THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 23, pp. 16383–16390, June 7, 2013

Antigen-specific Immunotherapy Regulates B Cell Activities in the Intestine

Received for publication, January 23, 2013, and in revised form, March 20, 2013 Published, JBC Papers in Press, April 15, 2013, DOI 10.1074/jbc.M113.456202

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Background: B cells play an important role in allergic diseases.
Results: Lower doses of antigen induce BCs to produce IL-10; higher doses result in high serum levels of soluble CD23.
Conclusion: Exposure to lower doses of antigen induces B cells to inhibit skewed Th2 responses.
Significance: Optimal dosage of antigen in specific antigen vaccination determines the clinical outcomes.

Mature B cells (BCs) express CD23 and B cell receptors. Whether activation of CD23 and B cell receptors has different effects on BC activities is unclear. This study aims to investigate the mechanism by which the specific antigen immunotherapy regulates the activation of BCs in the skewed Th2 responses. Mice were sensitized to ovalbumin. The specific antigen vaccination (SAV) at graded doses was employed to modulate the activities of BCs in which the expression of IL-10, IgE, matrix metalloproteinase-9 (MMP9), CD23, and serum soluble CD23 by BCs was evaluated. The immune regulatory effect of BCs primed by lower or higher SAV doses was observed with an adoptive transfer mouse experiment. SAV activated CD23 to produce IL-10 in BCs at lower doses. The higher doses of SAV increased the expression of MMP9 in BCs that reduced the amounts of CD23 in BCs and increased the serum levels of soluble CD23, which was abrogated by the pretreatment with MMP9 inhibitor. Adoptively transfer with BCs primed by lower doses of SAV inhibited the ongoing antigen-specific Th2 responses whereas the BCs primed by higher doses of SAV exacerbated the ongoing Th2 responses. Exposure to specific antigens at optimal doses can activate BCs to produce IL-10 to suppress the skewed antigen-specific Th2 responses. The antigen doses of SAV higher than the optimal doses may promote the production of soluble CD23 to exacerbate the ongoing immune responses.

The skewed T helper 2 (Th2)[2] responses play a critical role in the pathogenesis of a number of diseases, such as allergic asthma, food allergy, allergic dermatitis (1, 2), or a subset of inflammatory bowel disease (3). In these diseases, the frequency of Th2 cells increases; the cells produce high levels of Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 (2). Another feature of the skewed Th2 response is that high levels of immunoglobulin (Ig)E can be resulted in the local tissue that may be also detected in the serum (4, 5). Antigen-specific IgE may bind to the high affinity receptor, FcεRI, on effector cells, such as that IgE can bind FcεRI on mast cells to make mast cells sensitized; these mast cells can be activated to release chemical mediators to evoke allergic inflammation in the local tissue (6, 7). However, IgE may engage the low affinity receptor, CD23, to modulate target cell activities (8).

After priming by antigen information, naïve B cells may differentiate into plasma cells, featured as $CD38^{+}CD35^{-}$, or become $CD19^{+}CD35^{+}$ B cells (BCs) (9) to remain a quiescent status in the body (10). Reimmunization can activate the B cell receptor (BCR) to boost the BCs to develop into plasma cells to produce antibodies, to promote protective immunity or induce skewed immune reactions (11). Because of expressing the CD23 (8), BCs may be bound by IgE to form immune complexes on the surface of B cells, which can be further bound by specific antigens to form a triple immune complex; whether this triple immune complex modulates the BC activity is to be further elucidated.

The specific antigen vaccination (SAV) is the only specific therapeutic remedy for the treatment of allergic diseases currently. The mechanisms of SAV include reduction in mast cell reactivity, decreases in basophil responses, decreases in specific immunoglobulin (Ig)E, increases in IgG4, and induction of regulatory T cells (12). During SAV, small doses of specific antigens are introduced into patients with allergic disease. How the specific antigens interact with immune cells to result in clinical outcomes is not fully understood yet. Because B cells express both CD23 and BCR (8, 9, 13), the antigens may interact with B cells to regulate B cell activities directly besides via the antigen-induced T cell activation and further influence the ongoing allergic diseases. Here we report that after exposure to specific antigens, a triple immune complex, antigen-IgE-CD23, was formed on the surface of BCs; the BCs then produced IL-10 and showed immune suppressor activities on skewed Th2 responses.

MATERIALS AND METHODS

Reagents—Anti-ovalbumin antibody was obtained from AbBioTec (Guangzhou, China). Antibodies of IL-10 (M-18),

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[2] The abbreviations used are: Th2, T helper 2; BC, B cell; BCR, B cell receptor; CSFE, carboxyfluorescein diacetate succinimidyl ester; LPMC, lamina propria mononuclear cell; MMP9, matrix metalloproteinase-9; OVA, ovalbumin; qRT-PCR, quantitative real time RT-PCR; SAV, specific antigen vaccination.

recombinant CD23 protein, CD23 mAb (H-4), and CD23 pAb (M-282) were obtained from Santa Cruz Biotechnology. Cell isolating reagent kits were obtained from Miltenyi Biotech (Shanghai, China). Reagents for quantitative real time RT-PCR (qRT-PCR) and Western blotting were obtained from Invitrogen. Fluorescence-labeled antibodies for flow cytometry were obtained from BD Biosciences. The anti-IgE antibody, ELISA kits of IL-4, IL-10, TIM1 and IgE were obtained from Shanghai Transhold Tech (Shanghai, China). The immune precipitation reagents were obtained from Sigma-Aldrich. Btk inhibitor PCI-3276533 was obtained from Pharmacyclics (Sunnyvale, CA).

Mice—BALB/c, C57/Black 6 (B6) mice and CD23-deficient mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China) and maintained in a pathogen-free environment. The mice were allowed to access food and water freely. The experimental procedures of the animal experiments were approved by the Animal Care Committee at Chongqing Medical University.

Mouse Model of Intestinal Sensitization—Following published procedures (14) with modification, mice were treated with ovalbumin (OVA) (0.1 mg/mouse, mixed in 0.1 ml alum) via subcutaneous injection on day 0, day 3 and day 6. The mice were gavage-fed with OVA (0.5 mg/mouse in 0.3 ml saline) on day 9, 11 and 13 respectively.

Specific Antigen Vaccination—The sensitized mice were treated with the specific antigen OVA (at doses of 50, 100, 500, or 1000 ng/mouse in 0.1 ml of saline; saline or bovine serum albumin (BSA) was used as a control), via intraperitoneal daily for 7 consecutive days.

Assessment of Th2 Polarization in the Intestine—After sacrifice, the sera and intestinal segments were collected from the mice. The serum levels of IL-4, IL-13, specific IgE, and β-hexosaminidase, the frequencies of Th1 cell, Th2 cell, and $CD4^+$ T cell proliferation in the intestine were assessed, respectively; the methods were described separately.

Counts of Mast Cells and Eosinophils in the Intestinal Tissue—Intestinal segments were fixed by Carnoy solution (for mast cells) or formalin (for eosinophils) and embedded with paraffin. The sections were stained with 0.5% toluidine blue (for mast cells) or eosin and hematoxylin (for eosinophil). Mast cells and eosinophils in the sections were counted under a light microscope. Twenty randomly selected high power fields were counted for each sample. The slides were coded so that the observer was not aware of then to avoid the observer bias.

Enzyme-linked Immunosorbent Assay (ELISA)—The levels of IL-4, IFN-γ, IL-10, antigen-specific IgE, and TIM4 were determined by ELISA with reagent kits. The procedures were performed following the manufacturer's instruction.

Immune Cell Isolation from Intestinal Segments—The collected intestinal segments were cut into small pieces (2–3 mm) and shaken for 45 min at 37 °C in HBSS supplemented with 5% FBS and 2 mM EDTA. The tissue was incubated in the presence of 1.5 mg/ml collagenase VIII and 100 units of DNase I for 30 min at 37 °C. The supernatant was passed through a cell strainer (70 μm), and the cells were recovered by centrifugation. The lamina propria mononuclear cells (LPMCs) were isolated by the 40/70% Percoll discontinuous gradient, and LPMCs were recovered at the interface.

Flow Cytometry—The isolated cells were stained with fluorescence-labeled primary antibodies (0.5–1 μg/ml; using matched isotype IgG for control) for 30 min. After washing, the cells were fixed and permeabilized with Fix/Perm solution (eBioscience). The cells were then restained with the intracellular staining approach. Cells were analyzed with a flow cytometry (BD FACSCanto; BD Bioscience).

Immunoprecipitation—Total proteins were extracted from cells with Bio-Rad protein extraction kits. The proteins were precleared with protein A-agarose beads and protein G-agarose beads. The proteins were then immunoprecipitated by incubating with anti-CD23 antibody (or isotype IgG) and protein A-agarose beads at 4 °C for 24 h. The 2×SDS sample buffer was used to elute the precipitates. The precipitated fractions were separated by SDS-PAGE and then analyzed by Western blot analysis with antibodies of CD23, IgE, and OVA, respectively.

Western Blotting—The total proteins were extracted from the cells, separated by SDS-PAGE, and then transferred onto nitrocellulose membranes; the membranes were treated with 5% nonfat milk for 1 h and incubated with the primary antibodies for 1 h at room temperature followed by incubating with the horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. The membranes were visualized with the ECL chemiluminescence system. The results were recorded on x-ray films.

In Vitro T Cell Proliferation Assays—Isolated Th0 ($CD3^+CD4^+CD25^-$) cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with B regulatory cells (T cell:B cell = 10:1) in the presence of the specific antigen, Der p (20 ng/ml). After a 3-days incubation, the cultured cells were washed and analyzed by flow cytometry to identify CFSE-stained T cells.

Quantitative Real Time RT-PCR—The total RNA was extracted from cells using TRIzol Reagent. Template cDNA was reverse-transcribed from 1 μg of RNA using a cDNA synthesis kit. SYBR Green-based qRT-PCR was performed with a Bio-Rad MiniOpticon™ Real Time PCR Detection System. Expression of target genes was normalized to β-actin mRNA levels.

Btk Inhibitor Treatment—Btk inhibitor PCI-3276533 or saline was orally administered (25 mg/kg per day in water/5% mannitol/0.5% gelatin) to the mice daily for 1 week.

Assay for β-Hexosaminidase—The release of a preformed granular enzyme, β-hexosaminidase, in mast cells correlates well with histamine release (15). Finally, 40 μl of each supernatant was transferred to a fresh 96-well plate and mixed with 50 μl of substrate solution (5 mm 4-nitrophenyl *N*-acetyl-β-D-glucosaminide in 50 mM citric acid buffer, pH 4.5). After incubation at 37 °C for 90 min, the reactions were terminated by the addition of 50 μl of stop solution (0.5 M Na_2CO_3/$NaHCO_3$, pH 10.0). Absorbances at 405 nm were recorded using a microplate reader (Molecular Devices).

BCs Adoptive Transfer—The $CD19^+CD35^+$ BCs were isolated from the intestine of OVA-sensitized mice by MACS and cultured for 24 h in the presence of OVA at 100 ng/ml. The BCs were adoptively transferred to OVA-sensitized mice at 10^6 cells/mouse (naïve BCs were used as controls) via tail vein injection.

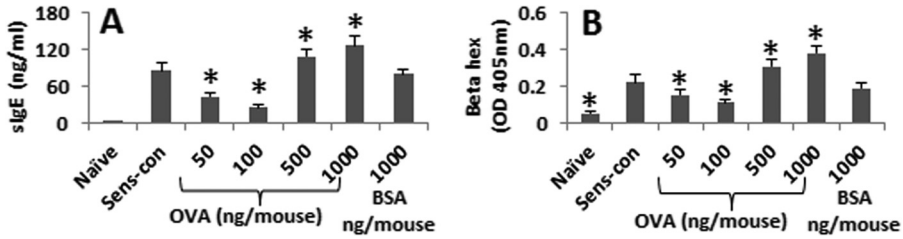

FIGURE 1. **SAV modulates IgE levels and mast cell activation in sensitized mice.** B6 mice were sensitized to OVA. SAV was administered intraperitoneally at the indicated doses of OVA (on *x* axes) daily for 1 week. The sera were collected at sacrifice and analyzed by ELISA (*A*) and enzyme assay (*B*). The *y* axes indicate the assessed parameters. The data in the *bars* are presented as mean ± S.D. (*error bars*). *, $p < 0.05$, compared with the sense-con group (mice were sensitized with OVA, challenged with saline; using as a control group). *Naïve*, naïve mice (using as a naïve control); *OVA* group, mice were sensitized and treated with SAV at the indicated doses. *BSA*, mice were sensitized to OVA, but challenged with BSA (using as an irrelevant protein). Each group consisted of six mice. The data represent six separate experiments.

Statistical Analysis—Results were expressed as mean value ± S.D. and analyzed by two-way analysis of variance and post hoc corrections or Student's *t* test. Differences were considered to be significant when $p < 0.05$.

RESULTS

SAV Modulates Serum Levels of Specific IgE and Mast Cell Activation in Sensitized Mice—The grouped mice were sensitized to OVA; the mice were then treated with SAV for 1 week mimicking the SAV in an allergy clinic. To understand the dosage effect of SAV on the immune regulation of SAV, the mice were treated with SAV at graded doses. After sacrificing the mice, the levels of IgE and β-hexosaminidase in the sera were determined by ELISA. The results showed that the levels of serum-specific IgE and β-hexosaminidase were markedly reduced in mice received the lower doses (50 and 100 ng/mouse) of SAV; however, the levels of serum-specific IgE and β-hexosaminidase were increased in mice that received higher doses (500 and 1000 ng/mouse) of SAV compared with the group of sensitized mice treated with saline (Fig. 1). The results imply that the dosage of the specific antigen in SAV plays an important role in the regulation of antigen-specific IgE and mast cell activation in sensitized subjects.

Regulatory Effect of SAV on BCs—Because IgE is produced by mature B cells, the data of Fig. 1 imply that SAV can regulate B cell properties. Production of IL-10 by regulatory B cells has been shown to modulate the severity of immune diseases (16). Thus, we collected the sera from mice treated with the same procedures in Fig. 1. Compared with naïve controls, the sensitized mice showed much lower serum IL-10 levels than controls, which were markedly increased after treated with lower doses of SAV; however, the higher doses of SAV suppressed the IL-10 levels (Fig. 2*A*).

Recent reports indicate that B regulatory cell-derived IL-10 plays a critical role in immune regulation (17). To see whether BCs in the sensitized mice also produce IL-10 that can be regulated by SAV, we isolated the LPMCs after SAV and analyzed by flow cytometry. The $CD19^+$ B cells were gated (Fig. 2*B*) and further analyzed the frequency of $CD35^+$ $IL\text{-}10^+$ B cells in the gated $CD19^+$ B cells. The results showed that in mice treated with lower doses of SAV, the frequency of $IL\text{-}10^+$ BCs significantly increased; however, the frequency of $IL\text{-}10^+$ BCs was decreased in mice treated with higher doses of SAV (Fig. 2*C*).

SAV Induces IL-10 Production by BCs via Activating CD23—Theoretically, during SAV, the specific antigens may bind to either IgE-CD23 complexes or BCR or both on the surface of B cells to modulate B cell activities. Because we observed that SAV induced BCs to produce IL-10, we next observed whether CD23 or BCR or both were involved in the process. Thus, we sensitized $CD23^{-/-}$ mice and the littermate B6 mice with OVA in the same procedures in Fig. 2 and then treated the mice with SAV (with the specific antigen OVA) for 1 week. The $IL\text{-}10^+$ $CD19^+CD35^+$ BCs in the intestine were analyzed. As shown by flow cytometry, the expression of IL-10 was detectable in naïve BCs that was increased markedly after exposure to the specific antigen at the lower doses; however, the IL-10 was not increased in $CD23^{-/-}$ BCs (Fig. 3). The results implied that the IgE-CD23 complexes existed on the surface of BCs; the specific antigen, OVA, bound on the IgE=CD23 complexes to activate BCs to produce IL-10. To test the hypothesis, in separate experiments, the BCs were stained with antibodies of IgE, CD23, and OVA. As shown by flow cytometry, indeed, a subset of the BCs from OVA-sensitized B6 mice were IgE^+ $CD23^+$ OVA^+ (Fig. 4, *A–D*). The results were further confirmed by the results of immune precipitation assay (Fig. 4*E*). The data demonstrate that specific antigen can bind to the IgE-CD23 complexes on the surface of BCs, which may activate BCs to produce IL-10. However, BCs also express BCR, which can be bound by specific antigens as well. To elucidate whether BCR was involved in the production of IL-10 by BCs upon exposure to specific antigens, in separate experiments, we pretreated the mice with the BCR signal inhibitor, the Btk inhibitor (PCI), then exposed the mice to the specific antigen, OVA, at lower doses. Such treatment did not reduce the production of IL-10 in BCs (Fig. 3). The results indicate that SAV at lower doses induces BCs to produce IL-10 via activating CD23.

High Doses of SAV Increase MMP9 Expression in BCs to Cleave CD23 on the Surface of BCs—CD23 can be cleaved to be sCD23; the latter can promote the production of IgE to exacerbate the ongoing allergic disorders (8). Whether the generation of sCD23 is associated with the dosage of SAV is unclear. Thus, we treated the sensitized mice with SAV at graded doses for 1 week. The levels of CD23 in spleen BCs and sCD23 in the sera were assessed in the mice. The results of Western blotting showed that the expression of CD23 was detected in BCs, which was decreased in BCs of sensitized mice and was recovered by SAV at lower doses, but the higher doses of SAV decreased it. Low levels of sCD23 were detected from the serum of naïve mice, which was markedly increased in sensitized mice, that

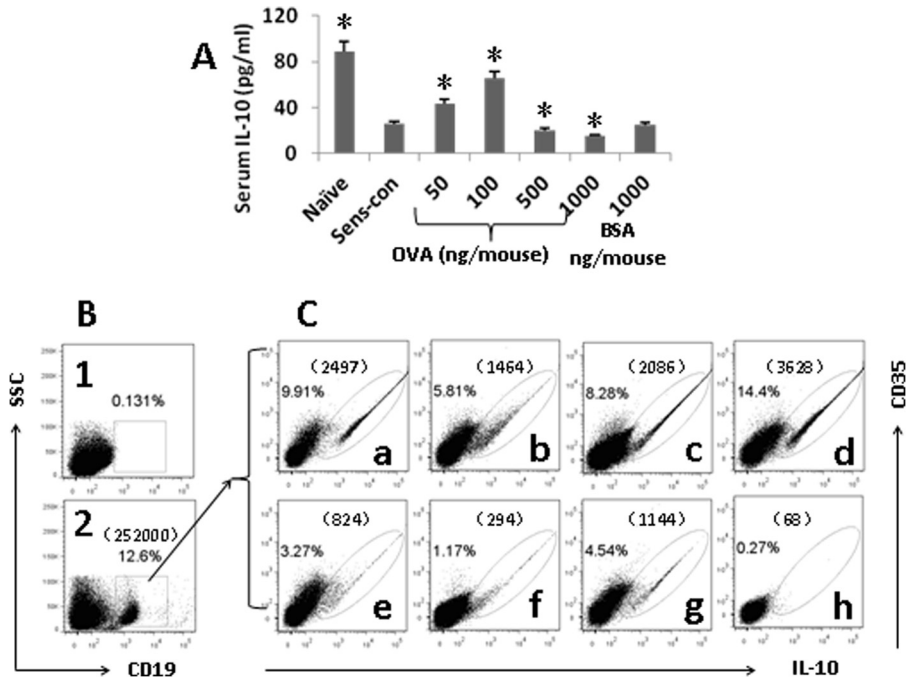

FIGURE 2. **SAV modulates the IL-10 production by antigen-specific BCs.** Mice were treated with OVA as described in Fig. 1. *A*, *bars* indicate the serum levels of IL-10 (by ELISA). *Error bars*, S.D. The group denotations are the same as Fig. 1. *B1*, isotype IgG control is shown. *B2*, dot plots indicating the frequency of $CD19^+$ B cells in isolated LPMCs (2×10^6 cells) isolated from mice treated with the same procedures of Fig. 1. *C*, dot plots show the frequencies of $CD35^+$ $IL\text{-}10^+$ BCs in the gated cells in B2. The absolute cell numbers are presented in each dot plot panel (in *parentheses*). Cell sources are shown in *C*, *a*, naïve mice; *b*–*g*, sensitized mice treated with SAV. OVA dosage (ng/mouse) was $b = 0$, $c = 50$, $d = 100$, $e = 500$, $f = 1000$; *g* is an isotype control. Each group consisted of six mice. The data represent six separate experiments.

was blocked by pretreatment with a BCR signal inhibitor. SAV at lower doses reduced the levels of serum sCD23 whereas higher doses of SAV further increased it (Fig. 5, *A* and *B*). The results imply that SAV at higher doses cleave CD23 from BCs that increases the sCD23 levels in the serum.

Previous reports indicate that activated B cells produce MMP9 that cleaves CD23 on the surface of B cells (13). To see whether the exposure to higher doses of SAV also increased the expression of MMP9 by B cells, we assessed the expression of MMP9 in the BCs. The results showed that the exposure to lower doses of SAV did not alter the expression of MMP9 whereas the exposure to higher doses of SAV markedly increased the expression of MMP9 in the BCs, which could be blocked by the BCR signal inhibitor, but not the anti-IL-10 antibody (Fig. 5, *C* and *D*). The results indicate that exposure to higher doses of specific antigen can increase the expression of MMP9 in BCs via the activation of BCRs.

Antigen-specific BCs Primed by Lower or Higher Dose of SAV Differentially Modulate Skewed Th2 Response—The data we have presented so far indicate that BCs have the potential either to inhibit or boost the skewed antigen-specific Th2 responses depending on the doses of SAV. To further confirm the results, the OVA-sensitized mice were treated with SAV at lower doses for 1 week, which significantly suppressed the T cell proliferation (Fig. 6, *A* and *B*), serum levels of IL-4 and IgE (Fig. 6*C*), mast cell, and eosinophil extravasation in the intestine (Fig. 6*D*) compared with the group of sensitized mice treated with saline, which were abolished by pretreatment with neutralizing anti-IL-10 antibody. The results indicate that upon exposure to lower doses of SAV, BCs produce IL-10 to suppress the skewed antigen-specific Th2 responses.

However, we adoptively transferred the BCs primed by higher doses of SAV to the sensitized mice; the treatment resulted in higher proliferation of T cells (Fig. 6, *A* and *B*), increases in the serum levels of IL-4 and specific IgE (Fig. 6*C*), mast cell and eosinophil extravasation in the intestine (Fig. 6*D*), which were abrogated by pretreatment with the BCR signal inhibitor. The results indicate that the SAV at higher doses activates the BCR and further boosts the ongoing antigen-specific Th2 responses.

DISCUSSION

Mice retain one of the functions of CD23, that is the engagement of IgE on the B cell surface (18). The present study has expanded the notion that specific antigens can bind the IgE-CD23 complexes on the surface of BCs; the binding activates the BCs to produce IL-10; the latter suppresses the ongoing antigen-specific Th2 responses. On the other hand, higher doses of specific antigens engage the BCR on BCs; the engagement induces BCs to produce MMP9; the latter cleaves CD23 from the BCs, followed by the increases in the serum levels of sCD23, which promotes the production of antigen-specific IgE and exacerbates the ongoing antigen-specific Th2 responses. Thus, checking the serum sCD23 levels during SAV may be an indicator of whether the antigen vaccine dose is optimal.

After invading into the body, specific antigens may form complexes with existing specific IgE; the complexes may bind to the CD23 on the surface of target cells, such as B cells, to activate the cells (8). Our data are in line with published data by showing the specific antigens binding the IgE-CD23 complexes on BCs. In terms of function, CD23 has two subforms, the soluble CD23 and the membrane CD23. Previous reports indicate

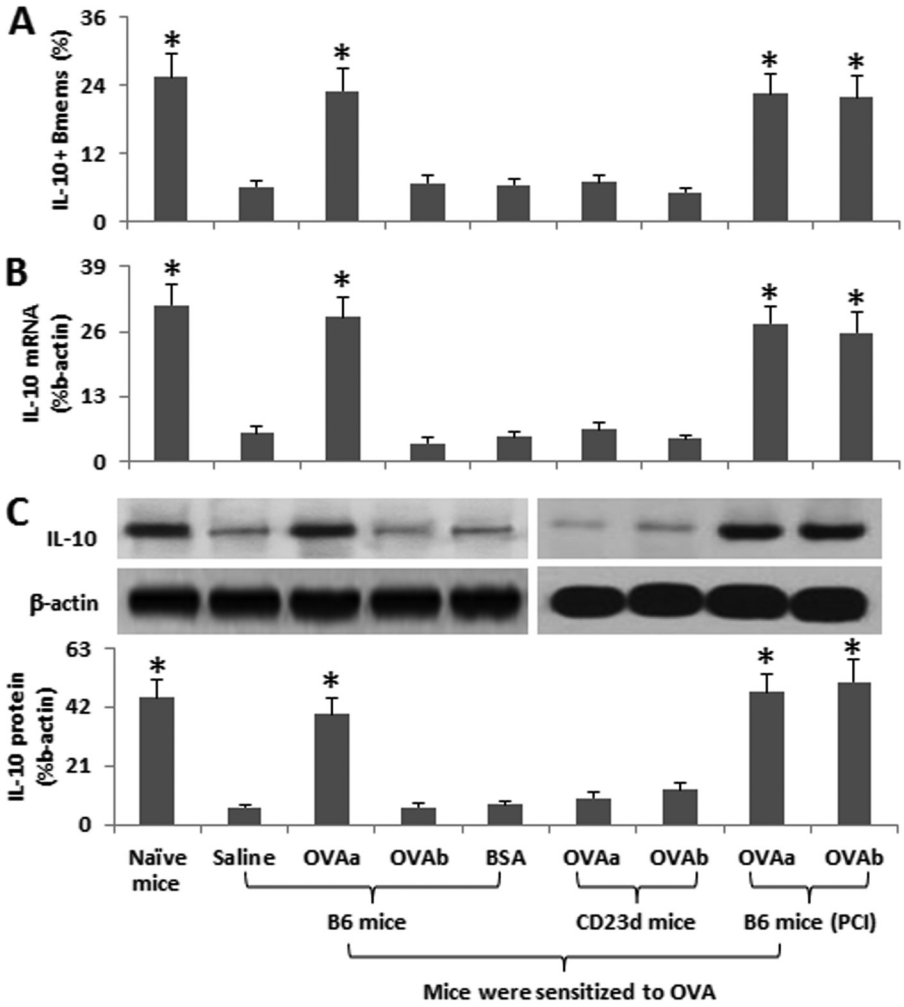

FIGURE 3. **BCs express IL-10 upon exposure to specific antigens.** OVA-sensitized B6 mice or CD23-deficient mice (*CD23d*) were treated with OVA (*A*, 100 ng/mouse; *B*, 1000 ng/mouse, intraperitoneally), or BSA (1000 ng/mouse) daily for 1 week (naïve B6 and sensitized mice were fed with saline using as controls). LPMCs were isolated and stained with antibodies of CD19, CD35, and IL-10. By flow cytometry, the $CD19^+CD35^+$ BCs were gated first (data not shown) and analyzed further for $IL\text{-}10^+$ BCs. *A*, the *bars* indicate the frequency of $IL\text{-}10^+$ cells in BCs. *Error bars*, S.D. *B* and *C*, a portion of $CD19^+CD35^+$ BCs was isolated from the LPMCs by MACS and analyzed by qRT-PCR and Western blotting. *B*, the *bars* indicate the levels of IL-10 mRNA in the cellular extracts of the BCs. *C*, the immune blots indicate the levels of IL-10 protein in the cellular extracts; the *bars* below the blots show the summarized integrated density of the immune blots. *PCI*, mice were pretreated with the Btk inhibitor, PCI-32765, to block the BCR signals. Each group consisted of six mice. The data represent six separate experiments.

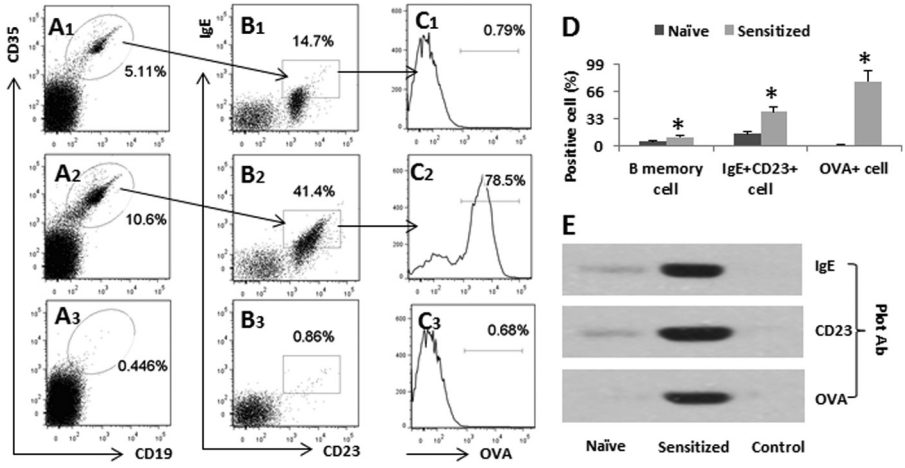

FIGURE 4. **Localization of antigen-IgE-CD23 on BCs.** *A*, the dot plots indicate the frequency of $CD19^+CD35^+$ BCs in LPMCs isolated from naïve mice (A_1) and mice sensitized to OVA (A_2). *B*, the dot plots indicate the frequency of $CD23^+IgE^+$ cells in the gated cells of *A* (pointed by *arrows*). *C*, the histograms indicate the frequency of OVA^+ cells in the gated cells in *B*. A_3, B_3, and C_3 are isotype control. *D*, the *bars* indicate the summarized data in *A–C*. *Error bars*, S.D. *E*, the immune blots indicate the immune precipitated IgE-CD23-OVA complex in the protein extracts from BCs isolated from LPMC by MACS. The data represent three separate experiments.

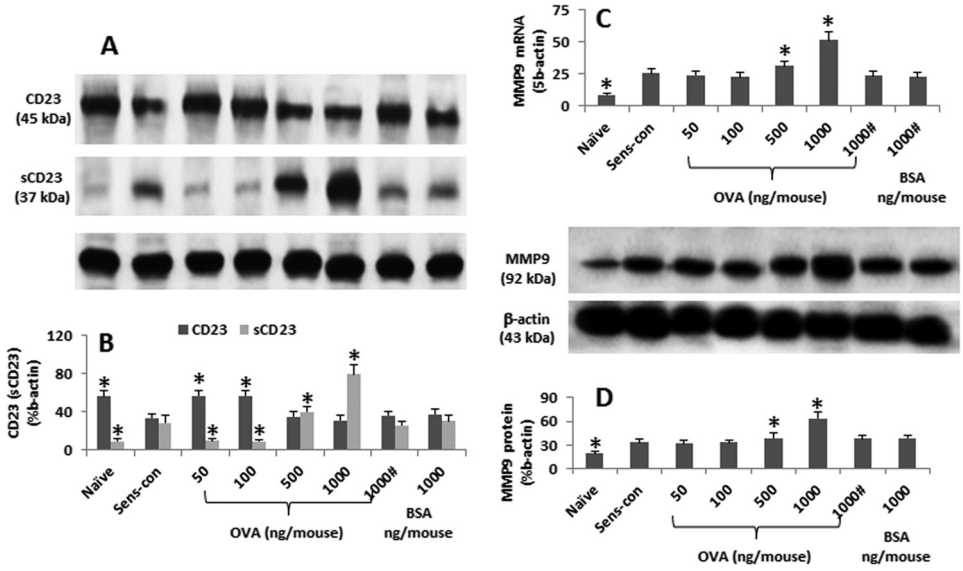

FIGURE 5. **SAV modulates expressions of MMP9 in BCs sCD23 in the serum.** Sensitized mice were treated with SAV at the indicated doses for 1 week. BCs were isolated from the spleen; the cellular extracts of BCs were prepared, and the sera were collected at sacrifice. *A*, the immune blots show the levels of CD23 in BCs and sCD23 in the sera, respectively. *B*, the *bars* indicate the summarized integrated density of the blots. *Error bars*, S.D. *C*, the *bars* indicate the levels of MMP9 mRNA in BCs (by qRT-PCR). *D*, the immune blots indicate the levels of MMP9 protein in BCs; the *bars* below the *blots* indicate the summarized integrated density of the blots. *1000#*, mice were pretreated with BCR signal inhibitor, PCI. *BSA*, sensitized mice were treated with BSA using as an irrelevant control protein. Each group consisted of six mice. The data represent six separate experiments.

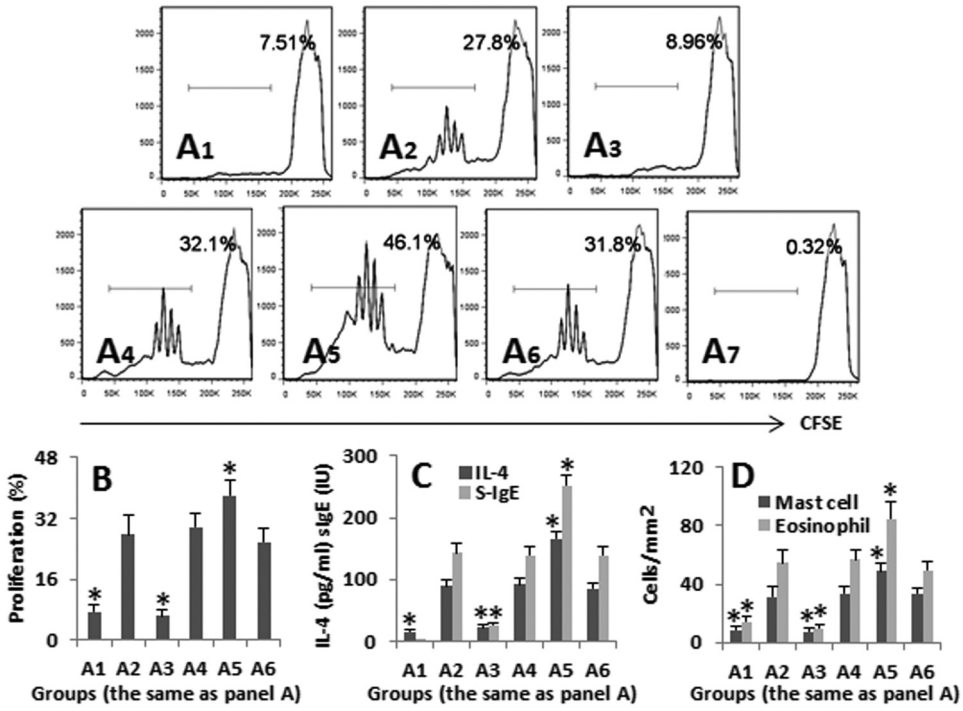

FIGURE 6. **Antigen-specific BCs primed by lower or higher dose of SAV differentially modulate skewed Th2 response.** Mice were sensitized to and challenged with OVA. The $CD3^+CD4^+CD25^-$ T cells were isolated from the intestine, labeled with CFSE, and cultured for 3 days in the presence of DCs (DC:T cell = 1:10). A_1–A_6, the flow cytometry histograms indicate the frequency of proliferating T cells. A_7 is a staining control. *B*, the *bars* indicate the summarized data in A_1–A_6. *C*, the *bars* indicate the serum levels of IL-4 and OVA-specific IgE (assessed by ELISA). *D*, the bars indicate the cell counts of the mast cell and eosinophil in the intestinal mucosa. Group annotations: A_1, naïve mice. A_2–A_6, sensitized mice were challenged with OVA. A_3 and A_4, mice were adoptively transferred with BCs (10^6/mouse; isolated from the intestine of sensitized mice; cultured for 24 h in the presence of OVA at 100 ng/ml). A_4, mice were injected with neutralizing anti-IL-10 antibody (100 ng/mouse) 30 min prior to each exposure to OVA. A_5 and A_6, mice were adoptively transferred with BCs (10^6/mouse; isolated from the intestine of sensitized mice and cultured for 24 h in the presence of OVA at 1000 ng/ml). A_4, mice were injected with PCI-3276533. Data in *bar graphs* are presented as mean ± S.D. (*error bars*). *, $p < 0.01$, compared with group A_2. Each group consisted of six mice. The data represent six separate experiments.

that activation of CD23 has an immune regulatory function. Cooper *et al.* indicate that soluble CD23 can up-regulate the production of IgE (8). Mossalayi *et al.* report that activation of CD23 can induce macrophages to release proinflammatory cytokines (19). Our data show that the forming antigen-IgE-CD23 complex induces the production of IL-10, an immune suppressor cytokine, by BCs; the results are in line with others' findings in different study systems, such as Uchimura *et al.*

indicate that T cell-derived IL-4 can activate CD23-bearing cells to overproduce IL-10 that may play an important role in Graves disease (20). Our data also indicate that the engagement of CD23 by specific antigens at lower doses increases the production of IL-10 by BCs that further inhibits the ongoing antigen-specific Th2 responses.

In addition to the expression of CD23, BCs also express the BCR, which has the potential to be bound by specific antigens and therefore to be activated, which is involved in the process of producing IgE (21). Different results also reported such as Jabara *et al.* indicated that BCR cross-linking inhibited IgE and IgG1 switching (22). Because we observed the production of IL-10 by BCs upon exposure to a specific antigen, we need to clarify whether the BCR or CD23 activation is involved in the production of IL-10 in our experimental system. By blocking the BCR signal, the IL-10 production in BCs was not affected whereas it was abrogated in CD23-deficient mice. The fact indicates that the specific antigen increased IL-10 production by BCs at the lower doses of SAV is via activation of CD23 pathway.

The SAV is currently used in the treatment of allergic diseases. The optimal dosage of SAV varies from patient to patient. Thus, to determine the optimal dosage of SAV is important for the therapeutic effect. In our SAV dose-response experiments, the lower doses and higher doses of SAV differentially influenced the outcome of antigen-specific Th2 cell response upon exposure to the specific antigens. A similar phenomenon was also noted in our clinical practice using SAV to treat allergic diseases; the doses of SAV are increased gradually; the maintaining SAV doses need to be adjusted accordingly to the clinical outcomes. The present study has also revealed the underlying mechanism; following the increases in SAV doses, the expression of MMP9 in BCs was induced. MMP9 can be produced by B cells upon the engagement of BCRs (13); our data are in line with the finding by showing that by pretreating with BCR signal inhibitor, the induced MMP9 was abrogated. MMP9 is a protease that can cleave the proteins on the cell surface (13), such as MMP9 can cleave CD23 from B cells; the cleaved part is sCD23. Indeed, after exposure to the higher doses of SAV, we observed that the CD23 amounts on BCs were reduced whereas the levels of sCD23 in the serum were increased. The finding implicates that the higher doses SAV increases the expression of MMP9 in BCs; the MMP9 cleaves CD23, the latter contributes the levels of sCD23 in the serum. The inference was supported by the subsequent results that pretreatment with a BCR signal inhibitor abolished the increase in the serum sCD23 levels.

The SAV is one of the major therapeutic remedies in the treatment of allergic diseases. The current understanding about the mechanism of SAV includes induction immune tolerance and generation of the blocking antibodies, particularly IgG4 and IgA2 subclasses, for the specific antigens (23). Our data have expanded the existing knowledge in SAV by showing that the optimal dosage of SAV induces BCs to produce IL-10 to regulate the ongoing skewed Th2 responses; the data were tested both *in vitro* and *in vivo* experiments. However, higher doses of SAV increase the expression of MMP9 in BCs, which further cleaves CD23 from BCs and increases the serum levels of sCD23; the latter contributes to the production of IgE and exacerbates the ongoing antigen-specific Th2 responses, a condition that should be avoided in SAV.

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J. Biol. Chem. 2013, 288:16383-16390.
doi: 10.1074/jbc.M113.456202 originally published online April 15, 2013