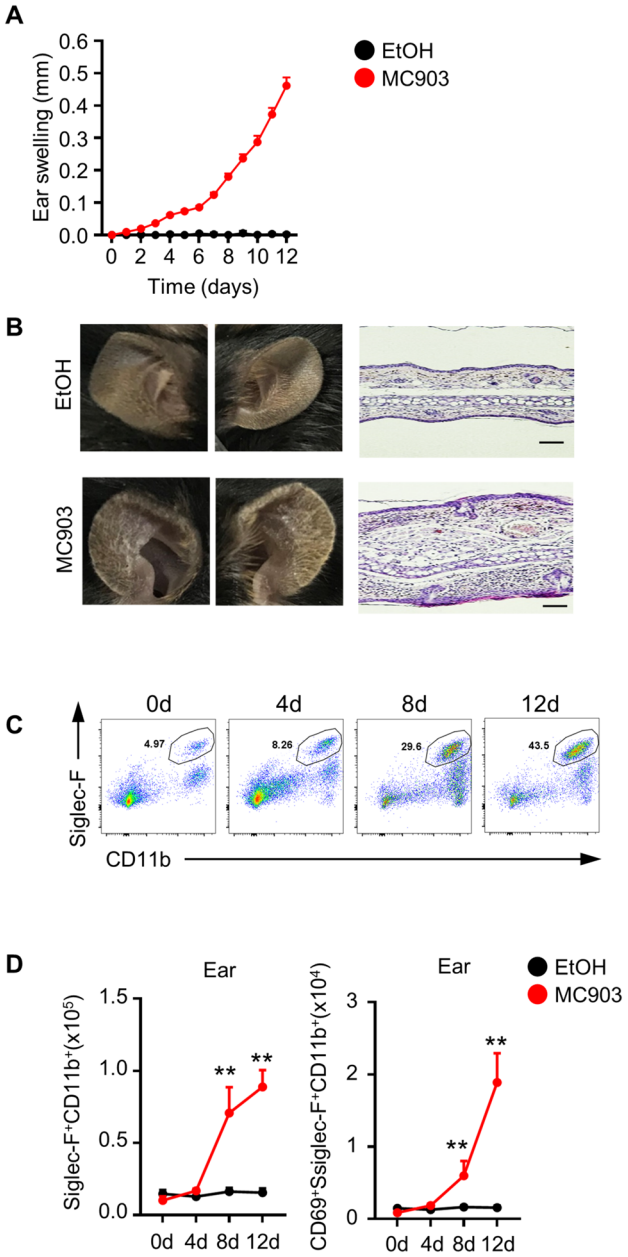

Figure 1. Increased Migration of Eosinophils to the Ear in Mice with AD. (**A**) Ear thickness measurements in WT mice induced with MC903 for 12 days. (**B**) Representative images of mouse symptoms and histology upon induction of AD. H&E staining; scale bar, 100 µm. (**C**) Assessment of eosinophil infiltration levels in the ear following induction of AD, determined by FACS data on a daily basis. (**D**) Graph depicting the numbers of total eosinophils and activated eosinophils infiltrating the ear upon induction of AD. Data are presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

Role of regulatory B cells in mice with Atopic Dermatitis

Next, we investigated the role of Breg cells in AD. It is well established that IL-10^{+} Breg cells are predominantly found within the CD1d^{hi}CD5^{+} Breg subsets in the mouse Spleen[12–14]. For this experiment, we utilized *Cd19Cre* mice, which lack IL-10^{+} Breg cells[14]. When AD was induced in *Cd19Cre* mice, there was a significant increase in ear swelling compared to WT mice (Fig. 2A). To assess the impact of adoptive transfer, we sorted and applied CD1d^{hi}CD5^{+} Breg cells (Fig. S3). Adoptive transfer of WT Breg cells into *Cd19Cre* mice significantly suppressed ear swelling compared to PBS-treated *Cd19Cre* AD mice (Fig. 2A). In contrast, the transfer of WT non-Breg cells (CD1d^{-}CD5^{-}) into *Cd19Cre* mice did not suppress ear swelling (Fig. 2A). These findings suggest that Breg cells possess the ability to alleviate AD symptoms.

Subsequently, we examined the suppressive effect of Breg cells on eosinophilia in the ear tissue induced by AD. When AD was induced in *Cd19Cre* mice, there was a significant increase in eosinophil infiltration into the

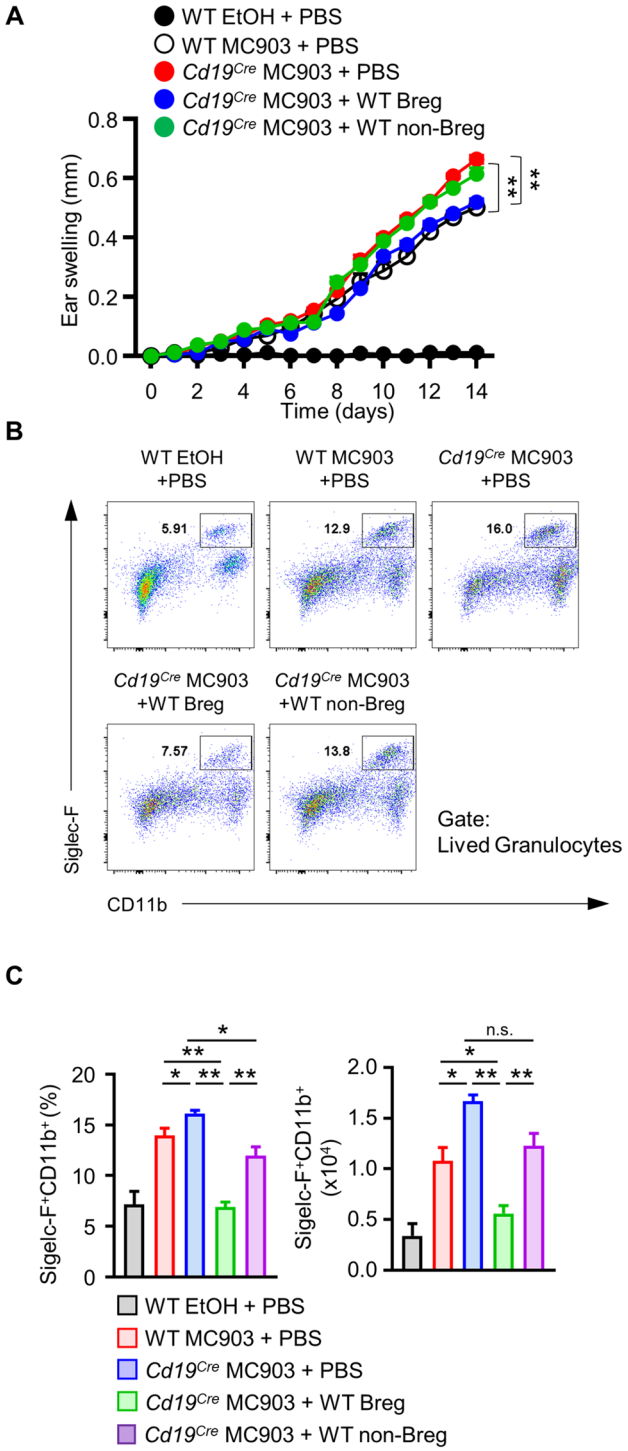

Figure 2. Regulatory B Cells Modulate Ear Swelling and Eosinophil Migration into the Ear in Mice with AD. (**A**) Ear thickness measurements in WT or *Cd19Cre* mice with AD. Additional groups include *Cd19Cre* mice with adoptive transfer of WT Breg cells or non-Breg cells followed by induction of AD. (**B**) Representative FACS images illustrating the extent of eosinophil infiltration in the ear of each group from three independent experiments. (**C**) Frequency and counts of infiltrating eosinophils from panel B. Data are presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

ear tissue compared to WT mice (Fig. 2B). However, as expected, adoptive transfer of Breg cells significantly reduced both the frequency and cell count of eosinophils. In contrast, the group receiving adoptive transfer of non-Breg cells showed no significant effect compared to PBS-treated *Cd19Cre* AD mice (Fig. 2C). These findings

indicate that Breg cells alleviate AD symptoms by suppressing eosinophil infiltration and activation in ear tissues induced by MC903.

Control of eosinophil activation by IL-10$^+$ Breg cells in Atopic Dermatitis mice

IL-10 is a well-known anti-inflammatory cytokine that suppresses symptoms in various immune disorders[36]. Therefore, to investigate whether IL-10 derived from Breg cells inhibits eosinophil infiltration and activation in the ear tissue of AD, we conducted experiments using *Il10*$^{-/-}$ mice. Additionally, we examined the roles of surface proteins, C–C chemokine receptor type 3 (CCR3) and CD49d (integrin alpha subunit), which are crucial for the migration of activated eosinophils to ear tissue in AD, as unique activated phenotypes[24,26,37,38]. Initially, *Il10*$^{-/-}$ AD mice exhibited increased severity of ear thickness compared to WT AD mice (Fig. 3A). This was accompanied by an increased number of eosinophils and active phenotypes within peripheral ear tissue (Fig. 3B and C). Further, we induced AD in *Il10*$^{-/-}$ mice and analyzed AD symptoms and eosinophil populations following the adoptive transfer of WT or *Il10*$^{-/-}$ Breg cells. The transfer of WT Breg cells significantly reduced the severity of AD symptoms; however, this inhibitory effect was not observed with the transfer of *Il10*$^{-/-}$ Breg cells, indicating that the in vivo regulatory effect of Bregs on AD is IL-10-dependent (Fig. 3A). Additionally, no significant difference was observed in eosinophil populations within the spleen, a secondary lymphoid organ, following the transfer of WT or *Il10*$^{-/-}$ Breg cells (Fig. 3B and C, upper panels). However, upon AD induction and the adoptive transfer of WT Breg cells into *Il10*$^{-/-}$ mice, significant reductions in both the frequency and number of total eosinophils and activated phenotypes (CCR3$^+$CD49d$^+$) were observed in the draining lymph nodes (cLN) (Figs. 3B and 3C, middle panels) and peripheral ear tissue (Fig. 3B and C, lower panels). Conversely, these inhibitory effects were not observed with the adoptive transfer of *Il10*$^{-/-}$ Breg cells (Fig. 3B and C). Taken together, these findings suggest that in vivo Breg cells regulate eosinophil proliferation and activation in an IL-10-dependent manner, thereby impacting the severity of AD.

IL-10$^+$ Breg cells directly suppress the expression of eosinophil migration markers

We further investigated the mechanism by which Breg cells inhibit eosinophil infiltration and activation through an in vitro co-culture experiment. Bone marrow-derived eosinophils were cultured with splenic CD1d^{hi}CD5$^+$ Breg cells at ratios ranging from 1:1 to 1:5 for 24 h, and changes in the distribution of CCR3$^+$CD49d$^+$ eosinophils were measured through flow cytometric analysis with or without IL-10 monoclonal antibodies (Fig. 4A). Our results demonstrated that the frequency of CCR3$^+$CD49d$^+$ eosinophils decreased in a Breg cell ratio-dependent manner (Fig. 4B). However, this inhibitory effect of Breg cells on CCR3$^+$CD49d$^+$ eosinophils was reversed by neutralization with IL-10 monoclonal antibodies (Fig. 4B). These findings provide evidence that IL-10 secreted by Breg cells directly regulates the expression of cell surface molecules involved in eosinophil homing to peripheral tissues.

Effect of Breg cells on eosinophil degranulation in mice

In the results presented in Fig. 3, we observed that IL-10$^+$ Breg cells reduce the severity of AD and suppress the infiltration of eosinophils homing to the peripheral tissues. To further investigate whether IL-10$^+$ Breg cells also suppress the effector function of eosinophils, specifically degranulation, we delivered WT or *Il10*$^{-/-}$ Breg cells into *Il10*$^{-/-}$ AD mice. Upon activation, eosinophils express CD69, CD44, L-selectin, and CD54 on their cell surface. Notably, CD69 is well-established as a representative marker for the degranulation of activated eosinophils[39,40]. We induced AD in *Il10*$^{-/-}$ mice, compared the systemic effects following the adoptive transfer of WT Breg cells, and observed a significant reduction in the frequencies and numbers of degranulated eosinophils primarily mainly in the cervical lymph nodes (cLN) and ear tissue. Conversely, significant suppression of eosinophil degranulation was not observed upon adoptive transfer of *Il10*$^{-/-}$ Breg cells (Fig. 5). These findings suggest that Breg cells not only suppress eosinophil infiltration into ear tissue but also inhibit their degranulation in mice.

Inhibition of eosinophil degranulation by Breg cells in an IL-10 dependent manner

To further investigate whether Breg cells exhibit inhibitory effects on eosinophil degranulation, we conducted in vitro co-culture experiments. Eosinophil peroxidase (EPO) is a representative protein released from intracellular granules upon eosinophil activation[21,24,41]. Extracellular secretion of EPO can induce inflammatory responses and damage normal tissues[42]. Additionally, EPO is known to stimulate mast cell degranulation and histamine release in mice[21,43]. First, we examined whether IL-10 has an inhibitory effect on eosinophil degranulation. IL-10 dose-dependently suppressed EPO secretion from eosinophils stimulated by platelet activating factor (PAF) (Fig. 6A). Furthermore, in co-culture experiments with WT or *Il10*$^{-/-}$ Breg cells, WT Breg cells inhibited EPO secretion from eosinophils in a ratio-dependent manner, whereas *Il10*$^{-/-}$ Breg cells did not exhibit such inhibitory effects (Fig. 6B). These findings indicate that the regulation of eosinophil degranulation is mediated by IL-10 derived from Breg cells.

Discussion

The prevalence of allergic diseases such as asthma, allergic rhinitis, food allergies, and AD is increasing worldwide[44,45]. An increase in the numbers of mast cells, which distribute to the target tissues of the disease, is commonly observed in both humans and animal models of allergic diseases[46]. According to previous studies, eosinophils have been reported to play a crucial role in the expulsion of intestinal parasites[3,46]. However, excessive accumulation of eosinophils in tissues can lead to chronic allergic inflammation and severe allergic reactions[24]. Consistent with previous reports, we observed an increase in the population of eosinophils in the ear tissue, a target site in MC903-induced AD mice (Fig. 1C and D). Deficiency of eosinophils in the MC903-induced AD model led to a significant suppression of disease symptoms (Fig. S3B). These results indicate the

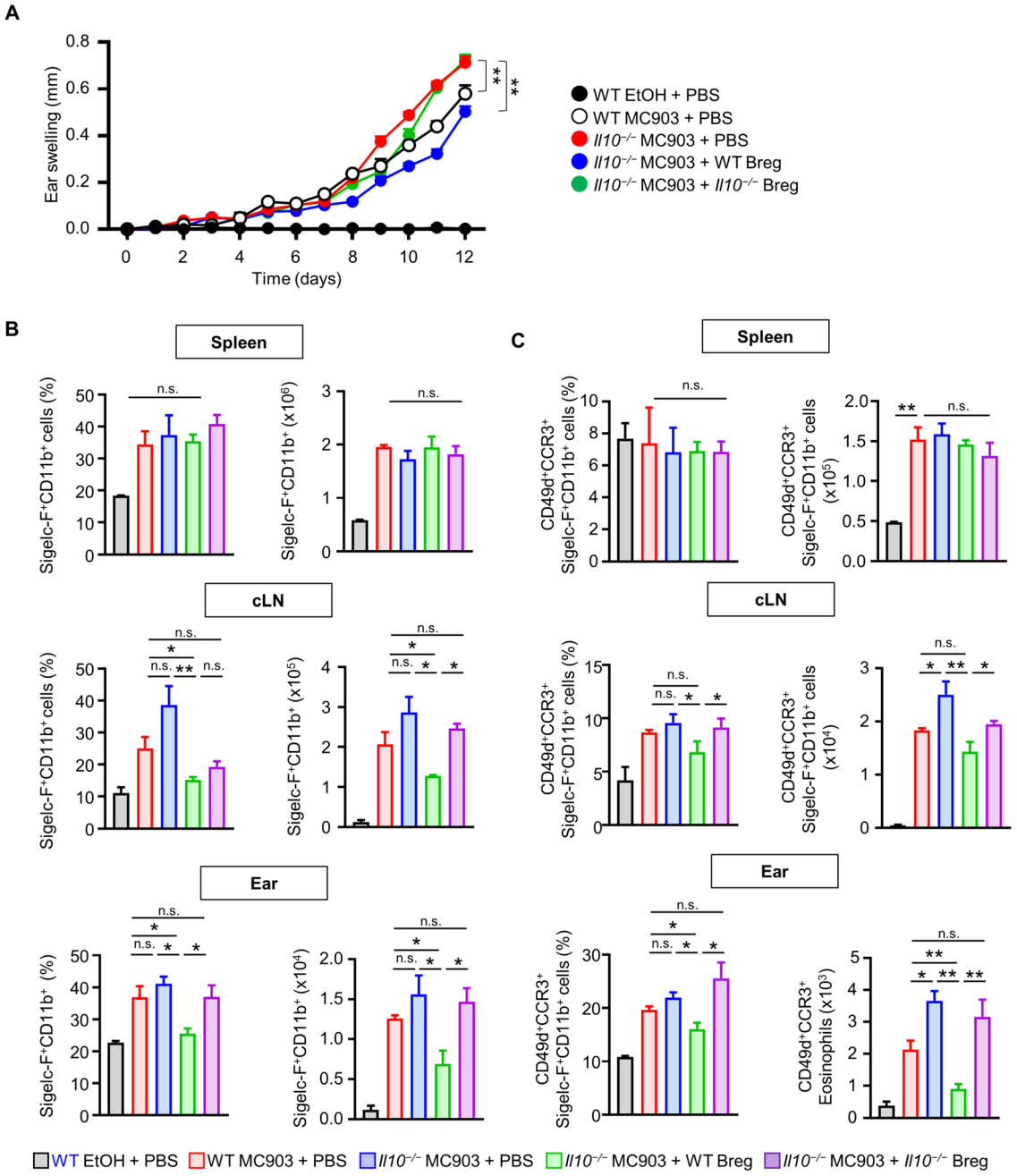

Figure 3. IL-10 Dependency in Regulatory B Cell-Mediated Inhibition of Ear Swelling and Eosinophil Migration in AD Mice. (**A**) Ear thicknesses in WT and *Il10*$^{-/-}$ mice with AD, with adoptive transfer of WT or *Il10*$^{-/-}$ Breg cells. (**B**) Graphs depicting changes in eosinophil frequency and counts in SP, cLN, and ear across groups. (**C**) Graphs showing changes in eosinophils expressing CD49d and CCR3, key infiltration markers, in SP, cLN, and ear. Data were presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. *$p < 0.05$; **$p < 0.01$.

crucial involvement of eosinophils in the development of AD, prompting several multinational pharmaceutical companies to conduct clinical trials targeting eosinophils for the treatment of AD and other allergic diseases[47]. However, therapies targeting the cytokine IL-5, particularly treatment involving Mepolizumab, have shown limited clinical efficacy[48]. Specifically, Oldhoff et al. reported that while Mepolizumab injections reduced circulating eosinophils, the decrease in eosinophil numbers in tissues was minimal[49]. Furthermore, our experimental results suggested that there are diverse mechanisms including eosinophil infiltration underlying ear AD symptoms induced by MC903 (Fig. S3). Therefore, there is an urgent need for the development of treatment strategies focusing not only on blocking IL-5 signaling but also on inhibiting other mechanisms that stimulate AD symptoms.

It has been reported that IL-10-producing Breg cells suppress the onset of various inflammatory murine diseases, such as anaphylaxis and experimental autoimmune encephalomyelitis (EAE)[50,51]. Similarly, we

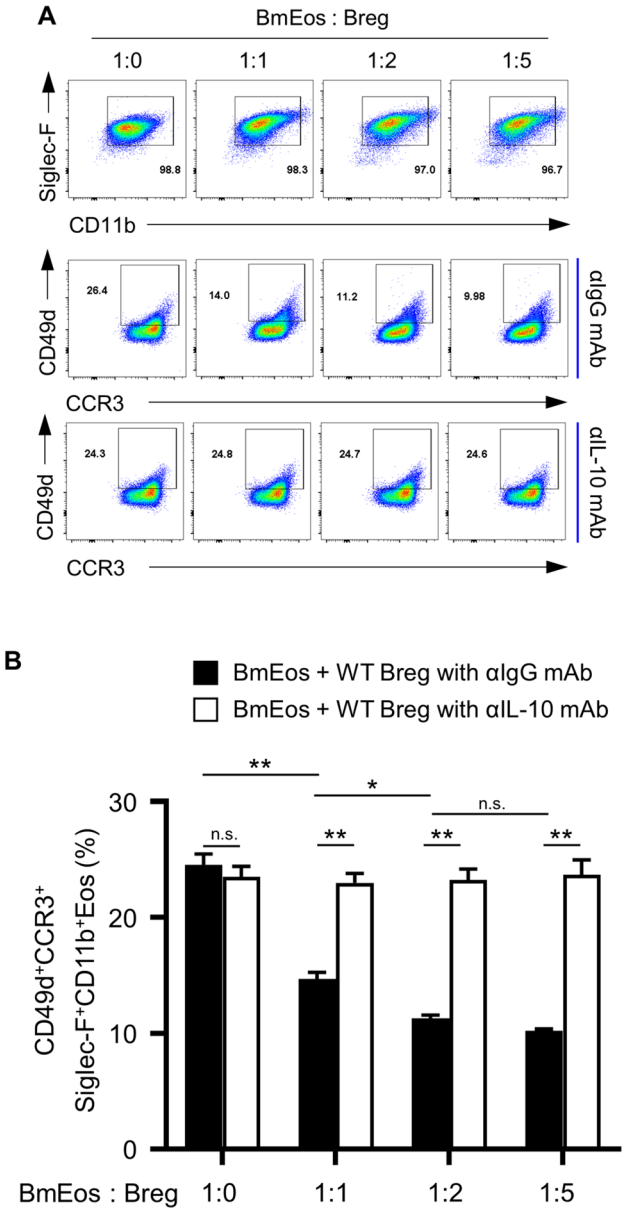

Figure 4. Inhibition of Eosinophil Migration Marker Expression by Breg Cells in an IL-10 Dependent Manner. (**A**) In vitro co-culture of bone marrow-derived eosinophils (BmEos) and sorted Breg cells at various ratios. Some cultures included the addition of anti-IL-10 antibody as indicated. Changes in eosinophil infiltration marker expression were assessed by FACS analysis. Representative images are shown from three independent experiments. (**B**) Graphical representation of the degree of activation of BmEos during co-culture with Breg cells from panel A. Data are presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

have observed that IL-10-producing Breg cells suppress the symptoms of allergic diseases, such as contact hypersensitivity and food allergies[52,53]. However, the correlation between IL-10-producing Breg cells and eosinophils in the manifestation of symptoms in AD still remains unclear. Therefore, we propose that IL-10-producing Breg cells may also play a significant role in suppressing symptoms of AD. CD19 is an essential surface marker protein for regulating B cell responses[54]. Unlike the normal follicular B cell subset generated in *Cd19Cre* mice, the IL-10-producing Breg cells subset is scarcely detectable in *Cd19Cre* (Breg cell-deficient) mice[55]. Based on this evidence, *Cd19Cre* mice have been utilized to ascertain the role of IL-10-producing B cells in various disorders exacerbated by the absence of such cells[51]. In this study, we observed that the symptoms of AD induced by MC903 were significantly more severe in *Cd19Cre* mice compared to WT mice (Fig. 2), suggesting that Breg cells inhibit the symptoms of AD induced by MC903. Furthermore, when Breg cells were adoptively transferred into *Cd19Cre* mice, the symptoms of AD were alleviated to the level observed in WT mice (Fig. 2B). Additionally, it was noted that both the infiltration and activation of eosinophils, which serve as one of effector

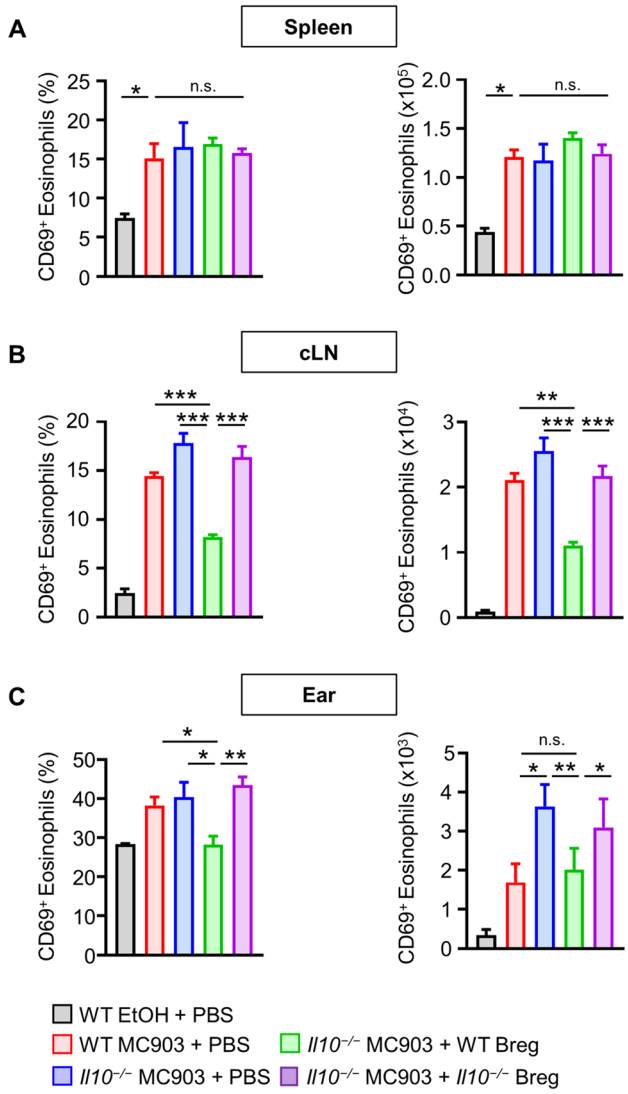

Figure 5. Inhibition of Eosinophil Degranulation Marker Protein Expression by Breg Cells in Mice. Following induction of AD by MC903 in each group except the EtOH control group, the frequency and counts of activated eosinophils were assessed in the SP (**A**), cLN (**B**), and ears (**C**) by FACS analysis. Data are presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

cells in the onset of AD, were inhibited by Breg cells (Fig. 2C). However, it also needs to note that compared to the group that was administered PBS in CD19 deficient mice, the percentage of the eosinophil population in the group that was administered non-Breg cells decreased slightly but significantly, but there was no significance in their numbers. Therefore, we speculated that the results could be due to the effects of a small number of other regulatory immune cells that may be included in the population of non-Breg cells. Anyway, $CD1d^{hi}CD5^{+}$ B cells containing IL-10^{+} Breg cells exhibited a mitigating effect on AD, whereas $CD1d^{-}CD5^{-}$ B cells (non-Breg) did not show any alleviation of AD symptoms (Fig. 2). These findings indicate that Breg cells contributes to the amelioration of AD symptoms.

IL-10, initially discovered as an inhibitory cytokine derived from T_H2 cells, is widely recognized for its pivotal role in attenuating inflammatory responses[56]. It has been reported to suppress the inflammatory effector functions of various immune cells, including T cells, macrophages, and others, in immune responses[57,58]. To explore this further, we conducted experiments inducing MC903-induced AD in *Il10*$^{-/-}$ mice. Our results demonstrated that the symptoms of AD were significantly more severe in *Il10*$^{-/-}$ mice compared to WT mice (Fig. 3A), indicating that IL-10 in mice has the activity of suppressing AD symptoms induced by MC903. Next, adoptive transfer of WT Breg cells suppressed symptoms, while transfer of *Il10*$^{-/-}$ Breg cells did not (Fig. 3A), emphasizing the essential role of IL-10 derived from Breg cells in mitigating the symptoms of AD.

We next examined the impact of IL-10 produced by Breg cells on eosinophils. Our findings revealed that the injection of Breg cells did not affect eosinophil levels in the spleen but significantly reduced their presence in the cLN and ear. Specifically, Breg cells, but not *Il10*$^{-/-}$ Breg cells, suppressed the expression of CD49d, a key protein

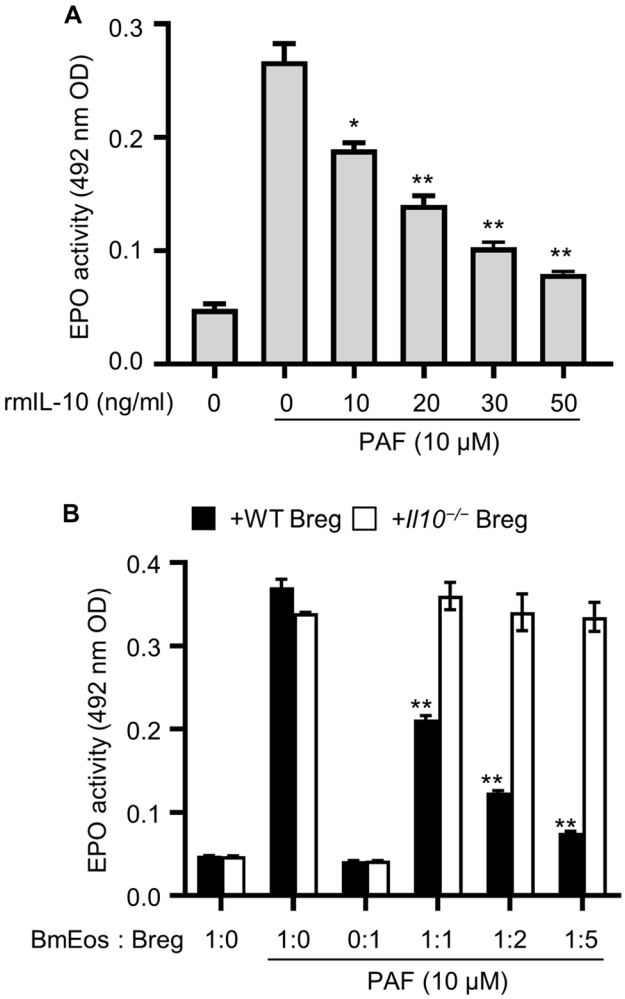

Figure 6. Suppression of Eosinophil Degranulation by Breg Cells in an IL-10 Dependent Manner. Breg cells and eosinophils were sorted out separately and subjected to in vitro experiments to assess the inhibitory response. (**A**) Graph depicting the change in eosinophil peroxidase (EPO) activity when eosinophils were treated with IL-10 as indicated. (**B**) Graph showing the change in EPO activity when bone marrow-derived eosinophils (BmEos) were co-cultured with sorted Breg cells from wild-type (WT) and *Il10*$^{-/-}$ mice. Data are presented as the mean ± SEM obtained from 3 individual experiments. n = 5 mice per group. *$p < 0.05$; **$p < 0.01$.

for eosinophil migration (Fig. 3C). These results suggest that IL-10 from Breg cells inhibits CD49d expression on eosinophils in the cLN, thereby reducing eosinophil migration to the ear tissue in AD.

Our findings indicate that Breg cells suppress AD symptoms by inhibiting eosinophil migration in an IL-10-dependent manner. We further investigated this inhibitory effect of Breg cells on eosinophils in vitro. CD49d and CCR3 are crucial for the migration of activated eosinophils to ear tissue in AD. Reduced expression of CD49d, an activation marker for eosinophils, has been associated with the alleviation of AD symptoms[59]. In our study, co-culturing BmEos with Breg cells led to decreased CD49d expression on eosinophils (Fig. 4). The inhibitory effect of Breg cells was dependent on IL-10, as it disappeared upon treatment with IL-10 monoclonal antibodies (Fig. 4). Furthermore, CD69, along with CD44, L-selectin, and CD54, is expressed on activated eosinophils as a surface marker. CD69 is particularly recognized as a marker protein for eosinophil degranulation upon activation, with its decrease indicating reduced activation and degranulation levels in eosinophils. In the mouse AD model, the adoptive transfer of WT Breg cells, but not *Il10*$^{-/-}$ Breg cells, suppressed the expression of CD69 on eosinophils in both the cLN and ear tissue (Fig. 5). This result underscores that Breg cells inhibit eosinophil activation in mouse AD in an IL-10 dependent mechanism.

To further elucidate whether Breg cells directly inhibit eosinophil degranulation, we conducted in vitro experiments on EPO secretion. EPO, a representative cytotoxic granule protein secreted by eosinophils upon stimulation, is well-known for inducing allergic inflammation[24]. Our EPO activity analysis showed that co-culturing WT Breg cells with BmEos resulted in the suppression of EPO secretion (Fig. 6B). However, this inhibitory effect was not observed when BmEos were co-cultured with *Il10*$^{-/-}$ Breg cells (Fig. 6). These cumulative findings indicate that IL-10 produced by Breg cells suppresses eosinophil degranulation and EPO secretion.

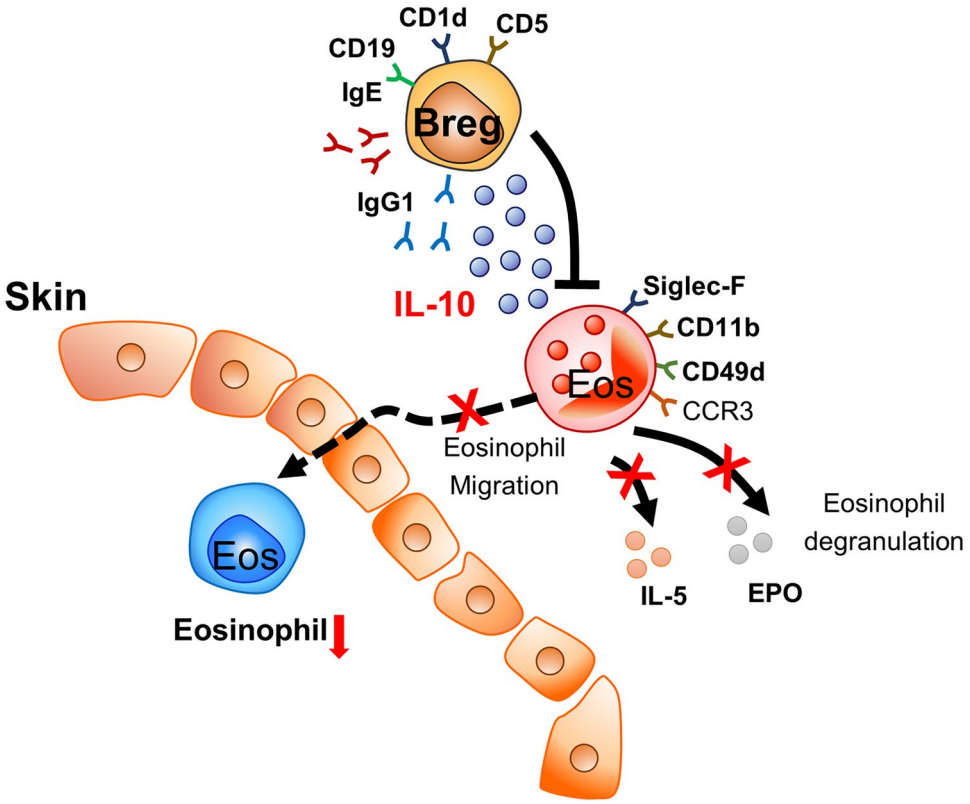

Figure 7. Proposed Mechanism of Eosinophil Inhibition by Regulatory B Cells in Mice with AD. Regulatory B cell-derived IL-10 suppresses eosinophil infiltration and degranulation into disease target tissues, thereby alleviating symptoms of AD.

Conclusions

In summary, our study reaffirmed the relevance of activated eosinophils in the development and severity of AD. Breg cells, through the secretion of IL-10, not only suppress eosinophil infiltration to dLN and skin but also inhibit eosinophil activation, including degranulation and EPO secretion. This ultimately leads to an alleviation of AD symptoms in the animal model (Fig. 7). However, considering that eosinophils only partially contribute to AD development, there may be limitations in using Breg cells for AD treatment. Therefore, enhancing the function of immune-regulatory cells, including Breg cells and regulatory T cells, could represent a novel approach for sustainable improvement and treatment of AD.

Data availability

All experimental data obtained and analyzed in this study are available from the corresponding author upon reasonable request.

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Author contributions
The study was conceptualized and designed by all authors. D. L., M. G. J., and K. Y. M. meticulously prepared the materials required for the study. D. L., M. Y. C., and Y. M. K. diligently collected the data. H. S. K., Y. M. K., and W. S. C. meticulously analyzed the collected data. D. L. and M. G. J. carefully crafted the experimental design. D. L. and M. G. J. took the lead in drafting the manuscript. All authors provided valuable feedback on subsequent revisions. The final manuscript was thoroughly reviewed and approved by all authors. All processes of this experiment were managed and directed by W. S. C.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to H.S.K. or W.S.C.