Letter to the Editor

IL-10-producing Regulatory B Cells Are Decreased in Patients with Atopic Dermatitis

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Running title: Regulatory B cells in atopic dermatitis

Word count: 999; tables: 0; figures: 2; references: 13

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Abbreviations used in this paper: AD, atopic dermatitis; SCORAD, Severity Scoring of Atopic Dermatitis; TARC, thymus and activation-regulated chemokine

Several B cell subsets that are potent negative regulators of inflammation and autoimmunity have been found; these are termed regulatory B cells (Bouaziz et al., 2008; Yanaba et al., 2008). Regulatory B cells that suppress inflammation through the secretion of the anti-inflammatory cytokine interleukin (IL)-10 (B10 cells) are the best described. The progenitors of human blood B10 cells are characterized as $CD24^{hi}CD38^{hi}$ B cells, although several other subsets have also been proposed (Blair et al., 2010; Tedder, 2015).

B cell depletion in humans using chimeric anti-CD20 monoclonal antibody ameliorates skin inflammation in atopic dermatitis (AD) (Simon et al., 2008), although the antibody failed to improve disease severity in another study (McDonald et al., 2016). Because anti-CD20 monoclonal antibody depletes not only pathogenic B cells, but also regulatory B cells, it does not always have a beneficial effect on autoimmune and inflammatory diseases. Therefore, we hypothesized that regulatory B cells might play an important role in AD.

We examined the frequencies of $CD19^{+}CD24^{hi}CD38^{hi}$ B10 progenitor cells in patients with AD and healthy controls. All experimental details are provided in Supplementary Materials and Methods online. Both patients with AD and healthy controls had similar frequencies of B10 progenitor cells (Supplementary Figure S1). Furthermore, the B10 progenitor cell frequency was comparable between patients with severe AD (Severity Scoring of AD [SCORAD] index > 50) and those with mild AD (SCORAD index ≤ 50; 3.1 ± 0.3 vs. 2.5 ± 0.3; $P = 0.18$). Thus, B10 progenitor cells showed normal frequencies in patients with AD. We next assessed whether the ability of B cells to produce IL-10 remained intact in patients with AD. The B10 cell frequency was significantly lower in patients with severe AD than in healthy controls (47% decrease; $P < 0.01$) and patients with mild AD (47% decrease; $P < 0.01$; Figure 1a, b). Moreover, the B10 cell frequency was negatively correlated with both the SCORAD index ($P < 0.05$, r = –0.50) and serum thymus and activation-regulated chemokine (TARC) levels ($P < 0.01$, r = –0.55) in patients with AD (Figure 1c). The B10 cell frequency was not significantly correlated with serum IgE levels ($P = 0.35$, r = –0.26; data not shown). Thus, the B10 cell frequency was negatively correlated with disease severity in patients with AD.

Serum levels of IL-4, IL-6, IL-10, and B cell-activating factor were analyzed in patients with AD and healthy controls by enzyme-linked immunosorbent assay. Patients with severe AD had significantly higher IL-6 levels compared with healthy controls ($P < 0.01$; Figure 2a). In contrast, there were no significant differences in serum IL-6 levels between patients with mild AD and healthy controls ($P = 0.17$). The levels of other cytokines were comparable between patients with AD and healthy controls (Supplementary Figure S2).

We next examined whether serum IL-6 levels were associated with disease severity and B10 cell frequency. Serum IL-6 levels were positively correlated with the SCORAD index ($P < 0.05$, r = 0.46) and serum TARC levels ($P < 0.05$, r = 0.43; Figure 2b). Furthermore, serum IL-6 levels were inversely correlated with B10 cell frequency ($P < 0.05$, r = –0.44). Thus, serum IL-6 levels were positively correlated with AD severity but negatively correlated with B10 cell frequency. We next verified the effect of IL-6 on B cell IL-10 production by culturing B cells for 48 hours with or without recombinant human (rh) IL-6. The addition of rhIL-6 significantly increased B10 cell frequencies in healthy controls (20% increase; Figure 2c). In contrast, the addition of rhIL-6 did not significantly increase B10 cell frequencies in patients with severe AD (5% decrease; Figure 2c). Thus, the addition of IL-6 increased B cell cytoplasmic IL-10 production in healthy controls, but not in patients with severe AD.

Studies of the role of regulatory B cells in allergic diseases are relatively limited compared with those of autoimmune diseases. Adoptive transfer of spleen B10 progenitor cells inhibits skin inflammation in murine contact hypersensitivity responses (Yanaba et al., 2008). In a hapten-induced mouse model of AD, B10 cells are decreased, whereas the suppressive function of B10 cells on IgE secretion from mice with AD is impaired (Li et al., 2015). Importantly, human B cells transfected with IL-10 secrete less IgE but suppress the antigen-specific proliferation of peripheral blood mononuclear cells (Stanic et al., 2015). In addition, patients with milk allergy have significantly decreased IL-10-producing regulatory B cells compared with milk-tolerant subjects (Lee et al., 2010). Collectively, B10 cells may contribute not only to the suppression of allergic diseases, but also to tolerance formation.

Furthermore, our results show that the frequency of B10 cells was decreased in patients with severe AD and inversely correlated with disease severity, suggesting that impaired B10 cell function may induce uncontrollable allergic inflammation, resulting in severe allergic diseases.

IL-6 production from peripheral blood mononuclear cells or peripheral blood T cells in patients with severe pediatric AD is significantly augmented compared with that in healthy controls (Bunikowski et al., 2001; Toshitani et al., 1993). Furthermore, systemic cyclosporine treatment reduces the peripheral blood mononuclear cell IL-6 production that accompanies the improved disease severity (Bunikowski et al., 2001), indicating that IL-6 may play a pathogenic role in AD. Interestingly, IL-6 directly binds to CD5 on B cells, thereby inducing STAT3 activation (Zhang et al., 2016). B cell IL-10 production is dependent on STAT3 activation (Liu et al., 2014). Moreover, most $CD19^{+}CD24^{hi}CD38^{hi}$ B10 progenitor cells are $CD5^{+}$ (Blair et al., 2010). Therefore, IL-6 is also suggested to be an inducer of IL-10 production from B10 cells through the CD5-STAT3 pathway. Importantly, our studies demonstrate that IL-6 enhanced B cell IL-10 production in healthy controls, but not in patients with severe AD. This implies that IL-6-dependent IL-10 production by B10 cells might be a safety valve to suppress excessive inflammation. In addition, patients with severe AD may have functional abnormalities in IL-10 production from B10 cells in response to IL-6. Further studies are necessary to clarify the precise mechanisms by which B10 cells regulate inflammation in response to IL-6.

CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

ACKNOWLEDGEMENT

This study was supported by a grant from Takeda Science foundation.

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FIGURE LEGENDS

Figure 1. The frequency of IL-10-producing B cells (B10 cells) is decreased in patients with severe AD. The frequency of B10 cells in peripheral blood. Total peripheral blood mononuclear cells were cultured in triplicate in complete medium and activated with 10 μg/ml CpG and 1 μg/ml CD40 ligand in 24-well plates at 2×10^6 cells/ml for 48 hours at 37°C. We added 50 ng/ml PMA, 1 μg/ml ionomycin, and 5 μg/ml brefeldin A for the last 5 hours of culture. The cells were stained for cell surface markers, permeabilized, and stained with anti-human IL-10 monoclonal antibody. (a,b) Frequencies of B10 cells in patients with mild AD, patients with severe AD, and healthy controls. Samples were analyzed from 26 patients with AD and 24 healthy controls. Bar graphs indicate the mean (± SEM) percentages of B10 cells. Significant differences between sample means are indicated: $*P < 0.05$, $**P < 0.01$. (c) Correlation of the frequencies of B10 cells with SCORAD scores and serum TARC levels in patients with AD. Samples were analyzed from 25 patients with AD. Serum TARC levels were measured by using enzyme-linked immunosorbent assay. Results represent one of two independent experiments with similar results.

Figure 2. Serum IL-6 levels are increased in patients with severe AD and are positively correlated with disease severity, but inversely with B10 cell frequencies. (a) Serum IL-6 levels in patients with AD. Samples were analyzed from 25 patients with AD and 22 healthy controls. Bar graphs indicate the mean (± SEM) serum IL-6 levels. Significant differences between sample means are indicated: $*P < 0.01$. Results represent one of two independent experiments with similar results. (b) Correlation of serum IL-6 levels with B10 cell frequencies, SCORAD, and serum IL-6 levels. (c) Frequencies of B10 cells in patients with severe AD (n = 5) and healthy controls (n = 7) after cultivation for 48 hours with or without 20 ng/ml rhIL-6. Each sample was subdivided into two groups, namely, cultivation (in triplicate) with or without rhIL-6. Bar graphs indicate the mean (± SEM) percentages of B10 cells. Significant differences between sample means are indicated: $*P < 0.05$.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. B10 progenitor cell frequencies are comparable between patients with AD and healthy controls. The frequencies of $CD19^{+}CD24^{hi}CD38^{hi}$ B10 progenitor cells in peripheral blood in patients with AD and healthy controls. (a) The frequencies of $CD19^{+}CD24^{hi}CD38^{hi}$ B10 progenitor cells in patients with AD and healthy controls were analyzed by flow cytometry. Samples were analyzed from 26 patients with AD and 25 healthy controls. (b) $CD19^{+}CD24^{hi}CD38^{hi}$ B10 progenitor cells were determined using the illustrated gates. Bar graphs indicate the mean (± SEM) percentages of B cells and B cell subsets. Significant differences are noted between the sample means: $^{**}P < 0.01$.

Supplementary Figure S2. Serum levels of IL-4, IL-10, and B cell-activating factor in patients with severe AD. Serum levels of IL-4, IL-10, and B cell-activating factor in patients with AD. Samples were analyzed from 25 patients with AD and 23 healthy controls. Bar graphs indicate the mean (± SEM) serum levels. Results represent one of two independent experiments with similar results.

MATERIALS AND METHODS

Subjects

This study comprised 26 patients with mild to severe atopic dermatitis (AD; 16 male and 10 female patients) with a mean age of 40 years (range, 17–68 years) and 25 healthy controls (10 male and 15 female) with a mean age of 37 years (range, 21–64 years). All patients had been diagnosed with AD based on clinical features and had not received any immunosuppressant in the previous 6 months. The Severity Scoring of AD (SCORAD) scale was used to assess disease severity. All participants provided written informed consent before study participation. The study was approved by the ethics committee of Jikei University in accordance with the Declaration of Helsinki.

Cell Isolation and Flow Cytometry

Peripheral blood mononuclear cells were isolated from whole blood using Lymphoprep (Axis-Shield PoC As, Oslo, Norway). The isolated peripheral blood mononuclear cells were resuspended (2×10^6 cells/ml) in triplicate in complete medium (RPMI 1640; Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (Sigma-Aldrich), 110 U/ml penicillin (Gibco, Auckland, New Zealand), 110 μg/ml streptomycin (Gibco), and 0.3212 mg/ml L-glutamine (Gibco). B cells were phenotyped by flow cytometry (MACSQuant; Miltenyi Biotec, Auburn, CA) using FITC-conjugated CD19 (BioLegend, San Diego, CA), PerCP-conjugated CD27 (BioLegend), PE/Cy7-conjugated CD24 (BioLegend), Pacific Blue-conjugated CD95 (BioLegend), Pacific Blue-conjugated CD86 (BioLegend), Pacific Blue-conjugated CD80 (EXIBO Pharma, Vestec u Prahy, Czech Republic), and PE-conjugated IgD monoclonal antibodies (BD Biosciences, San Diego, CA). Apoptotic cells were detected with an Annexin V-PE apoptosis detection kit (BD Biosciences) involving a specific marker for the early phase of apoptosis. Results are expressed as the percentage of positive cells. All analyses were performed using fresh blood samples.

Assessment of B10 Cells

Total peripheral blood mononuclear cells were cultured in triplicate in complete medium and activated with 10 μg/ml CpG (Toll-like receptor 9 ligand, ODN 2006 Type B; InvivoGen, San Diego, CA) and 1 μg/ml CD40 ligand (R&D Systems, Minneapolis, MN) in 24-well plates at 2×10^6 cells/ml for 48 hours at 37°C. We added 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), and 5 μg/ml brefeldin A (BioLegend) for the last 5 hours of culture. The cells were stained for cell surface markers, permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), and stained with anti-human PE-conjugated interleukin (IL)-10 monoclonal antibody (BioLegend). The extent of background staining was determined using unreactive isotype-matched control monoclonal antibody (eBioscience, San Diego, CA) with gates positioned to exclude ≥ 98% of unreactive cells.

Measurement of Circulating IL-4, IL-6, IL-10, TARC, and B cell-activating factor

IL-4, IL-6, IL-10, TARC, and B cell-activating factor levels were measured in serum samples obtained from patients with AD and healthy controls by using the appropriate enzyme-linked immunosorbent assay kits (R&D Systems) according to the manufacturer's instructions.

Stimulation and Analyses of Purified B Cells

B cells were stimulated with or without 20 ng/ml human recombinant human IL-6 (PeproTech, Rocky Hill, CT) for 48 hours. The frequencies of B10 cells were determined using flow cytometry.

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney *U* test for the comparison of two groups, the Kruskal–Wallis test and Bonferroni test for multiple comparisons, and Wilcoxon matched-pairs signed-rank test for paired comparisons. All values are expressed as the mean ± SEM. P values less than 0.05 were considered significant.

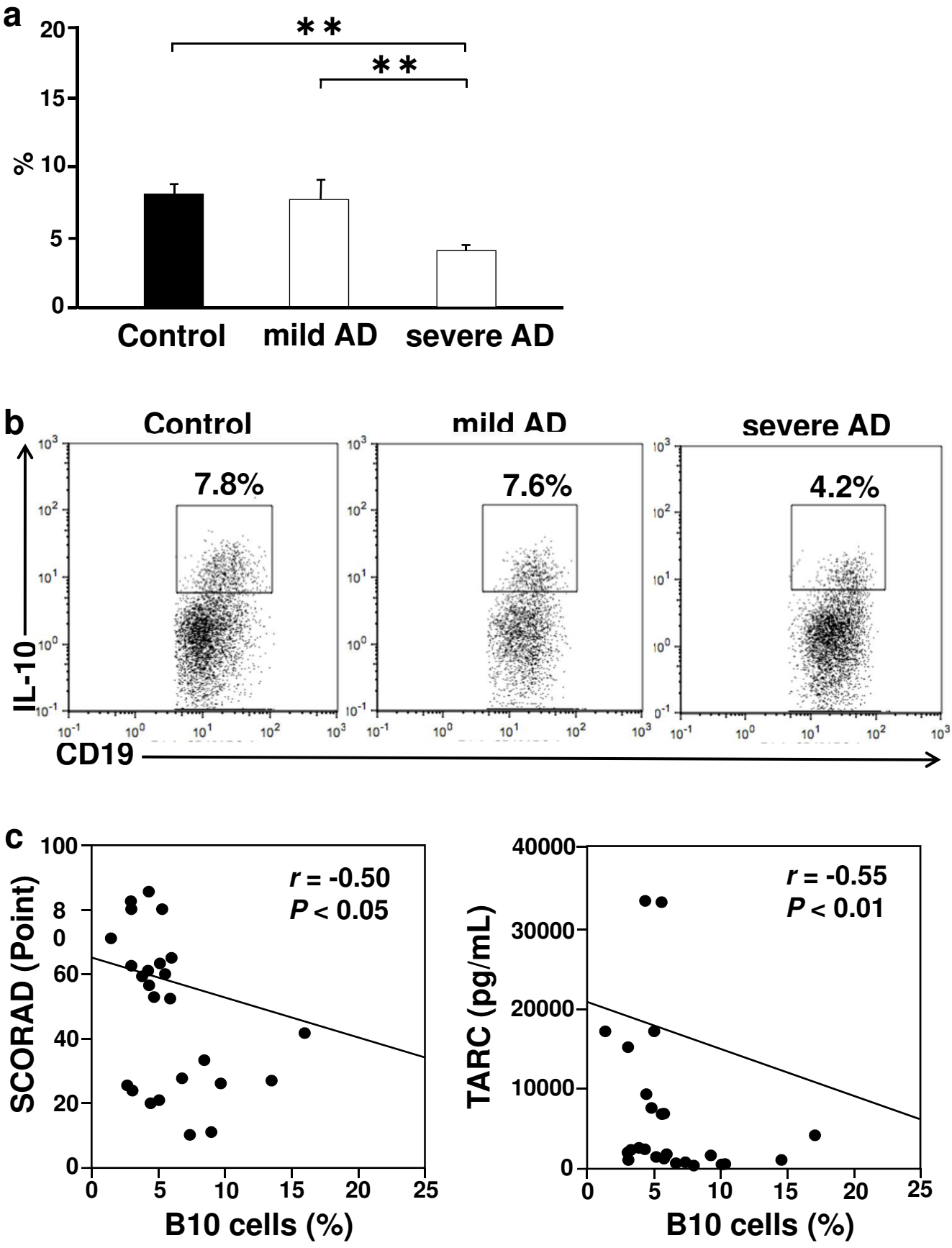

Figure 1
Yoshihara et al.

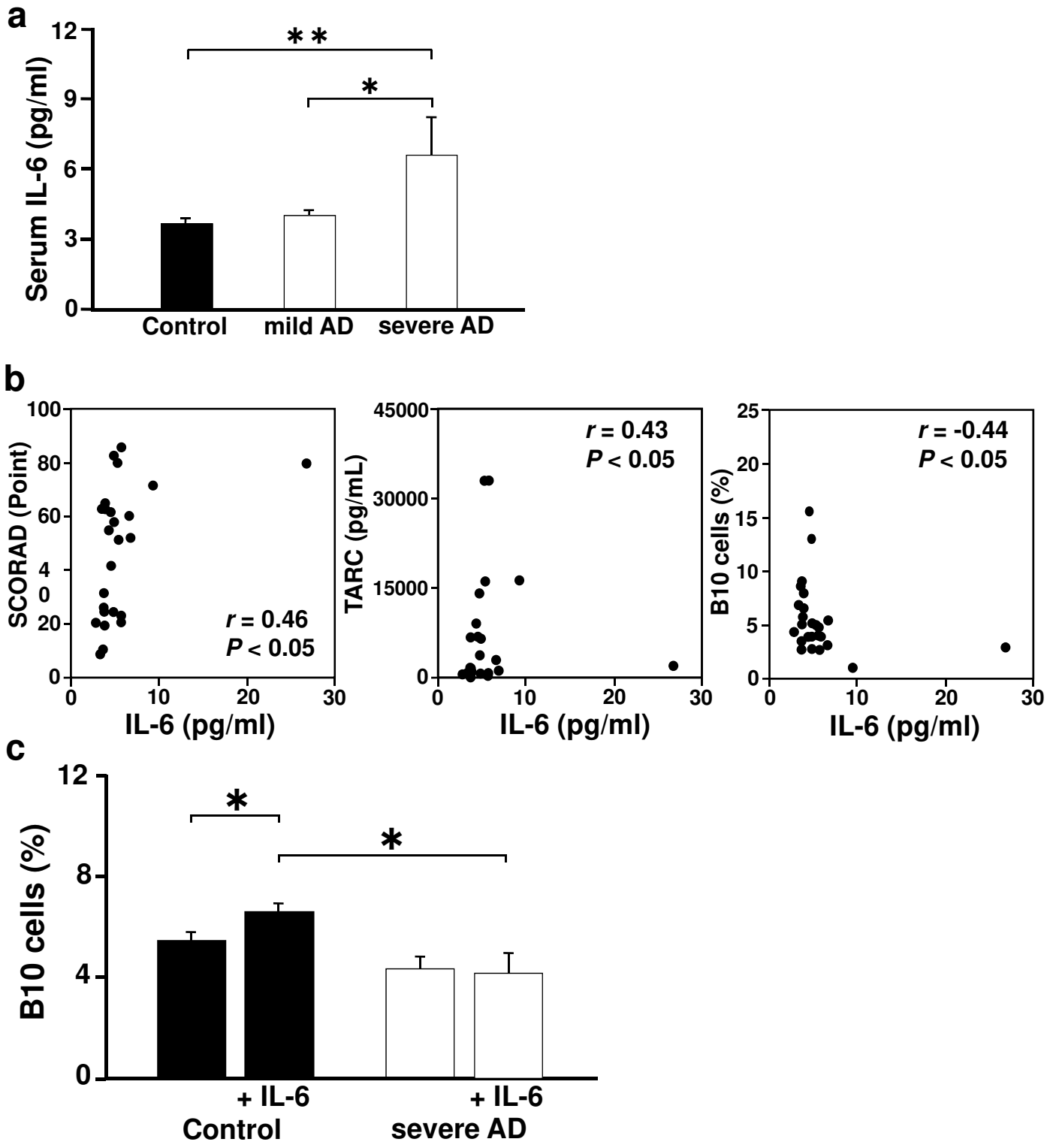

Figure 2
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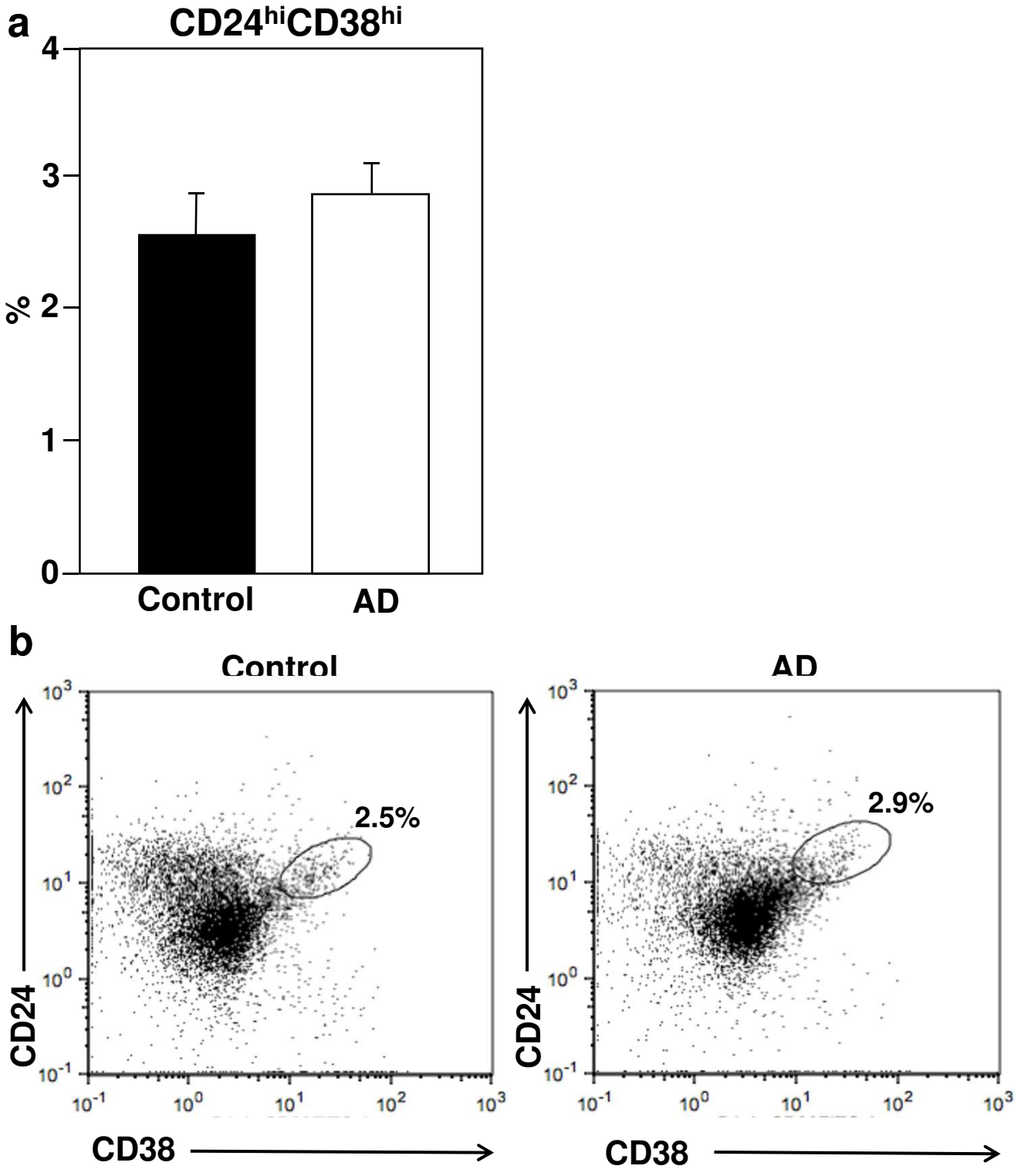

Supplementary Figure 1
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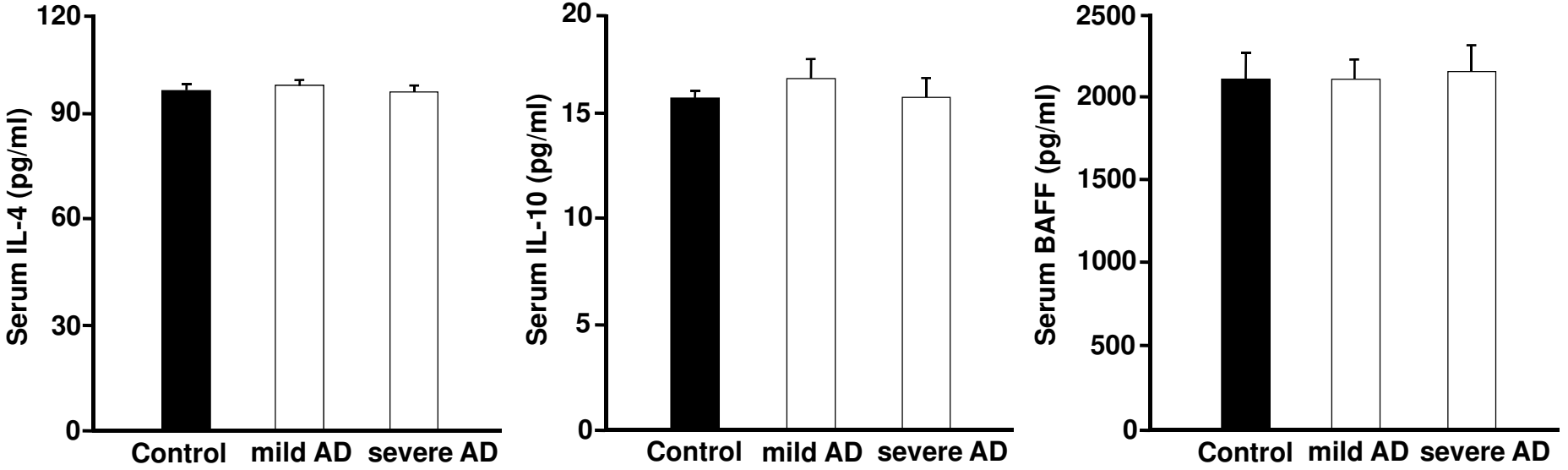

Supplementary Figure 2
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