

ORIGINAL ARTICLE

Asthma and Rhinitis

Phenotypic and functional alterations of regulatory B cell subsets in adult allergic asthma patients

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Abstract

Background: IL-10-producing regulatory B cells (Bregs) are widely ascribed immune regulatory functions. However, Breg subsets in human asthma have not been fully investigated.

Objective: We studied Breg subsets in adult allergic asthma patients by assessing two major parameters, frequency and IL-10 expression. We then investigated factors that affect these two parameters in patients.

Methods: Peripheral blood mononuclear cells (PBMCs) of adult allergic asthma patients (N = 26) and non-asthmatic controls (N = 28) were used to assess the frequency of five subsets of transitional B cells (TBs), three subsets of CD24^{high}CD27⁺ B cells and B1 cells. In addition to clinical data, IL-10 expression by individual Breg subsets was assessed by flow cytometry.

Results: Asthma patients had decreases of CD5⁺ and CD1d⁺CD5⁺, but an increase of CD27⁺ TBs which was significant in patients with moderate asthma (60 < FEV1 < 80). Regardless of asthma severity, there was no significant alteration in the frequencies of 6 other Breg subsets tested. However, we found that oral corticosteroid (OCS) significantly affected the frequency of Bregs in Breg subset-specific manners. OCS decreased CD5⁺ and CD1d⁺CD5⁺ TBs, but increased CD27⁺ TBs and CD10⁺CD24^{high}CD27⁺ cells. Furthermore, OCS decreased IL-10 expression by CD27⁺ TBs, all 3 CD24^{high}CD27⁺ B cell subsets (CD5⁺, CD10⁺ and CD1d⁺) and B1 cells. OCS-mediated inhibition of IL-10 expression was not observed in the other Breg subsets tested.

Conclusion & Clinical Relevance: Alterations in the frequency of Bregs and their ability to express IL-10 are Breg subset-specific. OCS treatment significantly affects the frequency as well as their ability to express IL-10 in Breg subset-specific manners.

KEYWORDS

allergy, asthma, B1, Bregs, CD24^{high}CD27⁺, IL-10, oral corticosteroid, regulatory B cell, transitional

Mathew Wiest, Katherine Upchurch, and Md Mahmudul Hasan contributed equally to this work.

[Correction added on 12 November 2019, after first online publication: Author name "HyeMee Joo" in the author contribution list has been corrected to "Md Mahmudul Hasan" in this version.]

1 | INTRODUCTION

B cells play a central role in the pathogenesis of allergic asthma, especially by secreting immunoglobulin E (IgE) in response to inhaled allergens. However, recent compelling evidence^{1,2} indicates that a subset of B cells collectively termed “regulatory B cells (Bregs)” can display immunosuppressive functions in chronic inflammatory diseases, including inflammatory bowel disease, rheumatoid arthritis and experimental autoimmune encephalomyelitis.^{3–6} In addition, Bregs are also capable of suppressing IgE-mediated murine models of allergic inflammation via IL-10 secretion.^{7–11} Breg deficiency is also known to result in increased type-2 responses with eosinophilia in the murine models of allergic airway diseases.^{8,12} Nonetheless, Bregs in human allergic asthma have not been fully investigated.^{13,14} This is partly due to the fact that human Bregs are composed of multiple subsets depending on their surface phenotypes.^{2,5,15} In addition, individual subsets of human Bregs may not be the same in terms of their ability to display immunosuppressive functions in allergic asthma patients who receive different types of therapeutic regimens.

In the adult, B cells are generated in the bone marrow and migrate to the periphery as transitional cells, defined as CD24^{high}CD38^{high}, when they are still short-lived and functionally immature. Fractions of immature transitional B cells (TBs) still express the B cell progenitor marker CD10, which is down-regulated as they mature. Although these immature TBs have the capacity to act in a regulatory manner in murine models of chronic inflammation,^{16,17} much is still unknown about this particular population of B cells in humans.^{18–20} When B cells mature, they down-regulate CD24 expression and begin expressing

IgD.²¹ As they encounter antigens, along with the presence of other activation signals, they can gain a memory phenotype and express CD27 in conjunction with immunoglobulin class-switching. However, a fraction of memory B cells do not express CD27.²² Apart from conventional B cells, B1 cells, considered innate or natural B cells, express CD27 and CD43. Although phenotypes and functions of human Bregs still need to be further characterized, it is generally agreed that fractions of human Bregs express CD24, CD27, CD38, CD1d and CD5.^{20,23} Human Bregs could thus include CD24^{high}CD27⁺ B cells² expressing IL-10.^{13,24,25} While almost all human B cells have the capacity to produce IL-10, the “most efficient” IL-10-producing B cells are similar to CD24^{high}CD38^{high} TBs.^{26,27} Associations between CD24^{high}CD38^{high} TBs and immune regulation have also been noted in the favourable clinical outcome of patients with autoimmune diseases.²⁸

In this study, we thus questioned whether adult allergic asthma patients had alterations in Breg frequency and their ability to express IL-10. To address this question, we tested nine different Breg subsets, including five subsets of TBs and three subsets of CD24^{high}CD27⁺ B cells and B1 cells, in asthma patients and compared them with those of non-asthmatic control subjects. We further investigated factors that could affect the frequencies of Breg subsets and their IL-10 expression in patients.

2 | METHODS

2.1 | Patients and control subjects

Adult allergic asthma patients (N = 26) and non-asthmatic control subjects (N = 28) were recruited in this study (Table 1). Lung function,

TABLE 1 Information of adult allergic asthma patients and non-asthmatic control subjects recruited in this study

Characteristics	Asthma patients	Non-asthmatic controls
Total population, n	26	28
Age (y)	51 (±12)	44 (±12)
Sex (M/F) (%M)	7/19 (27)	11/17 (39)
Caucasian, n (%)	25 (96)	21 (72)
African American, n (%)	0 (0)	4 (14)
Asian, n (%)	1 (4)	3 (10)
Severe asthma based on FEV	8	
No. of prescribed oral corticosteroid	1	
No. of prescribed leukotriene inhibitor	5	
No. of eosinophilic asthma	3	
Moderate asthma based on FEV	10	
No. of prescribed oral corticosteroid	2	
No. of prescribed leukotriene inhibitor	4	
Eosinophilic asthma	2	
Mild asthma based on FEV	8	
No. of prescribed oral corticosteroid	3	
No. of prescribed leukotriene inhibitor	4	
No. of eosinophilic asthma	4	

Note: All data are expressed as mean with SD (if applicable).

as defined by the forced expiratory volume in 1 second, (% predicted FEV1) was acquired. All patients showed positive responses to at least one allergen by a skin prick test, as measured by the assessment of hypersensitivity (weal—a raised white bump surrounded by a small circle of itchy red skin) to allergens. The majority of patients were being treated with either short- or long-acting β -agonists (24 of 26) and inhaled corticosteroid (23 of 26) at the time of blood draw. All patients treated with oral corticosteroid (OCS) also received inhaled corticosteroid. Untreated patients were defined as patients who were not prescribed the indicated medication for analysis at least 4 weeks before collecting blood samples. This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All subjects were enrolled under protocols approved by the Institutional Review Board (18006269 and 011-072). Donors were excluded if they were pregnant, under the age of 18, or if they had any other chronic diseases.

2.2 | Blood samples, PBMC isolation and flow cytometry

Blood was drawn twice over a one-week interval and average values from two separate experiments were used to determine frequency of Breg subsets. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). 1×10^6 PBMCs were stained with indicated antibodies that are listed in online repository Table S1 along with 50 μ L of Brilliant Stain Buffer (Becton Dickinson) for brilliant violet (BV) fluorochrome stability. Stained cells were analysed on an LSR Fortessa flow cytometer (Becton Dickinson) with approximately 2×10^5 B cells acquired per sample. Results were analysed with FlowJo (TreeStar).

2.3 | Analysis intracellular IL-10 expression by Breg subsets

2×10^6 PBMCs were cultured for 16 hours in the presence of 2.5 μ g/mL ODN2006 (CpG-B) (Invivogen) in RPMI 1640 (Invitrogen) supplemented with HEPES (Invitrogen), 1% non-essential amino acids, 2 mmol/L L-glutamate (Sigma-Aldrich), 50 μ g/mL penicillin and 50 μ g/mL streptomycin (Life Technologies). Cell Stimulation Cocktail (ThermoFisher) containing phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin was added according to the manufacturer's instructions for the last 5 hours. Cells were pre-incubated for 10 minutes with Fc blocker (Miltenyi Biotec), stained for surface markers, permeabilized, fixed and stained for intracellular IL-10 expression. All stained cells were run on an LSR Fortessa flow cytometer (Becton Dickinson) with more than 2×10^5 B cells acquired per sample. Reagents utilized in PBMC staining are listed in online repository Table S1.

2.4 | Eosinophil count

Eosinophilia in asthma patients was determined utilizing whole blood from patients. Whole blood (200 μ L) was stained with the indicated antibodies, and 50 μ L/well of brilliant stain buffer (Becton Dickinson)

for brilliant violet (BV) fluorochrome stability was added. Blood was lysed and cells were fixed with lysing solution (Becton Dickinson). Eosinophils were gated based on high side scatter as well as positive Siglec-8 (Clone: 7C9, Biolegend) and negative CD16 (Clone 3G8, BD) expression. To count eosinophils, 20 μ L of CountBright absolute counting beads (Life Technologies) was added to each sample directly before running on the flow cytometer. Cell counts were calculated using the number of cell events (A) divided by the number of bead events (B) multiplied by the assigned number of counting beads added based on lot (C) divided by the volume of the sample (D): $\frac{A}{B} \times \frac{C}{D}$ = concentration of sample

2.5 | Statistical analysis

Statistical significance for two group comparisons was determined using a non-parametric Mann-Whitney *U* test. Kruskal-Wallis one-way analysis of variance was utilized for multi-group analysis. In the presence of significant overall group difference, Dunn's post hoc test was used to determine statistical significance of pairwise differences. Spearman correlation was utilized to analyse the correlation between age and Breg subsets. Statistical significance analysis was performed with Prism 7 (GraphPad Software). Significance was set at $P < 0.05$.

3 | RESULTS

3.1 | Asthma patients have altered frequencies of CD5⁺ and CD27⁺ TBs, but not total TBs

Immature TBs (CD19⁺CD24^{high}CD38^{high}) can efficiently express IL-10.^{26,27} Associations between TB frequency and immune regulation have also been noted in the favourable clinical outcomes in chronic inflammatory diseases.^{29,30} We thus investigated whether adult allergic asthma patients have altered frequency of immature TBs.

Transitional B cells (CD19⁺CD24^{high}CD38^{high}) in PBMCs were gated as shown in Figure 1A. Both non-asthmatic control subjects and asthma patients had a similar frequency of CD24^{high}CD38^{high} TBs (Figure 1B). In addition, there was no significant difference in the frequency of CD19⁺ B cells in the two groups of subjects (data not shown).

We next divided CD24^{high}CD38^{high} TBs into five different subsets based on their surface expression of CD5, CD27, CD10, CD1d, and co-expression of CD1d and CD5 (Figure 1C). As shown in Figure 1C-G, significant fractions of TBs from both patients and control subjects expressed surface molecules tested. We found that patients had decreases of both CD5⁺ (Figure 1D) and CD1d⁺CD5⁺ TBs (Figure 1H), while they had an increase of CD27⁺ TBs (Figure 1E). The frequencies of CD10⁺ (Figure 1F) and CD1d⁺ TBs (Figure 1G) were similar in the two groups.

We thus concluded that the frequency of total CD24^{high}CD38^{high} TBs in adult allergic asthma patients was similar to non-asthmatic control subjects. However, patients had a decrease of CD5⁺ and CD1d⁺CD5⁺ TBs, but an increase of CD27⁺ TBs.

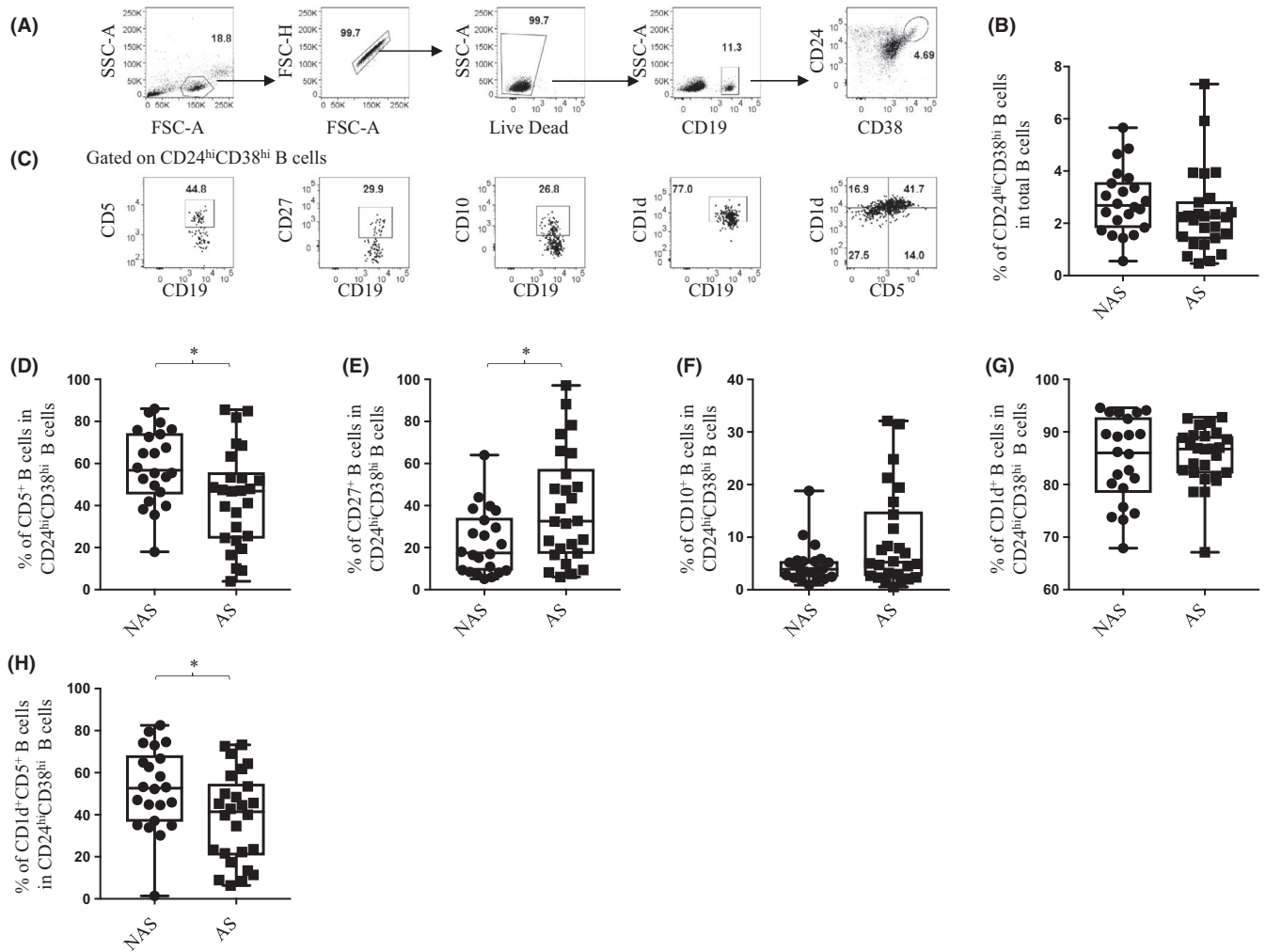


FIGURE 1 Comparison of the frequency of CD24^{high}CD38^{high} transitional B cell subsets in asthma patients (AS) and non-asthmatic subjects (NAS). A, Gating strategy for CD24^{high}CD38^{high} transitional B cells. B, Percentage of total transitional B cells. C, Representative flow cytometry data showing CD5⁺, CD27⁺, CD10⁺, CD1d⁺ and CD1d⁺CD5⁺ transitional B cells. Cells were gated based on isotype antibody staining. Percentages of CD5⁺ (D), CD27⁺ (E), CD10⁺ (F), CD1d⁺ (G) and CD1d⁺CD5⁺ (H) transitional B cells. Data are represented by box plots denoting median $\pm$ interquartile range. * $P < 0.05$ for the comparison between groups

3.2 | Asthma severity is associated with the altered frequencies of TB subsets

In order to gain a better understanding of the alterations in the frequencies of CD5⁺, CD1d⁺CD5⁺, CD27⁺ TBs in patients, we investigated whether such alterations are associated with asthma subtypes.

Patients were divided into three subgroups based on the lung function testing as a measurement of asthma severity (mild: FEV1 > 80, moderate: 60 < FEV1 < 80 and severe: FEV1 < 60). As shown in Figure 2A, patients with moderate asthma had a decrease of CD5⁺ TBs when compared to control subjects. In addition, moderate asthma patients had the lowest median frequency of CD5⁺ TBs among patients, although the differences were not statistically significant. In addition, Figure 2B also shows that patients with moderate asthma had a significant increase of CD27⁺ TBs when compared to control subjects. Patients with mild asthma also had an increased median frequency of CD27⁺ TBs, while patients with severe asthma were similar to non-asthmatic control subjects. All subgroups of

patients had similar frequencies of CD10⁺ (Figure 2C) and CD1d⁺ TBs (Figure 2D). Consistent with the data in Figure 2A, only patients with moderate asthma had significantly lower frequency of CD1d⁺CD5⁺ TBs when compared to non-asthmatic control subjects (Figure 2E). We further investigated whether the frequency of TB subsets in patients could be associated with blood eosinophilia (Figure S1). Patients with or without blood eosinophilia (eosinophil count $\geq$ or <450/ μ L blood, respectively) had a similar frequency of all 5 TB subsets tested in this study.

We thus concluded that the alterations in the frequency of CD5⁺ and CD27⁺ TBs in patients were largely due to the changes in patients with moderate asthma.

3.3 | OCS treatment affects the frequency of TB subsets

We next investigated whether any therapeutic regimens being taken by patients could affect the frequency of TB subsets. Patients

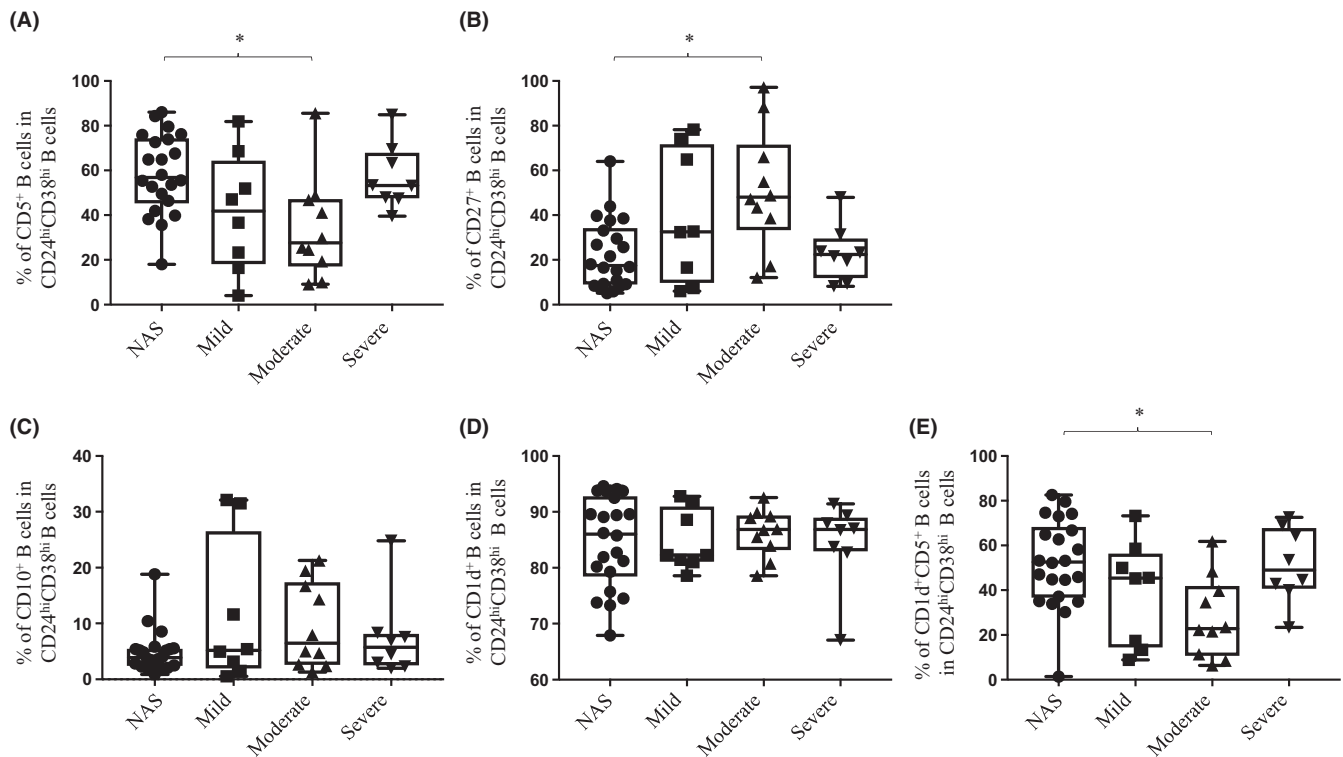


FIGURE 2 Frequency of CD24^{high}CD38^{high} transitional B cell subsets in asthma patients with different disease severities. Percentages of CD5⁺ (A), CD27⁺ (B), CD10⁺ (C), CD1d⁺ (D) and CD1d⁺CD5⁺ (E) transitional B cells. Data are represented by box plots denoting median $\pm$ interquartile range. * $P < 0.05$ for the comparison between groups

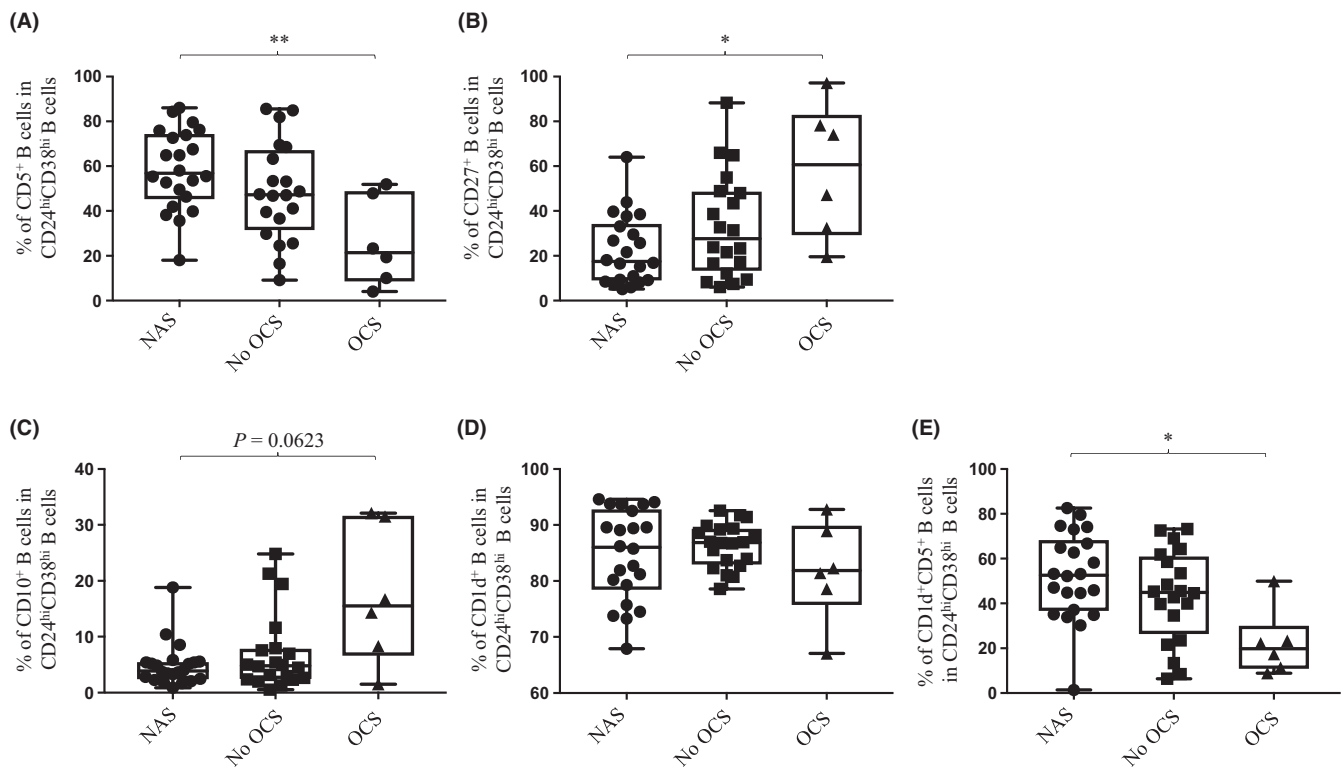


FIGURE 3 Comparison of the frequency of CD24^{high}CD38^{high} transitional B cell subsets in asthma patients (AS) with or without oral corticosteroid (OCS) treatment. Percentages of CD5⁺ (A), CD27⁺ (B), CD10⁺ (C), CD1d⁺ (D) and CD1d⁺CD5⁺ (E) transitional B cells. Data are represented by box plots denoting median $\pm$ interquartile range. * $P < 0.05$ and ** $P < 0.01$ for the comparison between groups

treated with leukotriene inhibitor did not have significant alteration of any TB subsets tested (Figure S2). However, patients without leukotriene inhibitor treatment had decreases of CD5⁺ and CD1d⁺CD5⁺ TBs and an increase of CD27⁺ TBs, suggesting that the alterations of such TB subsets in patients may not be due to leukotriene inhibitor.

We found that patients treated with OCS had significantly lower frequencies of CD5⁺ (Figure 3A) and CD1d⁺CD5⁺ TBs (Figure 3E) than control subjects and patients without OCS treatment. However, OCS treatment resulted in significant increases of CD27⁺ TB subsets (Figure 3B). CD10⁺ TB subsets were also increased in patients who were being treated with OCS although this was not statistically significant (Figure 3C). OCS did not significantly alter the frequency of CD1d⁺ TB subset (Figure 3D), suggesting that the decrease of CD1d⁺CD5⁺ TB by OCS was mainly due to the decrease of CD5⁺ TB subsets.

Therefore, we concluded that OCS treatment resulted in an alteration of TB frequency. However, the effect of OCS was TB subset-specific; OCS decreased CD5⁺ TBs, but increases CD27⁺ and CD10⁺ TB subsets. The use of OCS was not related to the severity of asthma in patients recruited. Therefore, these results suggest TB

subset alterations in moderate (Figure 2A,B) but not severe asthma may not be due to the use of OCS.

3.4 | Patient TBs are competent for IL-10 expression, but OCS suppresses IL-10 expression by CD27⁺ TBs

Immune regulatory function of Bregs is largely dependent on their ability to express IL-10.³¹ We thus investigated whether TBs in patients were competent for expressing IL-10.

As shown in Figure 4A, patients and non-asthmatic control subjects had similar frequencies of IL-10⁺ total TBs, which is in line with published data observed in asthmatic and allergic rhinitis patients.³² However, further analysis for individual subsets of TBs showed trends for increased frequencies of IL-10 expressing CD27⁺ and CD1d⁺CD5⁺ TB subsets in patients without OCS treatment (Figure 4B).

Patients treated with OCS had decreased CD5⁺, but increased frequencies of CD27⁺ and CD10⁺ TB subsets (Figure 3). To gain better insight of the biological relevance of such changes in the context of TB functions and asthma pathogenesis, we examined

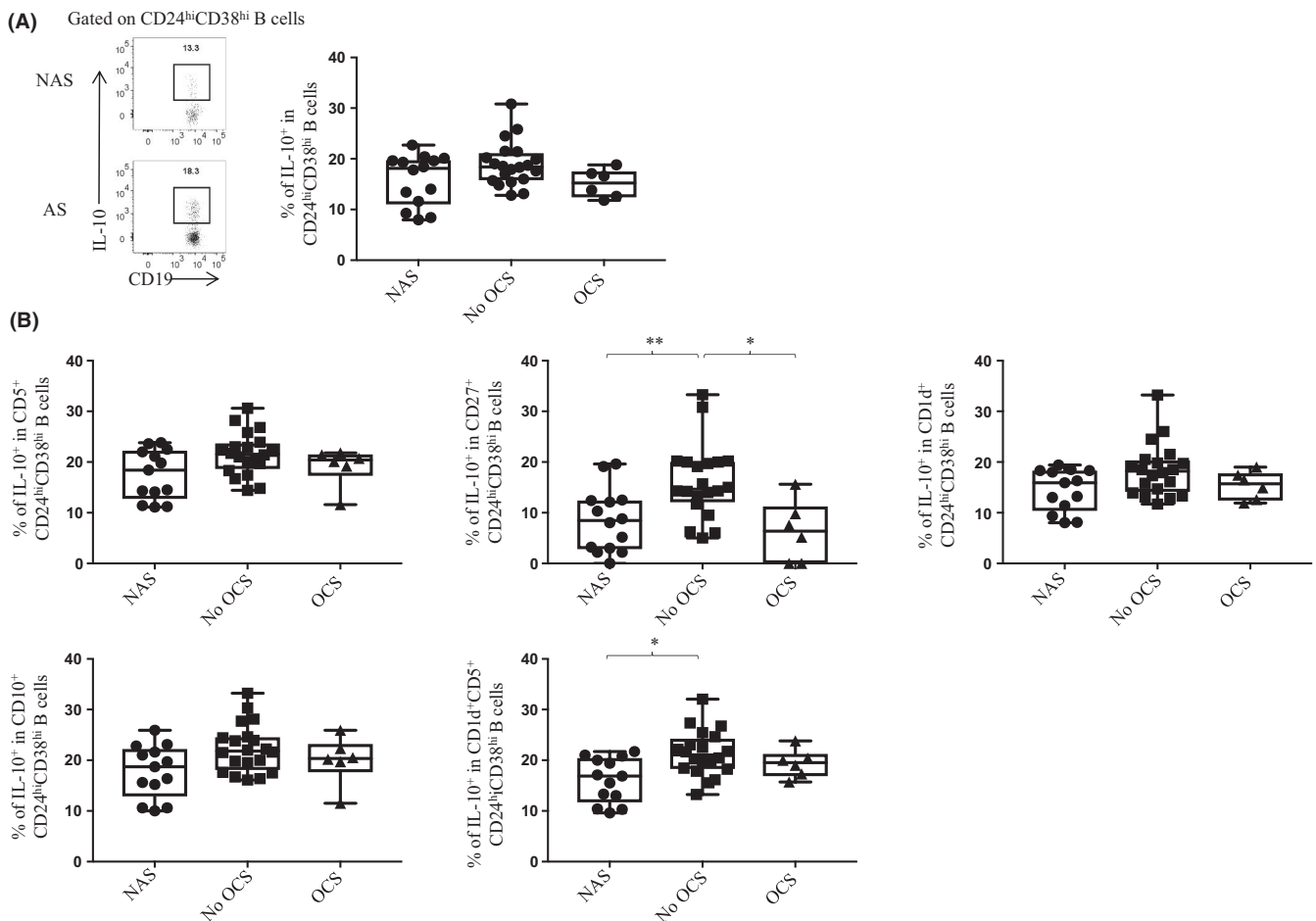


FIGURE 4 IL-10 expression by CD24^{high}CD38^{high} transitional B cell subsets in asthma patients (AS) and non-asthmatic subjects (NAS). Peripheral blood mononuclear cells from AS and NAS stimulated for 16h with CpG-B and intracellular IL-10 expression was analysed by flow cytometry. A, Percentage of IL-10⁺ transitional B cells. Cells were gated based on isotype antibody staining. B, Percentage of IL-10⁺ transitional B cell subsets (CD5⁺, CD27⁺, CD10⁺, CD1d⁺ and CD1d⁺CD5⁺). Data are represented by box plots denoting median ± interquartile range. *P<0.05 and **P<0.01 for the comparison between groups

whether OCS could also impact IL-10 expression by TB subsets. Figure 4B shows that OCS-treated asthma patients had similar frequencies of IL-10-expressing CD27⁺ TBs to control subjects. Leukotriene inhibitor-treated (Figure S3) patients had increases of IL-10-expressing CD27⁺ and CD1d⁺CD5⁺ TB subsets. Leukotriene inhibitor treatment did not significantly alter the frequency of IL-10-expressing TB subsets in asthma patients.

We thus concluded that TBs in adult allergic asthma patients are competent for IL-10 expression. However, OCS can significantly decrease IL-10 expression by TBs, particularly for CD27⁺ TB subset.

3.5 | OCS alters frequency and function of CD24^{high}CD27⁺ B cells

In mice, CD1d^{high}CD5⁺ B cells efficiently express IL-10 and thus display immune regulatory functions.^{33,34} In humans, the co-expression of CD24 and CD27 on B cells has been found to encompass a large subset of IL-10 producing B cells.^{13,24,25} We thus tested whether asthma patients had altered profiles of CD24^{high}CD27⁺ B cells.

Both asthma patients and control subjects had significant percentages of circulating CD24^{high}CD27⁺ B cells; however, the frequencies of CD24^{high}CD27⁺ B cells in the two groups of subjects were similar (Figure 5A). We further analysed the frequencies of CD5⁺, CD1d⁺ and CD10⁺ CD24^{high}CD27⁺ B cells (Figure 5B) but no difference was observed between asthma patients and control subjects (Figure S4). Frequencies of the three subsets of CD24^{high}CD27⁺ B cells were similar in all patients regardless of asthma severity and blood eosinophilia (Figure S5). OCS treatment did not significantly alter the frequencies

of CD5⁺ and CD1d⁺ CD24^{high}CD27⁺ B cells, but it increased the frequency of CD10⁺ CD24^{high}CD27⁺ B cells (Figure 5C).

We next assessed the frequency of total CD24^{high}CD27⁺ B cells expressing IL-10. We found that patients treated with OCS had a significant decrease of IL-10-producing CD24^{high}CD27⁺ B cells, when compared to control subjects and patients without OCS treatment (Figure 6A). We next divided CD24^{high}CD27⁺ B cells into three subsets depending on CD5, CD1d and CD10 expression and assessed their IL-10 expression. All three subsets in patients and control subjects were similar in terms of their IL-10 expression (Figure 6B). However, patients treated with OCS had significant decreases of IL-10-expressing CD5⁺, CD1d⁺ and CD10⁺ CD24^{high}CD27⁺ B cells. Leukotriene inhibitor treatment did not significantly alter CD24^{high}CD27⁺ B cell subsets ability to express IL-10 (data not shown).

Therefore, we concluded that the frequency of total CD24^{high}CD27⁺ B cells is similar in adult allergic asthma patients and control subjects. However, patients treated with OCS had an increase of CD10⁺ CD24^{high}CD27⁺ B cells. Nonetheless, OCS treatment resulted in a significant decrease of IL-10 expression by all three subsets, CD5⁺, CD10⁺ and CD1d⁺ CD24^{high}CD27⁺ B cells.

3.6 | Alterations of B1 cells in adult allergic asthma patients

Innate-like B1 cells (CD27⁺CD43⁺) also efficiently express IL-10 that can suppress inflammatory responses.^{35,36} We thus assessed the frequency of B1 cells in asthma patients and control subjects and also compared their ability to express IL-10.

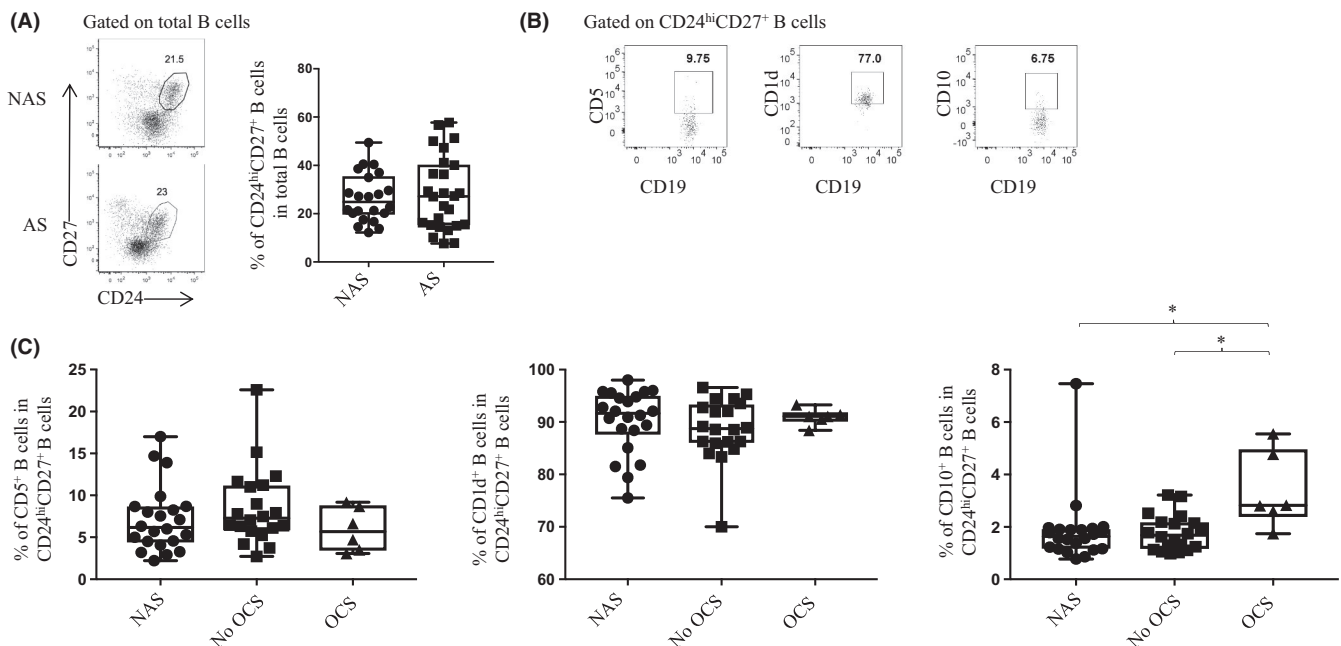


FIGURE 5 Frequency of CD24^{high}CD27⁺ B cell subsets in adult allergic asthma patients. A, Gating (left) and frequency of CD24^{high}CD27⁺ B cells in asthma patients (AS) and non-asthmatic subjects (NAS). B, Representative flow cytometry data showing CD24^{high}CD27⁺ B cells expressing CD5, CD1d and CD10. Cells were gated based on isotype antibody staining. C, Effects of oral corticosteroid (OCS) treatment on the frequency of CD24^{high}CD27⁺ B cell subsets. Data are represented by box plots denoting median $\pm$ interquartile range. * $P < 0.05$ for the comparison between groups

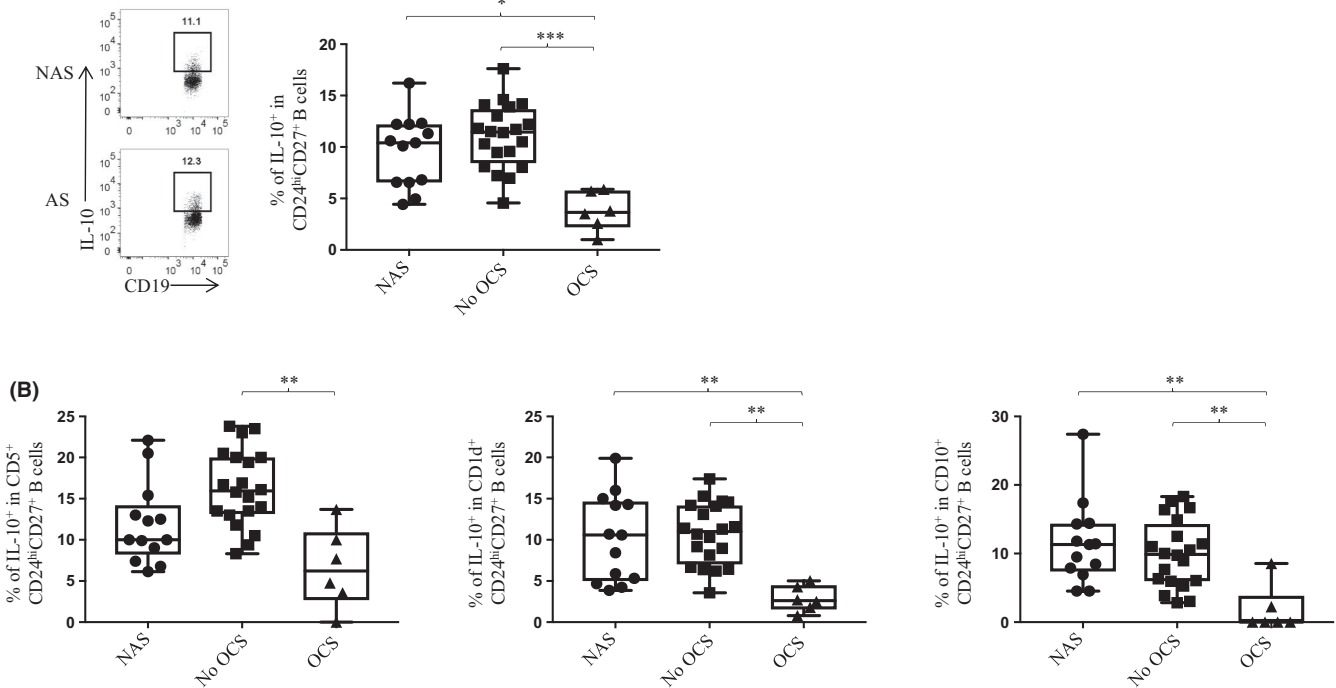
(A) Gated on CD24^{hi}CD27⁺ B cells

FIGURE 6 Frequency of IL-10⁺CD24^{high}CD27⁺ B cell subsets in adult allergic asthma patients. A, Representative flow cytometry data showing IL-10⁺ CD24^{high}CD27⁺ B cells in patients and control subjects (left). Summarized data (right); asthma patients (AS) and non-asthmatic subjects (NAS). B, Frequency of IL-10⁺ B cells in CD5⁺ (left), CD1d⁺ (middle) and CD10⁺ CD24^{high}CD27⁺ B cells in patients with or without OCS treatment. Data are represented by box plots denoting median $\pm$ interquartile range. *P < 0.05, **P < 0.01, and ***P < 0.001 for the comparison between groups

Similar to CD24^{high}CD27⁺ B cells (Figure 5A), there was no significant alteration in the frequency of total B1 cells in patients (Figure 7A). The frequency of B1 cells was also stable in different asthma subtypes, asthma severity and blood eosinophilia (Figure S6). In addition, OCS treatment also did not result in a significant alteration of B1 cell frequency (Figure 7A). We also found that B1 cells in patients are also competent for expressing IL-10 when compared to healthy controls (Figure 7B). However, patients treated with OCS had a decrease of IL-10 expression. Leukotriene inhibitor treatment did not alter the frequency or IL-10 expression by B1 cells (data not shown).

Therefore, we concluded that adult allergic asthma patients do not have an altered frequency of B1 cells. In addition, B1 cells in patients are competent for IL-10 expression. However, OCS treatment reduces IL-10 expression by B1 cells.

4 | DISCUSSION

This study demonstrates that the frequency and functions of Bregs in adult allergic asthma patients are Breg subset-specific. In addition, OCS is one of the major factors that affect both frequency and IL-10 expression by Bregs. Surprisingly, Bregs, including TBs, CD24^{high}CD27⁺ B cells and B1 cells, in asthma patients are competent for expressing IL-10, when compared to non-asthmatic control subject, unless they were treated with OCS. Although OCS does increase

the frequency of CD27⁺ TBs,²⁰ it dampens the ability of CD27⁺ TBs, CD24^{high}CD27⁺ B cells and B1 cells to express IL-10. Such alterations were not related to the ages of asthma patients (Figure S7).

Unlike in mice,³⁷⁻³⁹ phenotypic and functional features of human TBs, particularly in the context of allergic asthma, have not been fully elucidated. This study reports that adult allergic asthma patients have a decrease in CD5⁺ TBs, but an increase in CD27⁺ TBs when compared to non-asthmatic control subjects. This was despite similar frequencies of total TBs, as well as CD10⁺, and CD1d⁺ TBs. We also report decreases in CD1d⁺CD5⁺ TB cells in asthma patients. However, this was likely attributed to the decreases in CD5⁺ TB cells as CD1d⁺ TB cells are unaffected. Interestingly, such increase in CD27⁺ and decrease in CD5⁺ TBs were most profound in asthma patients with moderate asthma as defined by lung function testing and those with OCS treatment regimens. Furthermore, OCS also increased the frequency of CD10⁺ transitional B cells. While CD24^{high}CD27⁺ B cells and B1 cells have also been ascribed regulatory functions,^{4,5,36} we found that the frequency of these two Breg subsets was similar in both non-asthmatic control subjects and asthma patients when we assessed total CD24^{high}CD27⁺ B cells and B1 cells. However, OCS treatment significantly increased the frequency of CD10⁺ CD24^{high}CD27⁺ B cells. The frequency of the other CD24^{high}CD27⁺ subsets and B1 cells in patients were not significantly altered by any medications, including OCS. The frequencies of both CD24^{high}CD27⁺ B cells and B1 cells were also similar in different asthma subtypes examined in this study.

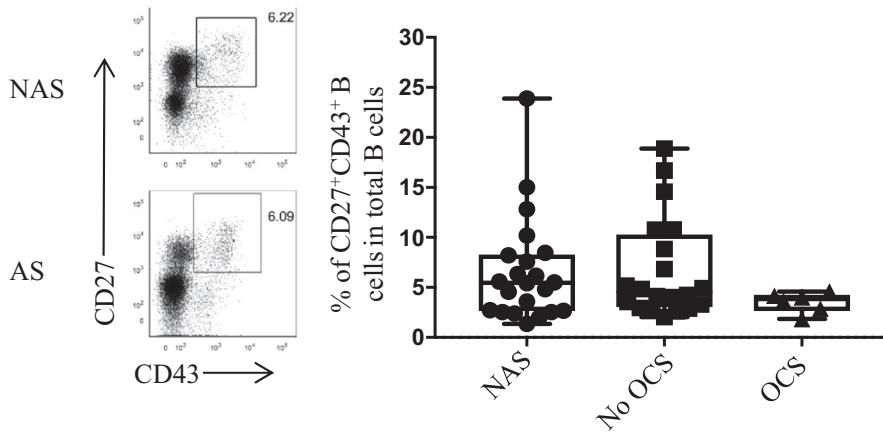
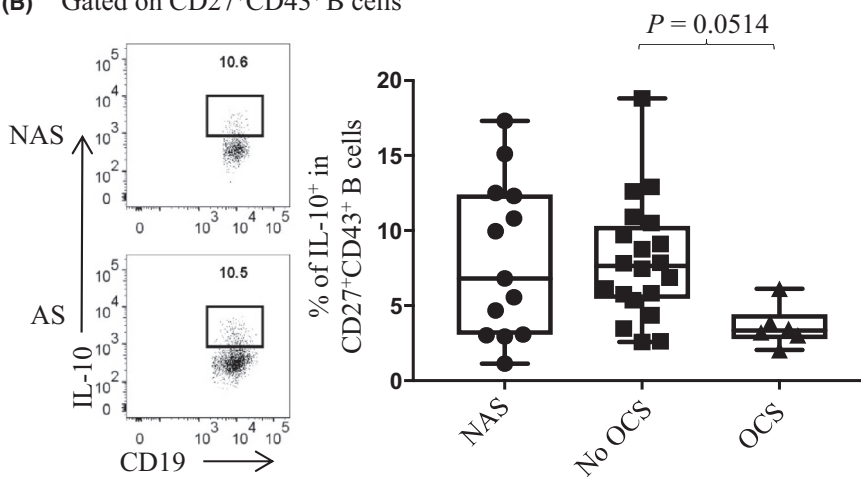
(A) Gated on CD19⁺ B cells

FIGURE 7 Frequency and function of CD27⁺CD43⁺ B1 cells in asthma patients. A, Gating strategy (left) and percentages of B1 cells (right) in non-asthmatic subjects (NAS) and asthma patients (AS) treated with or without oral corticosteroid (OCS). B, Representative flow cytometry data showing IL-10⁺ B1 cells in NAS and AS (left) and frequency of IL-10⁺ B1 cells (right) in NAS and AS treated with or without OCS. Data are represented by box plots denoting median $\pm$ interquartile range

(B) Gated on CD27⁺CD43⁺ B cells

Earlier studies reported that CD24^{high}CD38^{high} human TBs lack CD27 expression.^{20,40} However, a recent study has demonstrated that a significant fraction of CD24^{high}CD38^{high} TBs in patients with autoimmune diseases express CD27 on their surface.²⁰ CD27⁺ TBs were also more efficient than other TB subsets in the suppression of IFN γ and TNF α expression by T cells.²⁰ Although an overall frequency of CD27⁺ TBs is lower than that of CD5⁺ TBs in allergic asthma patients, the increase of CD27⁺ TBs in the asthma patients is in line with the previous observations.²⁰ We further found that such increase of CD27⁺ TBs in patients is observed particularly in patients treated with OCS. This might also explain the increase of CD27⁺ TBs in other inflammatory disease patients²⁰ who were treated with corticosteroid. The increase of CD27⁺ TBs in asthma patients was not due to the increase of total TBs (Figure 1B) or CD27⁺ total B cells (data not shown). In contrast to CD27⁺ and CD10⁺ TBs, OCS treatment resulted in a significant decrease of CD5⁺ TBs in asthma patients which might have an important relevance in the pathogenesis of human asthma⁴¹ as well as other inflammatory diseases.⁴² The lack of changes in frequency of total TBs, CD24^{high}CD27⁺ B cells and B1 cells in allergic asthma patients recruited in this study contrasts with previous findings in rheumatoid arthritis,^{29,43} systemic lupus erythematosus,²⁶ systemic sclerosis⁴⁴ and allergic rhinitis.^{14,45} This could be due to differences in the pathophysiology

of individual disease types, study design, and treatment regimens especially at the time of blood sample collection.¹⁵

IL-10 expressed by Bregs plays a key role in Breg-mediated immune regulation.^{10,27,31,46} We thus assessed the frequency of Breg subsets that express IL-10, and we have made two major observations. Similar to previously published data,³² subsets of Bregs in asthma patients in this study were competent for expressing IL-10 in response to CpG stimulus. However, this contrasts with a previous report of deficiency in IL-10 expression in Breg subsets of asthma patients in response to LPS stimulus.¹³ Our second major observation is that OCS treatment results in decreases of IL-10 expression by CD27⁺ TBs, CD24^{high}CD27⁺ B cells and B1 cells. It is thus worth to re-examine the previous published data on IL-10 expression by Bregs^{13,20,26,29,41,43,44} based on patient treatment regimens especially oral corticosteroid. Due to the limited numbers of cells collected from patients, we used IL-10 expression as a functional marker of Breg subsets in this study. In future, however, it might be important to test other cytokines (including TGF β , IL-35, granzyme B, IL-6 and TNF α) expressed by Breg subsets.

In conclusion, alterations of the frequency and function of Bregs in adult allergic asthma patients are Breg subset-specific. All Breg subsets tested from asthma patients are competent for expressing IL-10. However, OCS treatment reduced the ability of

CD27⁺ TBs, CD24^{high}CD27⁺ B cells and B1 cells to express IL-10. Data from this study provide us a new insight for the potential roles of individual Breg subsets in human asthma. Our data will also be important for the rational design of Breg-based therapeutic strategies for inflammatory diseases, including allergic asthma in the future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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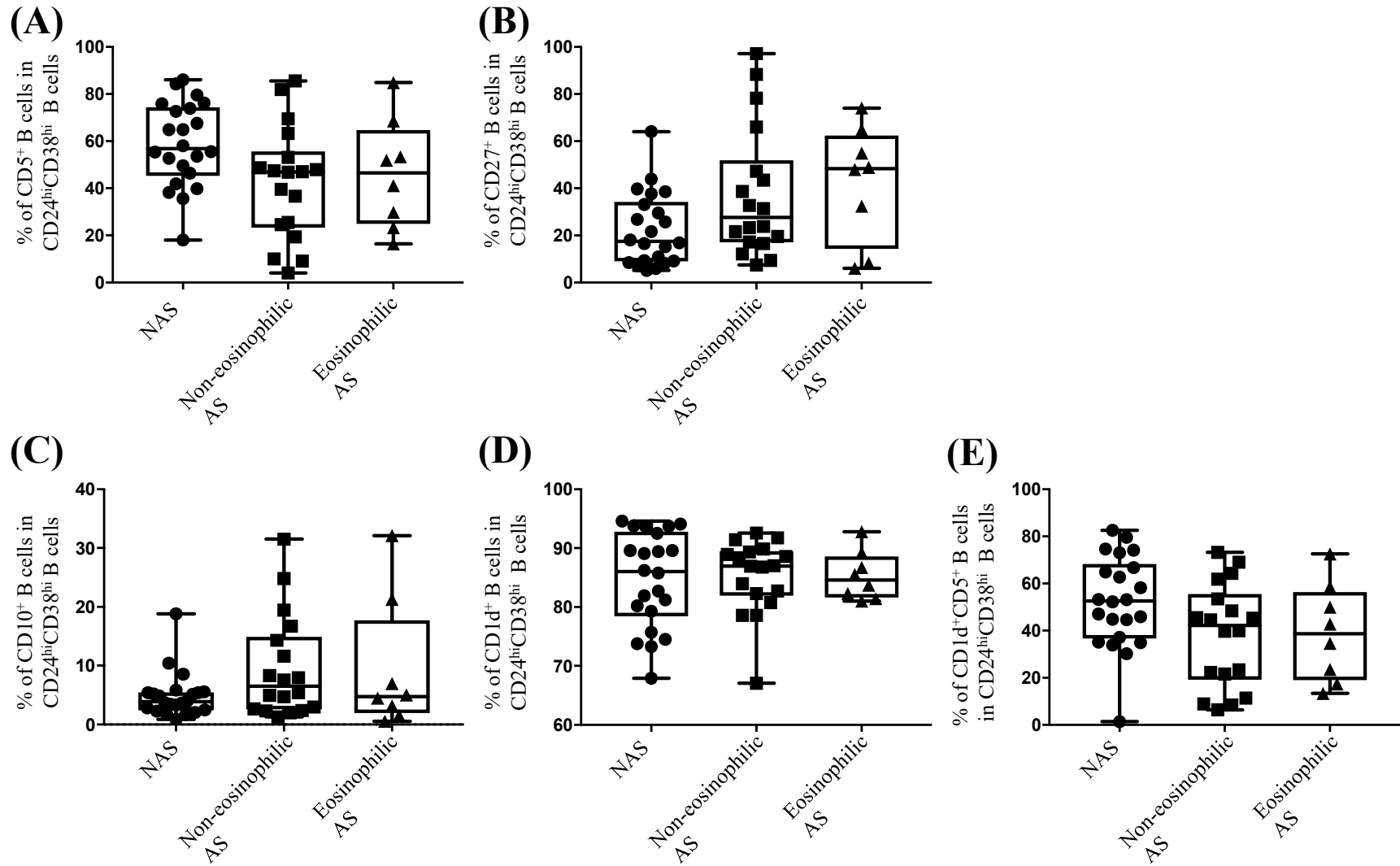


FIGURE S1 Comparison of CD24^{hi}CD38^{hi} transitional B cell subsets in non-asthmatic subjects (NAS) and asthma patients with or without blood eosinophilia. Percentages of CD5⁺ (A), CD27⁺ (B), CD10⁺ (C), CD1d⁺ (D), and CD1d⁺CD5⁺ (E) CD24^{hi}CD38^{hi} B cells. Data are represented by box plots denoting median $\pm$ interquartile range.

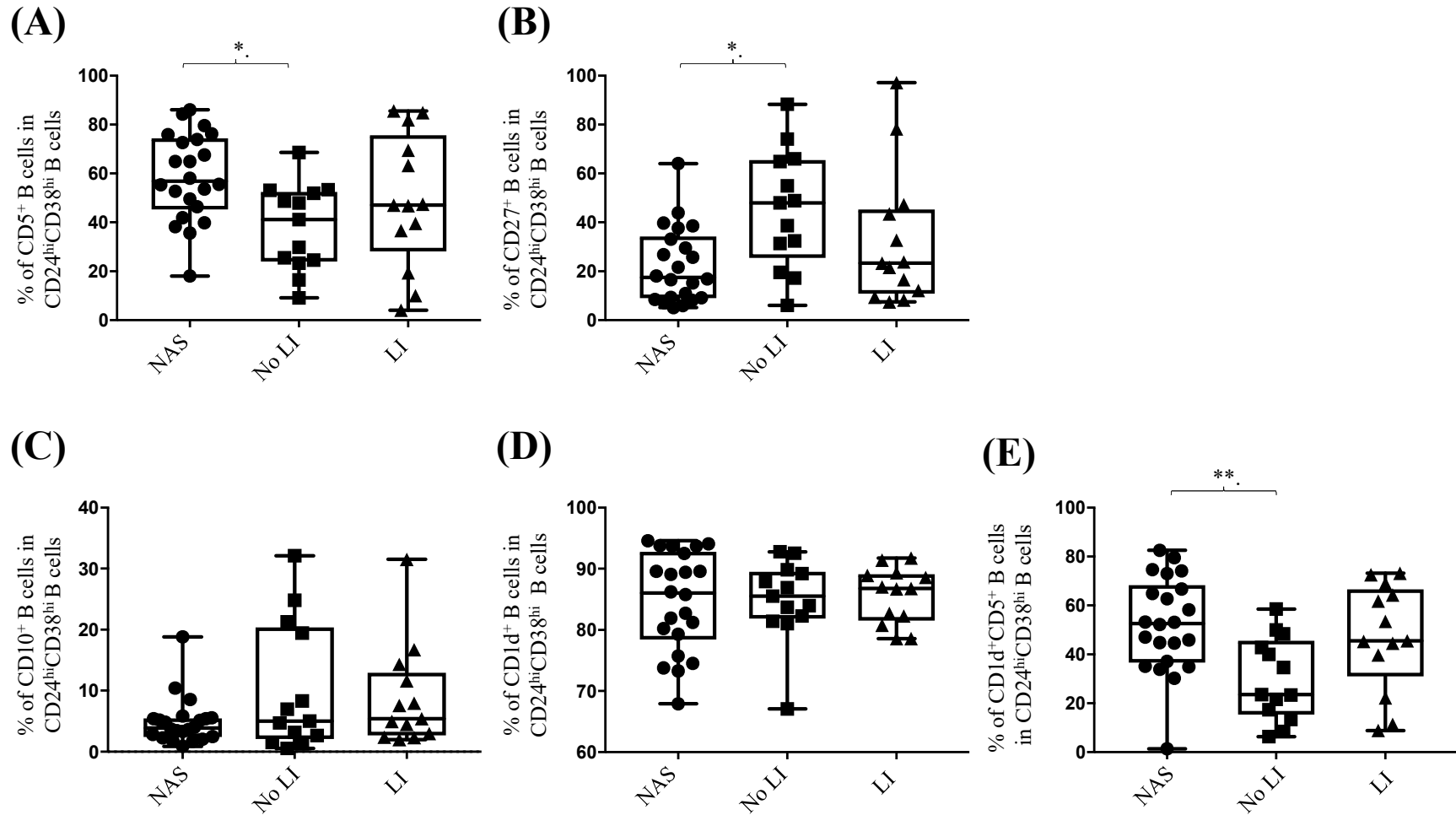


FIGURE S2 Comparison of CD24^{hi}CD38^{hi} transitional B cell subsets in non-asthmatic subjects (NAS) and asthma patients with or without leukotriene inhibitor (LI) treatment. Percentages of CD5⁺ (A), CD27⁺ (B), CD10⁺ (C), CD1d⁺ (D), and CD1d⁺CD5⁺ (E) CD24^{hi}CD38^{hi} B cells. Data are represented by box plots denoting median $\pm$ interquartile range. *P<.05 and **P<.01 for the comparison between groups.

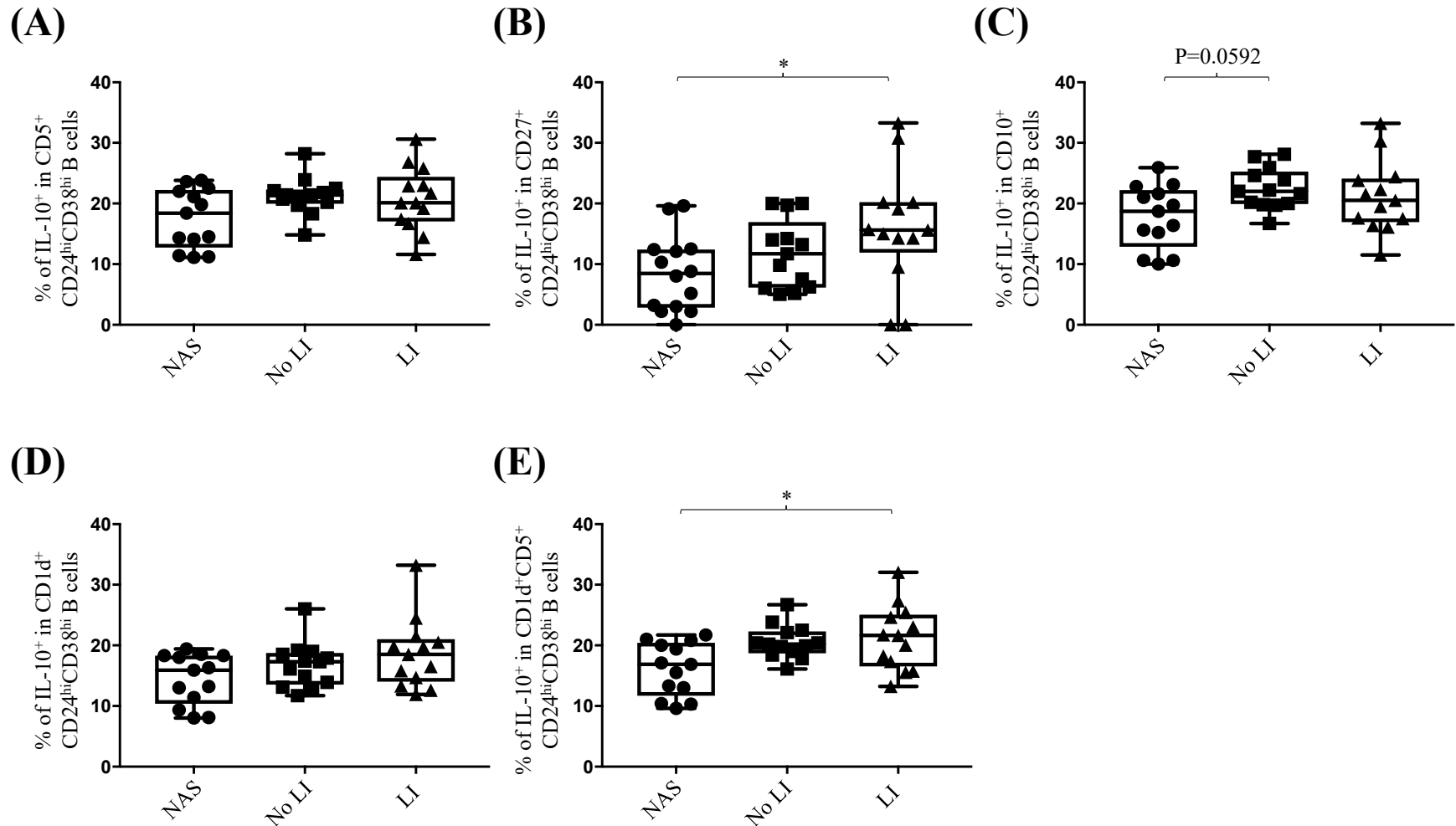


FIGURE S3 Effect of leukotriene inhibitor (LI) treatment on the frequency of IL-10⁺ CD24^{hi}CD38^{hi} B cell subsets. PBMCs from AS and NAS stimulated for 16h with CpG-B and intracellular IL-10 expression was analyzed by flow cytometry in CD5⁺ (A), CD27⁺ (B), CD10⁺ (C), CD1d⁺ (D), and CD1d⁺CD5⁺ (E) CD24^{hi}CD38^{hi} B cell subsets. Data are represented by box plots denoting median $\pm$ interquartile range. *P<.05 for the comparison between groups.

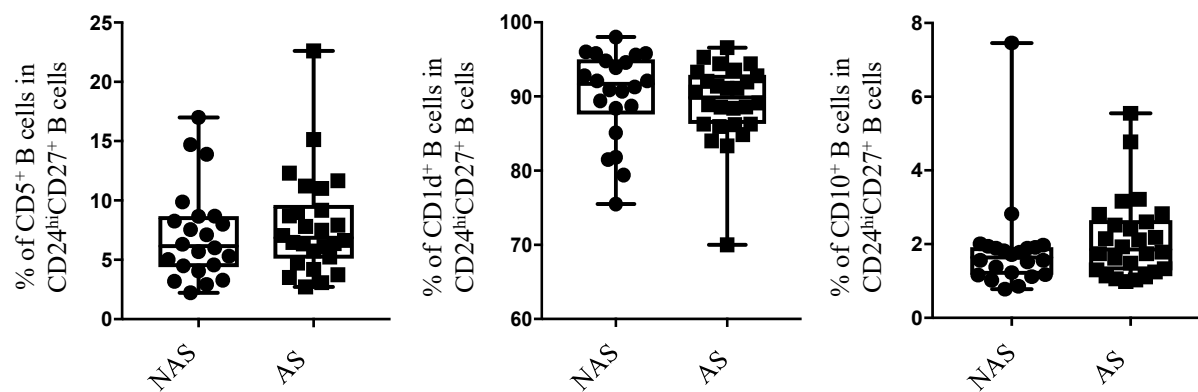


FIGURE S4 Frequency of CD24^{hi}CD27⁺ B cell subsets. PBMCs from asthma patients (AS) and non-asthmatic subjects (NAS) were analyzed by flow cytometry to assess the frequency of CD24^{hi}CD27⁺ B cell subsets. Data are represented by box plots denoting median $\pm$ interquartile range.

FIGURE S5

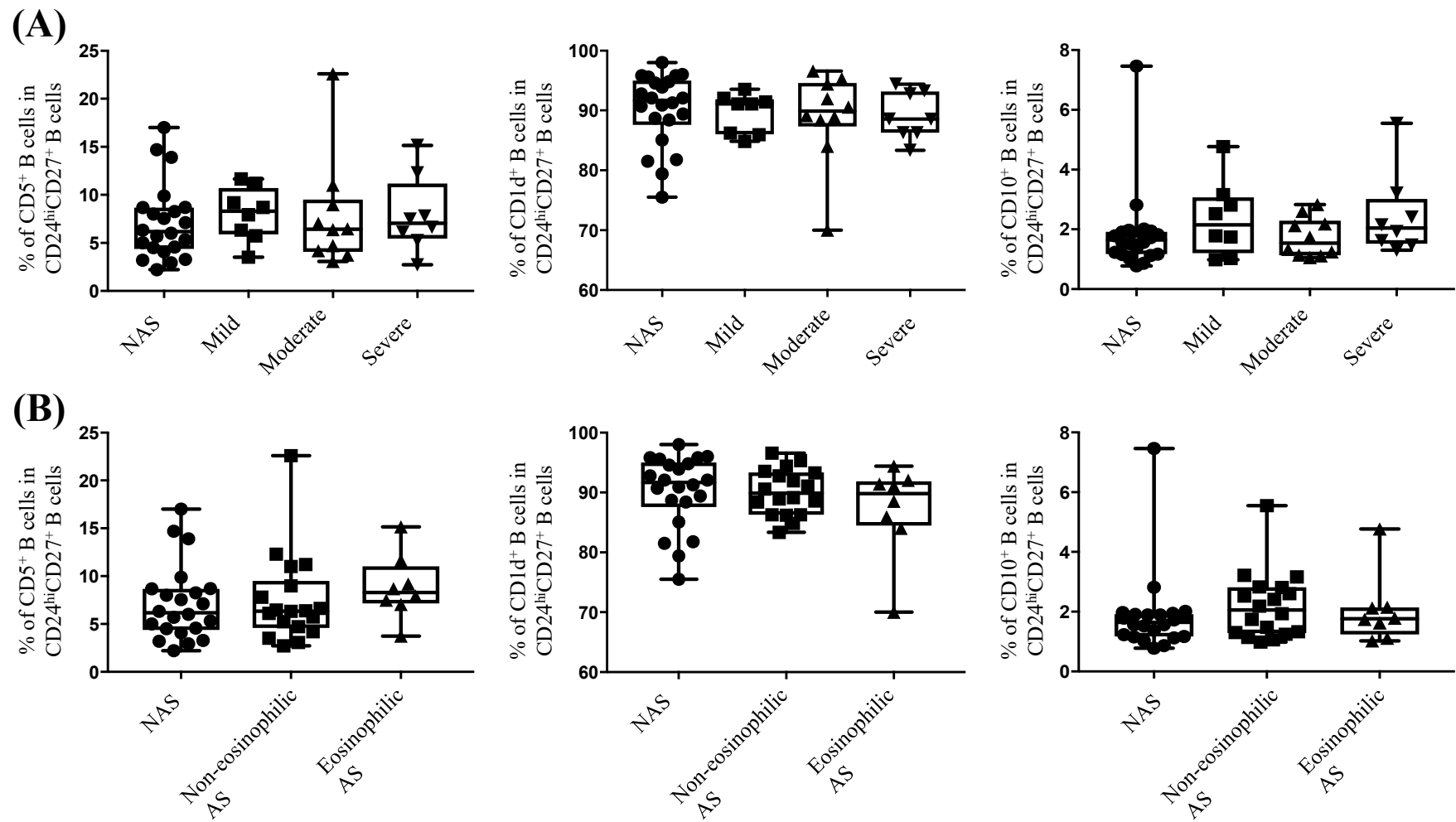


FIGURE S5 Frequency of CD24^{hi}CD27⁺ B cell subsets in different asthma subtypes. PBMCs from asthma patients (AS) and non-asthmatic subjects (NAS) were analyzed by flow cytometry to assess the frequency of CD24^{hi}CD27⁺ cell subsets in patients with mild, moderate, and severe asthma (A) and in patients with or without blood eosinophilia (B). Data are represented by box plots denoting median $\pm$ interquartile range.

FIGURE S6

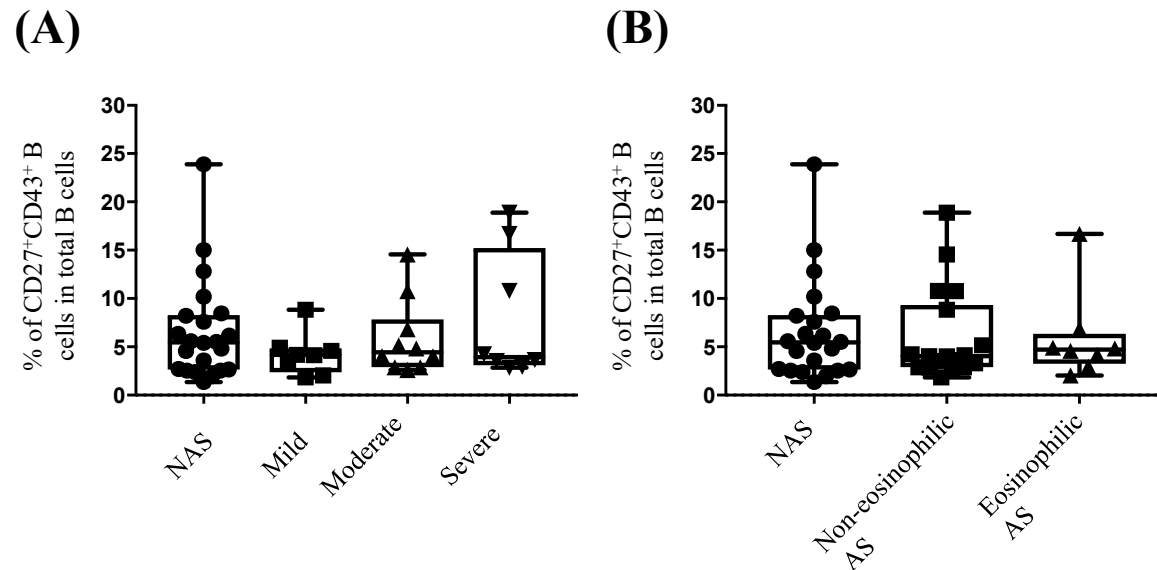


FIGURE S6 Frequency of CD27⁺CD43⁺ B cells in different asthma subtypes. PBMCs from asthma patients (AS) and non-asthmatic control subjects (NAS) were analyzed by flow cytometry to assess the frequency of CD27⁺CD43⁺ B cells in patients with mild, moderate, and severe asthma (A) and in patients with or without blood eosinophilia (B). Data are represented by box plots denoting median $\pm$ interquartile range.

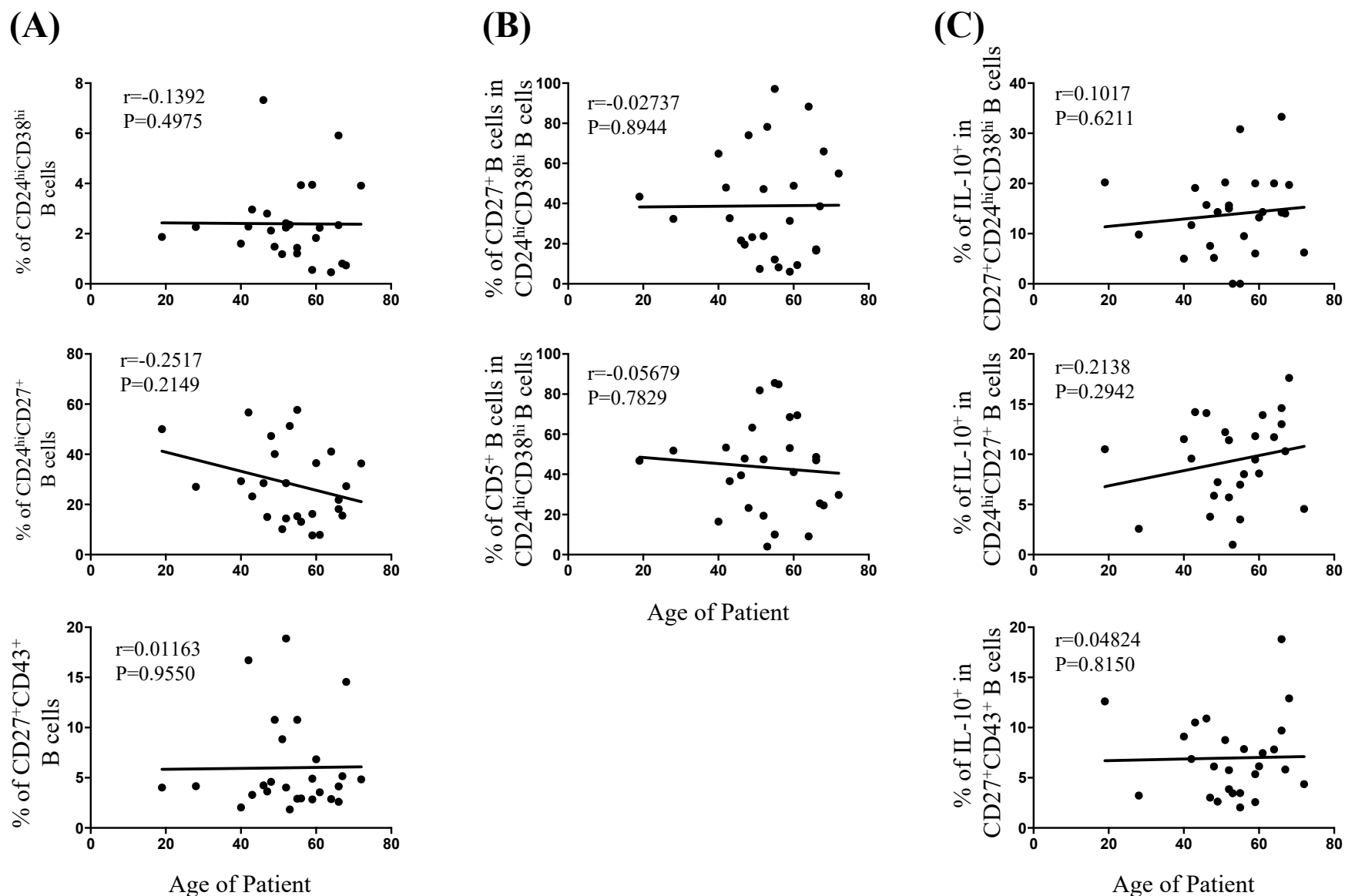


FIGURE S7 Frequency and function of Bregs are not associated with age of asthma patients. (A and B) Correlation analysis between age and frequency of Breg subsets in asthma patients. (C) Correlation analysis between age and frequency of IL-10⁺ Breg subsets. Data were analyzed with Spearman correlation.

TABLE S1 Antibodies used in this study

Antigens	Clones	Sources
CD19	SJ25C1	BD
CD20	2H7	BD
CD24	ML5	BD
CD27	M-T271	BD
CD27	O323	Biolegend
CD38	HIT2	BD
CD38	HB7	BD
CD1d	51.1	BioLegend
CD5	UCHT12	BD
CD10	HI10a	BD/Biolegend
CD43	L60	BD
CD43	IG10	BD
IL-10	JES3-9D7	eBioscience
live/dead	N/A	Invitrogen