Specific antigen vaccination modulates memory B cell activities

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Running title: SAV and B cell function

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Capsule
Background: B memory cells (Bmems) play an important role in allergic diseases. Lower doses of antigen induce Bmems to produce IL-10; higher doses result in high serum levels of soluble CD23.

Conclusions: Exposure to lower doses of antigen induces Bmems to inhibit skewed Th2 responses.

Significance: Suitable dosage of antigen in specific antigen vaccination determines the clinical outcomes.

Abstract
Background and aims: B memory cells (Bmem) express CD23 and B cell receptors. Whether activation of CD23 and B cell receptors has different effects on Bmems' activities is unclear. This study aims to investigate the activation of antigen specific Bmems in the regulation of skewed Th2 responses.

Methods: Mice were sensitized to ovalbumin (OVA) to generate the antigen specific Bmems. The specific antigen vaccination (SAV) at graded doses was employed to modulate the activities of Bmems in which the expression of IL-10, IgE, matrix metalloproteinase-9 (MMP9), CD23 and serum soluble CD23 by Bmems was evaluated. The immune regulatory effects of Bmems primed by lower or higher antigen doses was observed with an adoptive transfer mouse experiment.

Results: SAV at lower doses, but not at higher doses, activated CD23 to produce IL-10 in Bmems. Higher doses of SAV increased the expression of MMP9 in Bmems that reduced the amounts of CD23 in Bmems and increased the serum levels of soluble CD23, which were blocked by pretreatment with MMP9 inhibitor. Adoptive transfer with antigen specific Bmems primed by lower doses of SAV abrogated the ongoing antigen specific Th2 responses while the antigen specific Bmems primed by higher doses of SAV exacerbated the ongoing Th2 responses.

Conclusions: Exposure to specific antigens at optimal doses can activate antigen specific Bmems to produce IL-10 to suppress the skewed antigen specific Th2 responses. Higher doses of specific antigens may...
promote the production of soluble CD23 to exacerbate the ongoing immune responses.

Introduction

The skewed T helper (Th)2 responses play a critical role in the pathogenesis of a number of diseases, such as allergic asthma, food allergy, 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 skewed Th2 response is that high levels of immunoglobulin (Ig)E can be resulted in the local tissue that can be 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 may be activated to release chemical mediators to evoke inflammation in the local tissue (6,7). On the other hand, IgE can bind the low affinity receptor, CD23, to modulate the target cells activities (8).

Reagents

After priming by antigen, naïve B cells may differentiate to plasma cells, CD38+CD23- or become CD19+CD35+ B memory cells (Bmem) (9) to remain a quiescent status in the body (10). Re-immunization can activate the B cell receptor (BCR) to boost the Bmems to develop into plasma cells to produce antibodies, to promote protective immunity or induce skewed immune reactions (11). Because of expressing the CD23 (8), Bmems may be bound by IgE to form immune complexes on the surface of B cells, which can be further modulated by specific antigens to form a triple immune complex. Whether this triple immune complex modulates the Bmem's activity is to be further elucidated.

Materials and methods

Reagents

Anti-ovalbumin antibody was obtained from AbBioTec (Guangzhou, China). Antibodies of IL-10 (M-18), recombinant CD23 protein, CD23 mAb (H-4) and CD23 pAb (M-282) were obtained from Santa Cruz Biotech (Shanghai, China). Cell isolating reagent kits were obtained from Miltenyi Biotech (Shanghai, China). Cell isolating reagent kits were obtained from Millenyi Biotech. Reagents for quantitative real time RT-PCR (qRT-PCR) and Western blotting were obtained from Invitrogen (Shanghai, China). Fluorescence labeled antibodies for flow cytometry were obtained from BD Biosciences (Shanghai, China). The anti-IgE antibody, ELISA kits of IL-4, IL-10, TIM1 and IgE were obtained from Shanghai MANUSCRIPT WITHDRAWN BY THE AUTHOR.
Transhold Tech (Shanghai, China). The immune precipitation reagents were obtained from Sigma Aldrich (Shanghai, China). 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 animals 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 (SAV)**

The sensitized mice were treated with the specific antigen, OVA (at doses of 50, or 100, or 500, or 1000 ng/mouse in 0.1 ml saline; saline or bovine serum albumin, BSA, were used as controls) via i.p. 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 (β-hex), 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 that the observer were not aware of to avoid the observer bias.

**Enzyme-linked immunoassay (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 U 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 (LPMC) 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 re-stained with the intracellular staining approach. Cells were analyzed with a flow
cytometry (BD FACSCanto, BD Bioscience, Shanghai, China).

**Immunoprecipitation**

Total proteins were extracted from cells with Bio-Rad protein extraction kits. The proteins were pre-cleared 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 24h. The 2xSDS sample buffer was used to elute the precipitates. The precipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to CD23, IgE and OVA, respectively.

**Western blotting**

The total proteins were extracted from cells, separated by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 hour and incubated with the primary antibodies for 1 hour at room temperature, followed by incubating with the horseradish peroxidase-labeled secondary antibodies for 1 hour 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 succinimidy ester (CFSE) and cultured with Bregs (T cell:B cell = 10:1) in the presence of the specific antigen, Der p (20 ng/ml). After 3 days' incubation, the cultured cells were washed and analyzed by flow cytometry to identify CFSE-stained T cells.

**Real-time RT-PCR (qRT-PCR)**

The total RNA was extracted from cells using Trizol Reagents. 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. Primer sequences for IL-6, IL-10 and IgE were presented in Table 2.

**Btk inhibitor treatment**

Btk inhibitor PCI-3276533 or saline was orally administered (25 mg/kg/day in water/5% mannitol/0.5% gelatin) to the mice daily for one 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 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 Na2CO3/NaHCO3, pH 10.0). Absorbances at 405 nm were recorded using a microplate reader (Molecular Devices, USA).

**Bmems adoptive transfer**

The CD19+CD35+Bmems were isolated from the intestine of OVA-sensitized mice by MACS and cultured for 24h in the presence of OVA at 100 ng/ml. The Bmems were adoptively transferred to OVA-sensitized mice at 10^7 cells/mouse via tail vein injection.

**Statistical analysis**

Results were expressed as mean value ± SD and analyzed by ANOVA or student t test. Differences were considered to be significant when P<0.05.

**Results**

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SAV modulates serum levels of specific IgE and mast cell activation in sensitized mice

Grouped mice were sensitized to OVA; the mice were then treated with SAV for one week mimicking the SAV in an allergy clinic. In order 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-hex in the sera were examined by ELISA. The results showed that the levels of serum specific IgE and β-hex were markedly reduced in mice treated with lower doses (50 and 100 ng/mouse) of SAV; however, the levels of specific IgE and β-hex were increased in mice treated with higher doses (500 and 1000 ng/mouse) of SAV as compared with the sensitized mice treated with saline (Fig.1). These results indicated that the dosage of specific antigen in SAV may play an important role in the regulation of specific IgE and mast cell activation in sensitized subjects.

Regulatory effect of SAV on Bmems

Since IgE is produced by mature B cells, the data of Fig.1 imply that SAV can regulate B cells’ 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 and compared with naïve controls. The sensitized mice showed much lower serum IL-10 levels, which were markedly increased after treated with lower doses of SAV; however, the higher doses of SAV suppressed the IL-10 levels (Fig.2A).

Recent reports indicate that B regulatory cell (Breg)-derived IL-10 plays a critical role in immune regulation (17). To see if Bmems 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.2B) and further analyzed the frequency of CD35⁺ IL-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-10⁺ Bmems significantly increased; however, the frequency of IL-10⁺ Bmems was decreased in mice treated with higher doses of SAV (Fig.2C).

SAV induces IL-10 production by Bmems via activating CD23

Theoretically, during SAV, the specific antigens may bind to the IgE/CD23 complexes or BCR or both on the surface of Bmems and activate the B cells. Since we observed that SAV induced Bmems to produce IL-10, we next observed whether CD23 or BCR or both were involved in the process. Thus, we sensitized CD23⁻/⁻ mice and littermate B6 mice with OVA in the same procedures in Fig.2; and then treated the mice with SAV. After one week, the IL-10⁺ CD19⁺ CD35⁺ Bmems in the intestine were analyzed. As shown by flow cytometry, the expression of IL-10 was detected in Bmems that was increased significantly after exposure to the specific antigen, however, the IL-10 was not increased in CD23⁻ Bmems (Fig.3). The data demonstrated that the IgE/CD23 complexes exist on the surface of Bmems; the specific antigen, OVA, bound on the CD23⁻ Bmems to produce IL-10. In a separate experiment, the Bmems were stained with antibodies of IgE, CD23 and OVA. As shown by flow cytometry, indeed, a subset of the Bmems from OVA-sensitized B6 mice were IgE⁺ CD23⁺ OVA⁺ (Fig.4A-D). The results were further confirmed by the results of immune precipitation assay (Fig.4E). The data demonstrated that specific antigen can bind to the IgE/CD23 complexes on the surface of Bmems, which may activate Bmems to produce IL-10. However, Bmems
also express BCR, which can be bound by specific antigens as well. To elucidate if BCR was involved in the production of IL-10 by Bmems 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 Bmems (Fig.3). The results indicate that SAV at lower doses induces Bmems to produce IL-10 via activating CD23.

Higher doses of SAV increases MMP9 expression in Bmems and cleave CD23 from the Bmem surfaces. CD23 can be cleaved to sCD23, the latter can promote the production of IgE to exacerbate the ongoing allergic disorders (8). Whether the cleavage of CD23 is associated with the engagement of CD23 or CD23 is engaged with the engagement of SAV. We treated sensitized mice with SAV at graded doses for one week. The levels of CD23 in spleen Bmems and sCD23 in the sera were assessed in the mice. The results of Western blotting showed that the expression of CD23 was detected in Bmems which was decreased in Bmems from 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 was blocked by pretreatment with a BCR signal inhibitor. The results imply that SAV at higher doses cleave CD23 from Bmems that increases the sCD23 levels in the serum.

**Antigen specific Bmems primed by lower or higher dose of SAV differentially modulate skewed Th2 response**

The data we have presented so far indicate that antigen specific Bmems have the potential to either inhibit or boost the skewed antigen specific Th2 responses depending on the doses of SAV. To further confirm the results, OVA-sensitized mice were treated with SAV at lower doses for one week, which significantly suppressed the T cell proliferation (Fig.6A, 6B), serum levels of IL-4 and IgE (Fig.6C), mast cell and eosinophil extravasation in the intestine (Fig.6D) as compared with 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, specific Bmems produce IL-10 to suppress the skewed antigen specific Th2 responses. On the other hand, we adoptively transferred the Bmms primed by higher doses of SAV to the sensitized mice; the treatment resulted in higher proliferation of T cells (Fig.6A, 6B), increased in serum levels of IL-4 and specific IgE (Fig.6C), increased mast cell and eosinophil extravasation in the intestine (Fig.6D), 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 Bmems; the binding activates the Bmems 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 Bmems; the engagement induces Bmems to produce MMP9; the latter cleaves CD23 from the
Bmems, 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 if 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 on how specific antigens may form complexes with IgE and activate CD23 on B cells. CD23 has two sub forms, the soluble CD23 and the membrane CD23. Previous reports indicate that activation of CD23 has an immune regulatory function. Soluble CD23 can regulate the production of cytokines by B cells, whereas membrane 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 Bmems. Our data 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 allergies (20). Our data also indicate that the engagement of CD23 by specific antigens at lower doses increases the production of IL-10 by antigen specific Bmems that further inhibits the ongoing antigen specific Th2 responses.

In addition to the expression of CD23, Bmems 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). Since we observed the production of IL-10 by Bmems 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 Bmems was not affected whereas it was abrogated in CD23-deficient mice. The fact indicates that the specific antigen-increased IL-10 production by Bmems at the lower doses of SAV is via activation of CD23 pathway.

The SAV is currently used as the treatment of allergic diseases. The optimal dose of SAV varies from patients to patients. Thus, to determine the optimal dosage of SAV is important for the therapeutic effect. In our study, we observed that the lower doses of SAV had a higher incidence of IL-10 production, whereas the higher doses of SAV did not affect the production of IL-10. We also observed a similar phenomenon 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. Our present study has also revealed the underlying mechanism; following the increases in SAV doses, the expression of MMP9 in Bmems was induced. 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 Bmems were reduced while the levels of sCD23 in the serum were increased. The finding implicates that the higher doses SAV increases the expression of MMP9 in Bmems;
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 antigen specific Bmems 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 increases the expression of MMP9 in Bmems, that further cleaves CD23 from Bmems and increases the serum levels of sCD23, the latter contributes to the production of IgE and exacerbate the ongoing antigen specific Th2 responses, a condition should be avoided in SAV.

Reference List


Figure legends

Figure 1. SAV modulates IgE levels and mast cell activation in sensitized mice. B6 mice were sensitized to OVA. SAV was administered by i.p. at the indicated doses of OVA (in X axis) daily for one 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 were presented as mean ± SD. *, 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 6 mice. The data represent 6 separate experiments.
Figure 2. SAV modulates the IL-10 production by antigen specific Bmems. Mice were treated with OVA as described in Fig. 1. A, the bars indicate the serum levels of IL-10 (by ELISA). The group denotations are the same as Fig. 1. B1, isotype IgG control. B2, the dot plots indicate the frequency of CD19+ B cells in isolated LPMCs isolated from mice treated with the same procedures of Fig. 1. C, the dot plots show the frequencies of CD27+ IL-10+ Bmems in the gated cells in B2. Cell source in C: a, naive mice; b-g, sensitized mice were 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 6 mice. The data represent 6 separate experiments.
Figure 3. Bmems 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; i.p.), or BSA (1000 ng/mouse) daily for one week (naïve B6 and sensitized mice were fed with saline using as controls). LPMCs were isolated and stained with antibodies of CD19, CD27 and IL-10. By flow cytometry, the CD19+CD27+Bmems were gated first (not shown) and further analyzed for IL-10+Bmems. A, the bars indicate the frequency of IL-10+Bmems. B-C, a portion of CD19+CD27+Bmems 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 Bmems. 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 6 mice. The data represent six separate experiments.

Figure 4. Localization of antigen/IgE/CD23 on Bmems. A, the dot plots indicate the frequency of CD19<sup>+</sup> CD27<sup>+</sup> Bmems in LPMCs isolated from naïve mice (A1) and mice sensitized to OVA (A2). B, the dot plots indicate the frequency of the gated cells in the cells of panel A (pointed by arrows). The histograms indicate the frequency of OVA positive cells in the gated cells in panel B. A3 and C3 are isotype controls. D, the bars indicate the summarized data in A-C. E, the immune blots indicate the immune precipitated IgE/CD23/OVA complex in the protein extracts from Bmems isolated from LPMC by MACS. The data represent 3 separate experiments.
Figure 5. SSV modulates expression of MMP9 in the serum. Sensitized mice were treated with SAV at the indicated doses for one week. Mice were sacrificed, the cellular extracts of Bmems were prepared, the sera were collected at sacrifice. A, the immune blots show the levels of CD23 in Bmems and sCD23 in the sera respectively. B, the bars indicate the summarized integrated density of the blots. C, the bars indicate the levels of MMP9 mRNA in Bmems (by qRT-PCR). D, the immune blots indicate the levels of MMP9 protein in Bmems; the bars below the blots indicate the summarized integrated density of the blots. #, 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 6 mice. The data represent 6 separate experiments.

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Figure 6. Antigen specific Bmems 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 and labeled with CFSE and cultured for 3 days in the presence of DCs (DC:T cell = 1:10). A1-A6, the flow cytometry histograms indicate the frequency of proliferating T cells. The A7 is a staining control. B, the bars indicate the summarized data in A1-A6. 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. A, naïve mice. A2-A6, sensitized mice were challenged with OVA. A3-A4, mice were adoptively transferred with Bmems (10^7/mouse; isolated from the intestine of sensitized mice; cultured for 24h in the presence of OVA at 100 ng/ml). A4, mice were injected with neutralizing anti-IL-10 antibody (100 μg/mouse) 30 min prior to each exposure to OVA. A5-A6, mice were adoptively transferred with Bmems (10^7/mouse; isolated from the intestine of sensitized mice; cultured for 24h in the presence of OVA at 1000 ng/ml). A4, mice were injected with PCI-3276533. Data in bar graphs were presented as mean ± SD. *p<0.01, compared with group A2. Each group consisted of 6 mice. The data represent 6 separate experiments.
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