

Regulation of antigen-specific versus by-stander IgE production after antigen sensitization

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Abstract

IgE is critical in the pathogenesis of allergic disorders. In this report, we investigated the differential regulation of antigen-specific and by-stander IgE. Ovalbumin (OVA) immunization did not increase IgE producing cells in the spleen, but significantly enhanced the intracellular IgE content of all IgE⁺ cells. In contrast, OVA induced a significant increase of IgE⁺ cells in the draining lymph nodes (LN). Furthermore, OVA-specific IgE was detected only in the ex vivo cultures of the draining LN but not the spleen cells, while total IgE was increased in both cultures. These results indicated that antigen-specific IgE was mainly produced in the draining LN, while the spleen was a major source for by-stander IgE. Anti-IL-4, but not anti-IL-13, antibody blocked the expansion of IgE producing cells in the draining LN as well as systemic OVA-specific and total IgE levels, indicating IL-4 was important in both antigen-specific IgE generation and total IgE upregulation

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1. Introduction

IgE plays a pivotal role in allergic disorders. After being cross-linked by the corresponding antigens, the receptor bound IgE triggers degranulation of mast cells and basophils. These cells then release a variety of inflammatory mediators that cause immediate-type hypersensitivity reactions in allergy and asthma [1]. It has been well documented that serum IgE levels, including both allergen specific and by-stander (Ag unidentified), are usually much higher in patients with allergic disorders compared to those in normal individuals [2–4]. Therefore, it is important to understand the pathological sources of the elevated serum IgE. The current notion regarding IgE production is based on

results generated from in vitro cell culture or serum assays. It is believed that after Ag sensitization, IgM⁺ B cells specific to the Ag undergo activation, IgE isotype-switch and clonal expansion in the germinal centers of the spleen and the draining lymph nodes (LN) [5]. However, it is not clear whether the production of Ag-specific IgE is similarly regulated as that of by-stander IgE in terms of anatomic locations and cytokine involvement.

IgE production is up-regulated by cytokines such as IL-4 and IL-13 [2–4,6]. Both cytokines enhance proliferation of human B cells, and induce immunoglobulin class switch to IgG4 and IgE [7–9]. Unlike the human system, the effect of mouse IL-13 on mouse B cells is controversial. Originally, mouse IL-13 was reported to have no effects on IgE production [10], which was consistent with the observation that IL-4-deficient mice did not produce IgE in response to nematode infection [11]. However, IgE was produced in these mice upon malaria infection [12], which

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indicated an IL-4-independent mechanism for IgE production and raised the possibility for IL-13 in regulating IgE in mice. Furthermore, IL-13 transgenic mice expressed 10- to 100-fold higher serum IgE level than that of their littermate controls, and elevated IgE levels were also detected in sera from IL-4-deficient mice carrying IL-13 transgenes [13]. A better understanding of the involvement of IL-13 in mouse IgE production would provide better application with results obtained from mouse allergy and asthma models.

In this report, using a standard ovalbumin (OVA)/alum immunization protocol and single cell intracellular staining for IgE, we investigated the anatomic locations and cytokine involvement in Ag-specific IgE production versus by-stander IgE upregulation. We have demonstrated that unlike other Ig isotypes, antigen-specific IgE is mainly generated from the draining LN through antigen driven B cell expansion, while the spleen serves as a major source for by-stander IgE. In addition, both the Ag-specific and the by-stander IgE production were regulated by IL-4 but not IL-13.

2. Materials and methods

2.1. Animals

Female, 6–8 weeks, BALB/C mice (Charles Rivers Laboratories, Raleigh, NC, USA) were used in all the experiments. Mice were maintained under specific pathogen-free conditions and provided with ovalbumin (OVA) free food and water. All in vivo procedures performed were approved by Centocor's Animal Care and Use Committee.

2.2. Immunization protocol

Animals were immunized i.p. with 50 µg OVA (Sigma Chemical Company, St. Louis, MO, USA) in Alum solution (Pierce Chemical Company, Rockford, IL, USA) or PBS in Alum solution on day 1 and day 8. On days 5, 11, 15, 22, and 29, four mice from each group were euthanized (CO₂ asphyxiation), and their serum, spleen and the mesenteric LN (the draining LN) were collected and analyzed as described below.

2.3. Staining for intracellular IgE and IgG1

Cells isolated from spleen or the mesenteric LN were fixed with Cytofix buffer (B.D. Pharmingen, San Diego, CA, USA) for 15 min at room temperature, and permeabilized with Cytoperm buffer (B.D. Pharmingen) plus 5% BSA (Sigma Chemical) for 1 h on ice. The cells were then incubated with FITC-labeled anti-IgE mAb and PE-labeled anti-CD3 mAb, or FITC-labeled anti-IgG1 mAb and PE-labeled anti-CD3 mAb (B.D. Pharmingen)

for 30 min on ice. The cell content of IgE or IgG1 was then analyzed on a FACSCalibur (BD Biosciences) using CellQuest Software. In the analysis, T cells were gated out according to their anti-CD3 staining. The background staining for intracellular IgE and IgG1 staining was determined using an isotype control mAb (BD Biosciences). The specificity of the IgE staining has been confirmed using excess amount of soluble mouse IgE.

2.4. Ex vivo culture of cells from spleen or the draining LN

The ex vivo culture of spleen and the LN cells were conducted as described previously [14]. Briefly, cells (5×10^6) from spleen or the mesenteric draining LN were cultured for 72 h (a time point suitable for IgE pro-

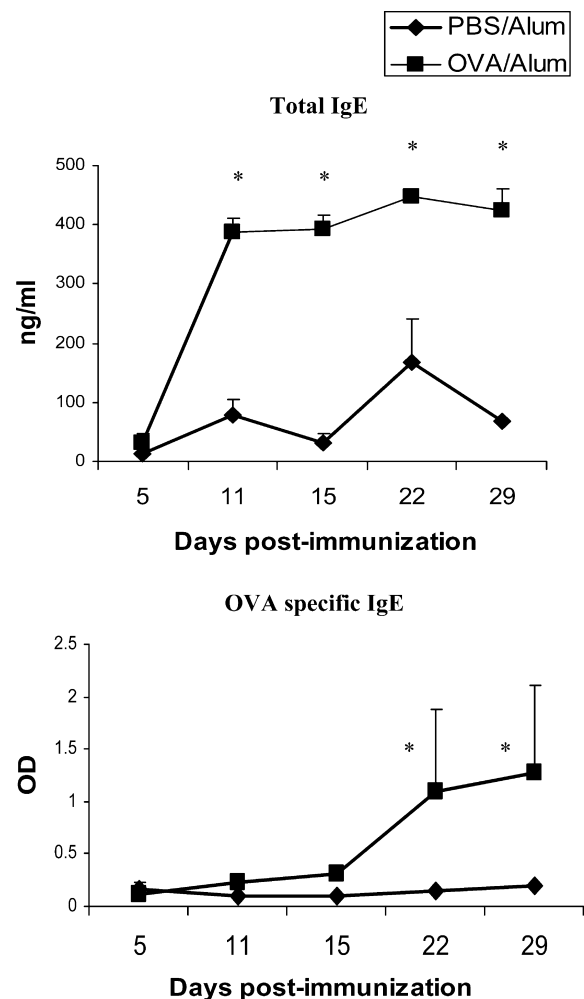


Fig. 1. OVA-specific and total IgE levels were increased in serum after OVA/alum immunization. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Serum samples were collected on different days post-immunization. The OVA-specific and total IgE levels in the serum were detected using ELISA. Results were expressed as means $\pm$ SD, $n = 4$. * $p < 0.05$ compared to PBS/alum immunized groups.

duction) in RPMI-1640 medium (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (Intergen, Purchase, NY), 10 mM glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin (RPMI-fetal bovine serum) in the presence of 10 µg/ml OVA. Supernates were collected and stored at -70°C for IgE and cytokine detections.

2.5. Cytokine and IgE detection

Interleukin (IL)-4 and IL-13 were measured using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Total IgE was measured using ELISA (B.D. Pharmingen). To measure OVA-specific IgE, plates were coated overnight at 4°C with 20 µg/ml OVA in PBS. After being blocked with 20% FBS, serum samples or cell culture supernatants were added and incubated for 2 h at room temperature followed by detection with biotinylated anti-mouse IgE (B.D. Pharmingen), avidin-HRP (B.D. Pharmingen), and

tetramethylbenzidine substrate. Purified mouse IgE was used as relative standard (B.D. Pharmingen).

2.6. In vivo neutralization of IL-4 and IL-13

Rat mAb with neutralizing activity for murine IL-4 was obtained from the hybridoma line 11B11 (ATCC, Rockville, MD). A rat anti-IL-13 mAb (C531) was generated in house using standard hybridoma technology. The IC_{50} of this antibody to mouse IL-13 (at 5 ng/ml) is 100 ng/ml in an IL-13-induced B9 (ATCC) cell proliferation assay. Normal rat IgG, purchased from Jackson ImmunoResearch, was used as control.

Mice were immunized as described above, and were given intravenous injections of 0.5 mg/mouse mAb to IL-4, IL-13 or in combination, on days 1, 4, 8, 10, and 12 after the first OVA immunization. On day 15, all mice were euthanized (CO_2 asphyxiation), and their serum, spleen, and the mesenteric LN were collected and analyzed as described above.

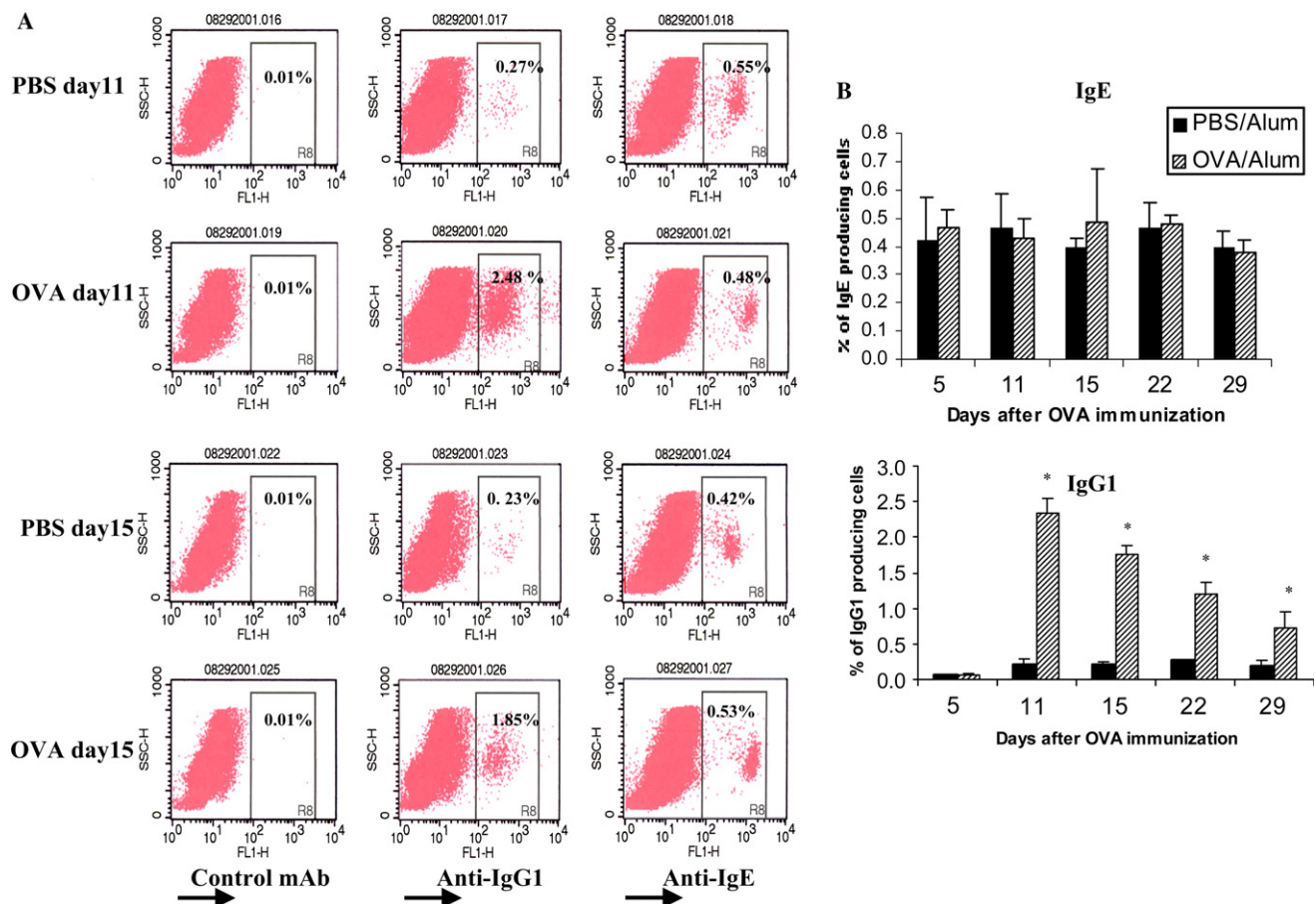


Fig. 2. The percentages of IgG1 positive B cells but not IgE positive B cells were increased in the spleen. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on different days as indicated and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD3 and IgE or CD3 and IgG1. The $\text{CD}3^{+}$ cells were gated out and the percentages of the $\text{CD}3^{+}\text{IgE}^{+}$ or the $\text{CD}3^{+}\text{IgG1}^{+}$ population were analyzed using FACS. (A) A set of representative plots. (B) The summary results of four mice in each group. Each bar represents the mean $\pm$ SD, $n = 4$. * $p < 0.05$ comparing to the corresponding PBS/alum immunized groups.

2.7. Statistics

Student's *t* test (two-tailed) was used for statistical analyses, and $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. OVA sensitization increased the intracellular IgE content of IgE⁺ cells in the spleen but not the percentage of these cells

It has been demonstrated that OVA/alum immunization induces elevated serum levels of IgE and IgG1 [15], which are regulated by Th2 cytokines [16]. Using this model, in combination with intracellular staining for IgE and IgG1, we investigated the production and regulation of Ag-specific vs. by-stander polyclonal IgE after OVA sensitization. Mice were immunized with OVA/Al-

um once a week for two weeks. Serum, spleen, and the draining LN samples from these mice were harvested at various time points as described in Materials and methods. As expected, both OVA-specific and the total IgE levels in the serum were elevated after OVA/alum immunization (Fig. 1), and the serum IgG1 levels were also increased (data not shown).

To study the cell source of IgE production, spleen cells were isolated and stained for their intracellular IgE, as well as IgG1 content. To our surprise, although the percentage of IgG1⁺ cells was dramatically increased in the sensitized mice, the percentage of IgE⁺ cells was not changed (Fig. 2). However, when the fluorescence intensity of the IgE⁺ cells was examined, we found that the intracellular IgE content of the IgE⁺ cells in the OVA sensitized mouse was significantly higher than that of the sham sensitized mice, and the increase was observed on day 11 through 29 after the OVA immunization (Fig. 3). Interestingly, although OVA immunization increased the percentage of IgG1⁺ cells in the spleen, it

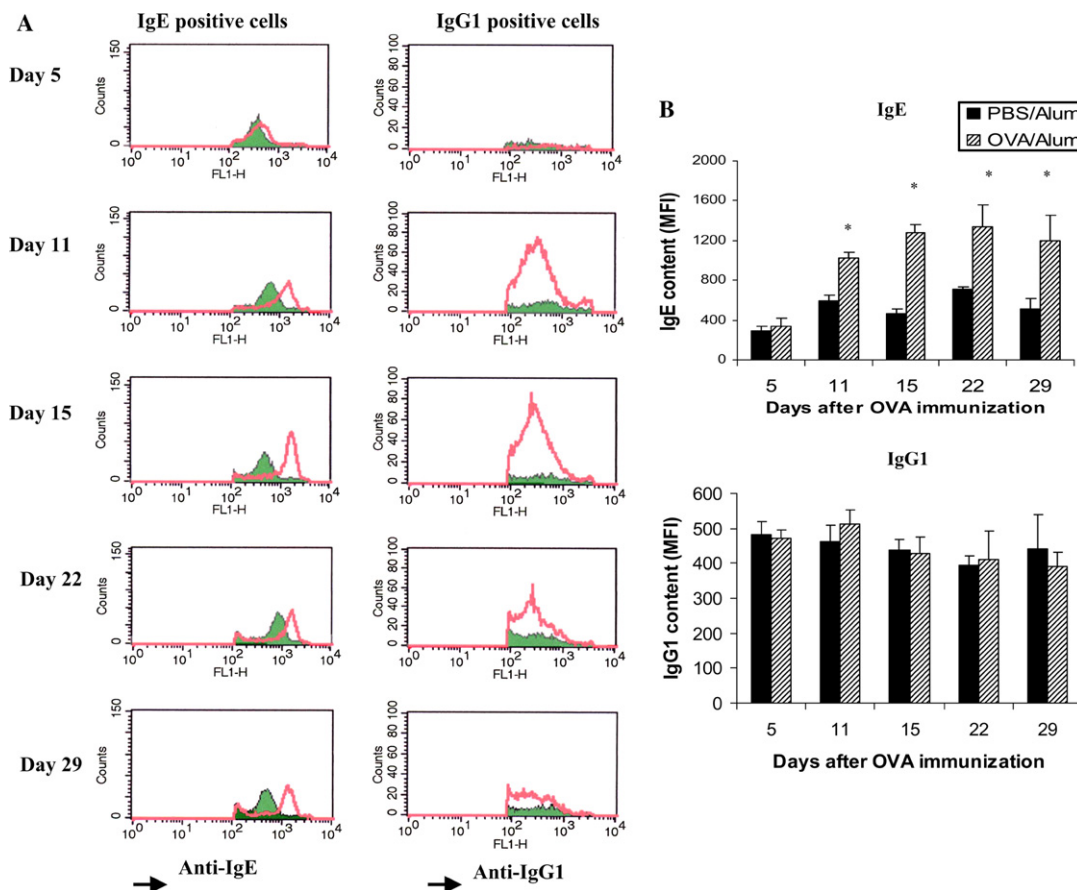


Fig. 3. OVA/alum immunization increased IgE but not IgG1 producing capacity at single cell levels of the spleen cells. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on different days as indicated and single cell suspensions were prepared. The cells were then fixed, permeabilized and stained for CD3 and IgE or CD3 and IgG1. The CD3⁺ cells were gated out and the mean fluorescence intensity (MFI) of the CD3⁺ IgE⁺ or the CD3⁺ IgG1⁺ population was measured. (A) A set of representative plots of IgE or IgG1 positive cells in OVA immunized (open histogram) and sham immunized (filled histogram) group. (B) The summary results of four mice in each group. Each bar represents the mean $\pm$ SD, $n = 4$. * $p < 0.05$ comparing to the corresponding PBS/alum immunized groups.

did not change the intracellular IgG1 content in these cells (Fig. 3). These results demonstrated a clear difference in terms of the production and regulation of IgG1 and IgE after Ag sensitization. The increased percentage of IgG1⁺ cells in the spleen may represent antigen driven B cell expansion. However, this expansion did not seem to occur with the IgE⁺ cells in the spleen. On the other hand, all pre-existing IgE⁺ cells in the spleen increased their capability for IgE production.

3.2. Antigen-specific IgE was generated in the draining LN through antigen driven B cell expansion

Since the antigen driven IgE⁺ B cell expansion was not observed in the spleen, the draining LNs were examined. Cells from mesenteric LN were isolated and stained for CD3 and intracellular IgE and IgG1, and the CD3⁺ negative cells were gated and the IgE and IgG1 producing cells were analyzed. As shown in Fig. 4, The percentages

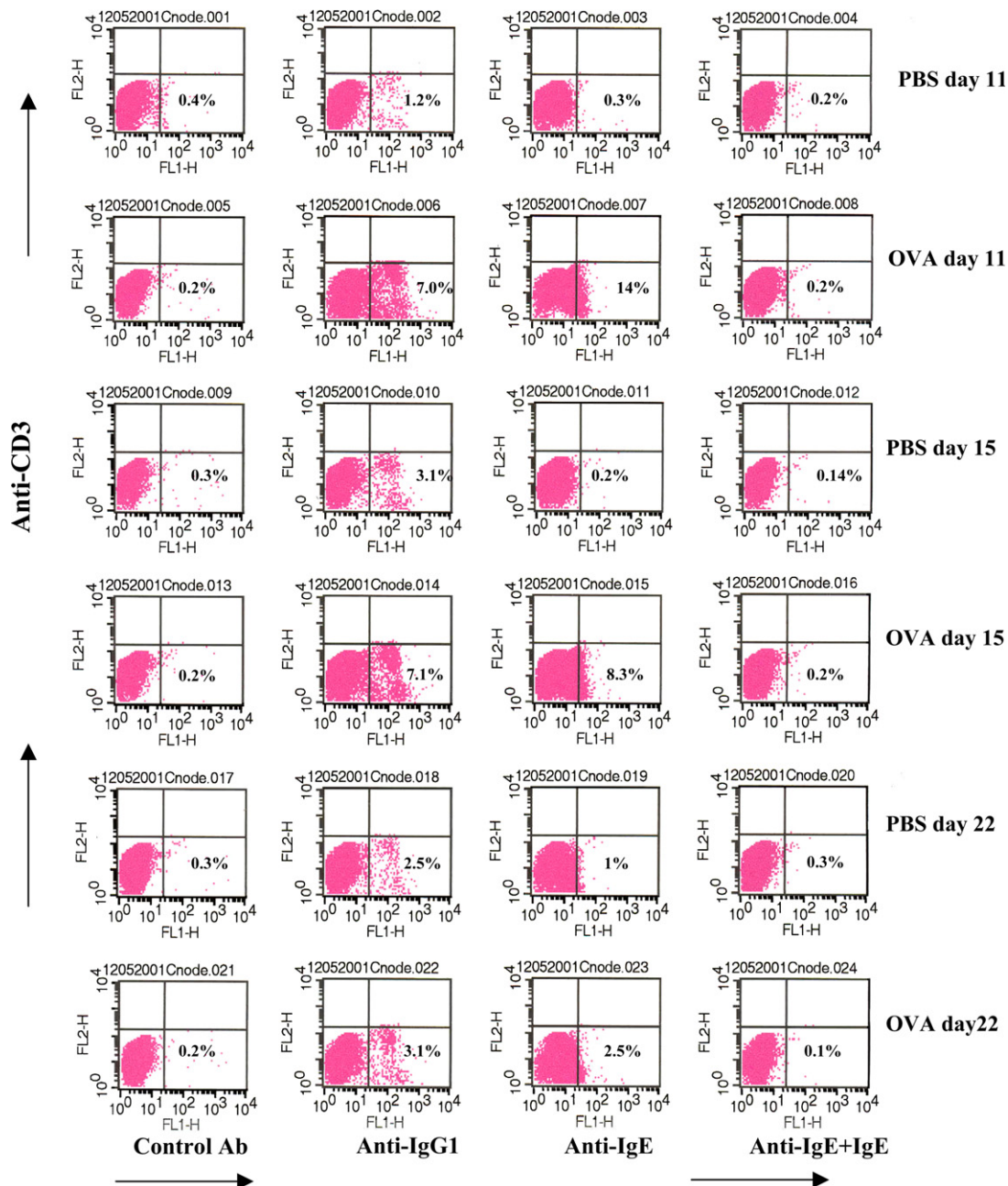


Fig. 4. Both IgE and IgG1 positive B cells were increased in the lymph nodes after OVA/alum immunization. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Mesenteric lymph nodes were collected on different days as indicated and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD3 and IgE or CD3 and IgG1. The CD3⁺ cells were gated out and not shown in the graph. The percentages of the CD3⁺IgE⁺ or the CD3⁺IgG1⁺ population were analyzed using FACS. The specificity of IgE staining was further confirmed by pre-incubating the antibody with excess amounts of IgE.

of both IgG1 and IgE producing cells were quite low in the mesenteric LN of sham sensitized mice, but were dramatically increased in those of OVA immunized mice, especially on days 11 and 15 post-immunization. Thus, unlike what was observed in the spleen, OVA immunization induced expansion of Ag-specific IgE producing B cells in the draining LN.

To confirm our hypothesis that the draining LN but not the spleen was the lymphoid organ that the OVA-specific IgE was produced, cells from the mesenteric LN and the spleen were collected on days 11, 15, 22, and 29 post-immunization and stimulated ex vivo with OVA for 72 h. The culture supernatants were collected, and the OVA-specific as well as the total IgE was measured. As shown in Fig. 5, although elevated levels of total IgE were detected in both the spleen and LN cell cultures from OVA immunized mice, significantly increased levels ($p < 0.05$) of OVA-specific IgE was only detected in the LN and not in the spleen cultures. Taken together, these results strongly suggest that after OVA sensitization, the Ag-specific IgE was mainly secreted in the draining LN probably through antigen driven B cell expansion, while the spleen produced by-stander IgE through the enhancement of IgE producing capability of individual IgE producing cells.

3.3. IL-4 but not IL-13 was involved in regulating both the Ag-specific and the by-stander IgE production after Ag sensitization

Both IL-4 and IL-13 have been reported to be involved in IgE regulation in mice [13]. To study their potential contribution to Ag-specific and by-stander total IgE production after OVA sensitization, we measured the IL-4 and IL-13 levels in the ex vivo spleen and draining LN cell cultures. Cells from the spleen and the draining LN were collected on days 11, 15, and 22 after OVA immunization, and were stimulated ex vivo with OVA for 72 h. The levels of IL-4 and IL-13 in the culture supernatants were measured by ELISA. As shown in Fig. 6, high levels of IL-4 and IL-13 were detected in the culture supernatants of both the spleen and the draining LN from the OVA immunized mice. IL-4 and IL-13 were transiently elevated in the day-15 LN culture, but sustained for days in the spleen cultures. Furthermore, increased level of IFN- γ was only detected in the spleen, but not in draining LNs (data not shown). These results suggested that the OVA-induced immune responses were probably regulated differently in these two lymphoid organs.

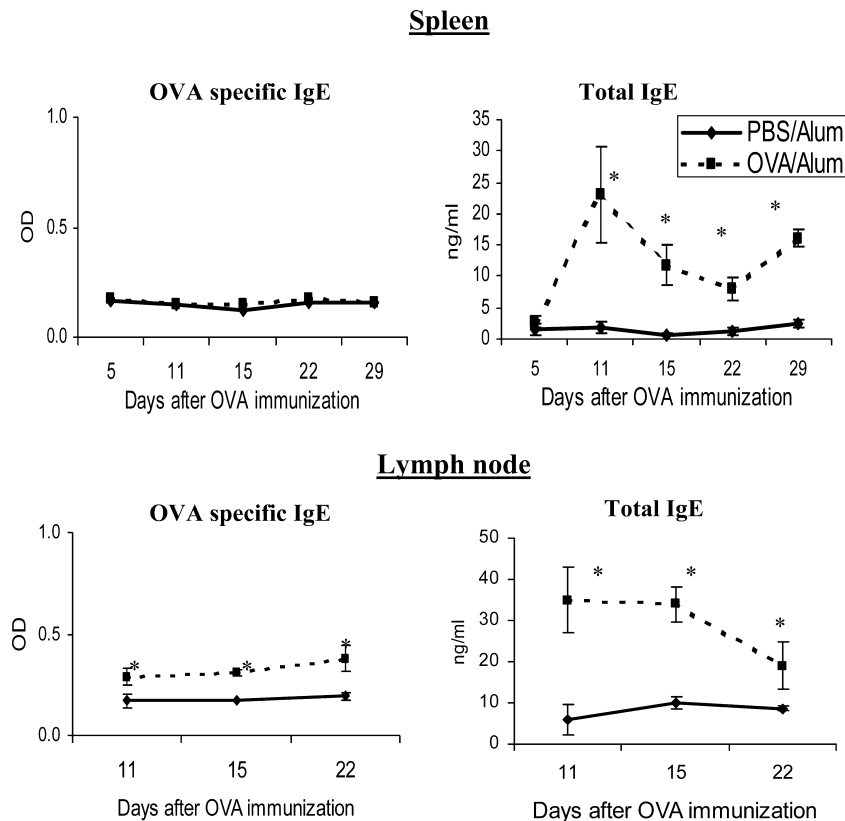


Fig. 5. OVA-specific IgE was detected in the ex vivo cultures of the draining lymph node but not the spleen cells. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. On different days post-immunization, the mesenteric lymph nodes and the spleens were collected and single cell suspensions were prepared. The cells ($5 \times 10^6/\text{ml}$) were cultured with OVA ($10 \mu\text{g}/\text{ml}$) for 72 h. The supernatants were collected and OVA-specific and total IgE were detected using ELISA. Each bar represents the mean $\pm$ SD of 4 mice in each group. * $p < 0.05$ comparing with the corresponding PBS/alum immunized groups.

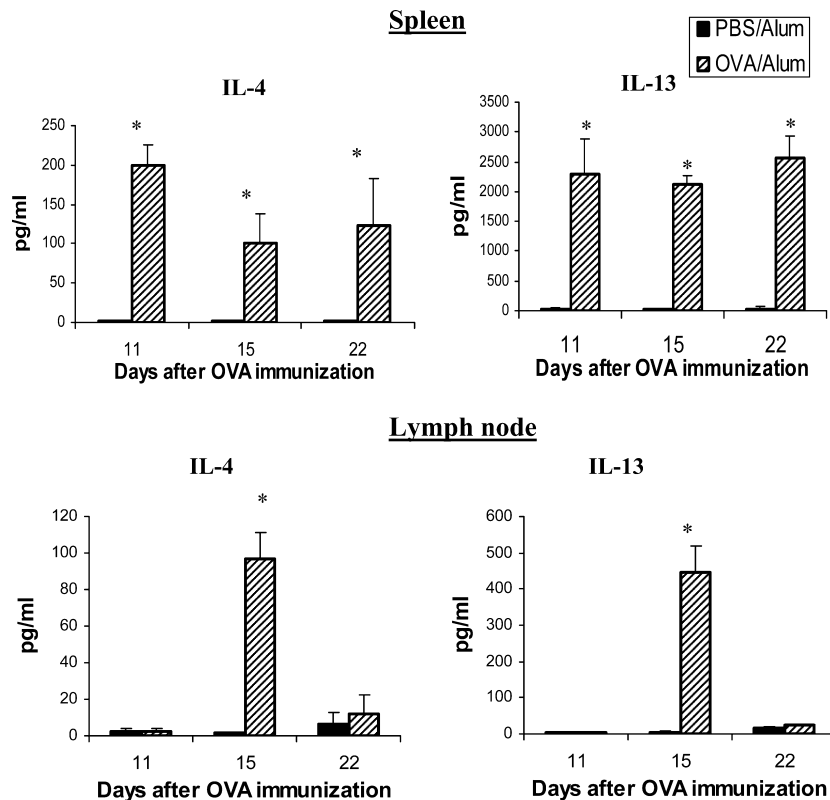


Fig. 6. IL-4 and IL-13 were elevated in the ex vivo cultures of both spleen and lymph node cells. BALB/c mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. The mesenteric lymph nodes and spleens were collected on different days post-immunization and single cell suspensions were prepared. The cells (5×10^6 /ml) were cultured with OVA ($10 \mu\text{g}/\text{ml}$) for 72 h. The supernatants were collected and IL-4 and IL-13 levels were measured using ELISA. Each bar represents the mean $\pm$ SD of 4 mice in each group. * $p < 0.05$ comparing to the corresponding PBS/alum immunized groups.

To examine the contribution of IL-4 and IL-13 to OVA-specific and total IgE production, mice were administered with neutralizing mAbs to IL-4, IL-13 or in combination during and after OVA sensitization. On day 15 post-OVA immunization, the spleen and the draining LN were collected and the percentage of IgE positive cells and their intracellular IgE content at the single cell level were analyzed. Neutralization of IL-4 significantly prevented the enhancement of the intracellular IgE content in the IgE⁺ cells in spleen, whereas, neutralization of IL-13 did not show a significant effect (Figs. 7A and B). No synergistic effect was observed when both IL-4 and IL-13 were neutralized together (Figs. 7A and B), and neither of the antibodies affected the percentage of the IgE producing cells in the spleen (Fig. 7C). In marked contrast, neutralization of IL-4 strongly suppressed the expansion of the IgE producing cells in the draining LN (Fig. 8). Again neutralization of IL-13 did not show the same effect, and no synergistic effect was observed when both IL-4 and IL-13 were neutralized (Fig. 8). In agreement with the above results, neutralization of IL-4 but not IL-13 also reduced the serum levels of the Ag-specific as well as the total IgE (Fig. 9). These data indicated that IL-4, but not IL-13,

appeared to play a critical role in regulating both the Ag-specific and the total IgE levels after allergen challenge.

4. Discussion

Using a murine model with OVA/alum immunization, we have shown that the spleen and the draining LN (mesenteric lymph node) functioned differently in regulating IgE production. Antigen-specific IgE is produced mainly in the draining LN, but not in the spleen, and the spleen appears to be only involved in by-stander total IgE upregulation. This conclusion is supported by the evidence from IgE intracellular staining of the spleen and the draining LN cells. IgE producing B cells pre-exist in the spleen of normal or sham (PBS/alum) immunized mice. OVA/alum immunization did not induce expansion and isotype switch of IgE producing B cell in the spleen, but enhanced the production of all the IgE producing B cells. In contrast, although the number of IgE producing B cells was low in the draining LN of normal or sham immunized mice, OVA/alum immunization induced significant expansion

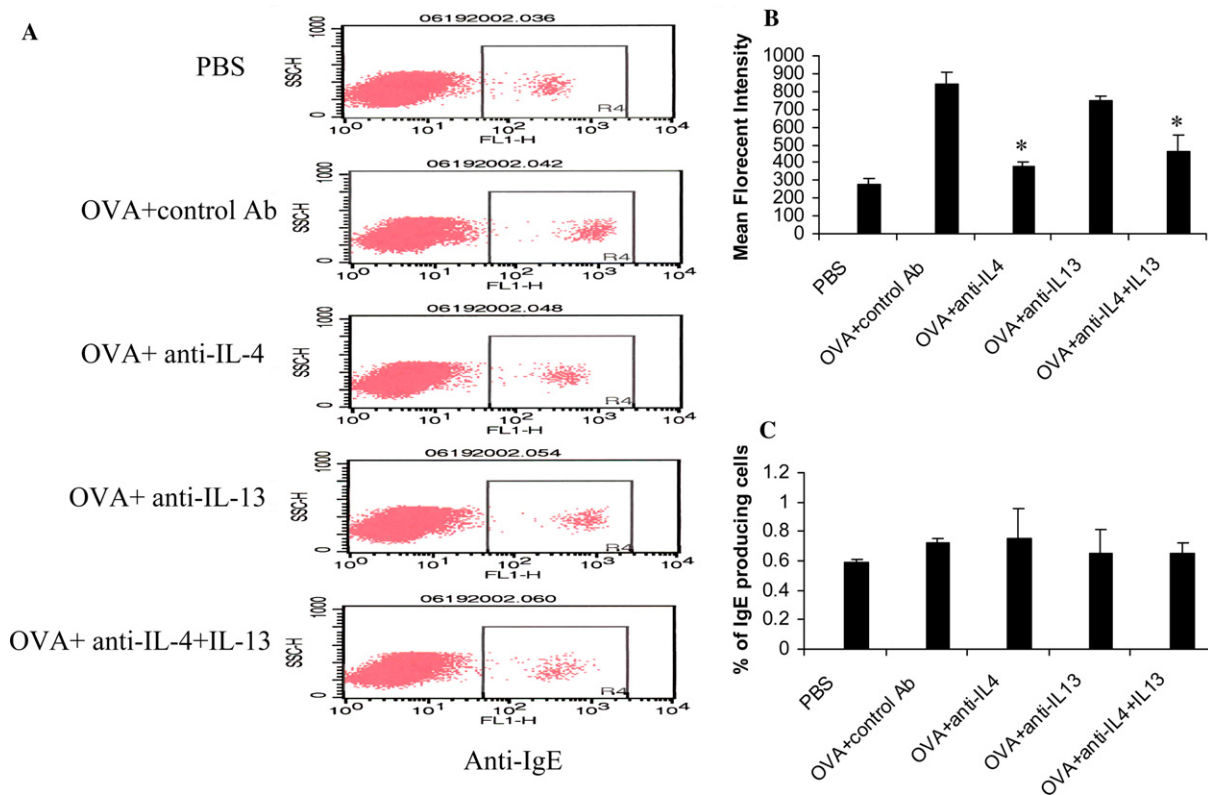


Fig. 7. Enhancement of IgE production capacity in the spleen was IL-4 dependent. Mice were given an intravenous injection of 0.5mg/mouse mAb to IL-4, IL-13 or in combination as indicated on days 1, 4, 8, 10, 12 after OVA immunization. Normal rat IgG was used as a control. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized and stained for CD3 and IgE. The CD3⁺ cells were gated out and the percentages and the MFI of the CD3⁺IgE⁺ population was analyzed using FACS. (A) A set of representative plots. (B,C) The summary results of four mice in each group. Each bar represents the mean $\pm$ SD, $n = 4$. * $p < 0.05$ comparing to control Ab-treated groups.

and IgE isotype switch, thereby increasing the number of IgE producing B cells. In addition, OVA-specific IgE was only detected in the culture of the draining LN but not the spleen further confirming that the draining LN but not the spleen was the lymphoid organ for Ag-specific IgE generation. Similar phenomena were described by an earlier study, which indicated the differential localization of antigen specific and by-stander components of IgE response to inhaled antigen in a rat model [17]. The different location of Ag-specific versus by-stander IgE production provides evidence favoring topical drug delivery, which could be more effective to suppress allergen-specific IgE generation in the local draining LN.

Although allergy is often triggered by certain allergens and allergen-specific IgE, by-stander IgE also plays a role in the pathogenesis of allergy. By-stander polyclonal IgE responses could result from non-cognate interactions between allergen activated T-helper cells and B cells, under the influence of IL-4 or other Th2 cytokines. [18,19]. Indeed, high levels of serum IgE, with known or unknown allergens, were detected in patients with allergic disorders [2]. These IgE molecules, when bound to its high affinity receptors on

mast cells and basophils further increase the receptor intensity on these cells, thereby increase their sensitivity to IgE-mediated activation [20,21]. In the clinical studies with Xolair, an anti-IgE monoclonal antibody, IgE-mediated pathological events were suppressed only when more than 99% of the free IgE was neutralized [22] strongly suggesting the functionality of by-stander IgE. In this study, we provide evidence that during each allergen-specific response, by-stander IgE was also elevated in the serum, and the spleen is one major lymphoid organ contributing to the increased by-stander IgE.

In this murine OVA model, IL-4, but not IL-13, appeared to regulate both the by-stander polyclonal and the Ag-specific IgE production. IL-4 has been shown to be required for the generation of IgE both in vitro and in vivo [23–27]. But the involvement of IL-13 in IgE production in the murine system is controversial. Until recently, murine B cells were shown to be unresponsive to IL-13 since IL-13 receptors were not detected on murine B cells [10,28–30]. In addition, some observations showed that IL-13 did not stimulate spleen B cells to produce IgE in vitro in the presence of anti-CD40 mAb [31]. Recently, Poudrier et al. [32]

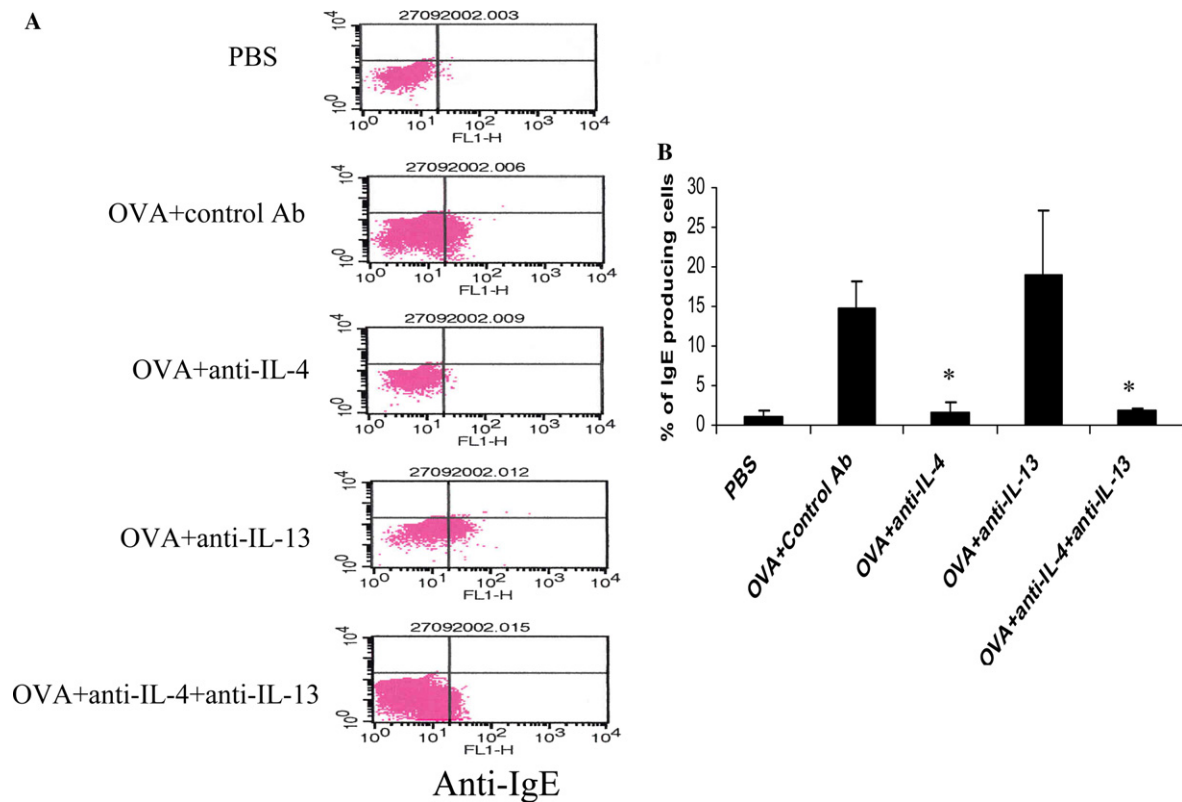


Fig. 8. Isotype switch and clonal expansion of IgE producing B cells in the draining LN were IL-4 dependent. Mice were given an intravenous injection of 0.5mg/mouse mAb to IL-4, IL-13 or in combination on days 1, 4, 8, 10, 12 after OVA immunization. Normal rat IgG was used as a control. Mesenteric LNs were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized and stained for CD3 and IgE. The CD3⁺ cells were gated out and the percentages of the CD3⁺IgE⁺ population were analyzed using FACS. (A) A set of representative plots. (B) The summary results of four mice in each group. Each bar represents the mean $\pm$ SD, $n = 4$. * $p < 0.05$ comparing to control Ab-treated groups.

demonstrated that murine B cells expressed IL-13R α 1 and responded to IL-13 in the presence of a CD40 signal. In addition, elevated IgE was detected in IL-4-deficient mice carrying IL-13 transgenes [13], and blockade of IL-13 using a soluble IL-13R α 2-Fc fusion protein significantly reduced total serum IgE levels in a pulmonary granuloma model triggered by schistosome eggs [33]. Furthermore, an anti-IL-13 mAb reduced total serum IgE levels in a chronic mouse model of asthma (Yang, et al unpublished observation). However, these in vivo observations were from relative long-term models, and it is not clear if the effect of IL-13 on IgE production is direct or indirect. In this report, we showed that IL-13 did not play an obvious role in either the antigen-specific or by-stander polyclonal IgE production in a relatively acute model, which may suggest that IL-13 might not exert direct effects on IgE production.

Although the spleen does not produce Ag-specific IgE, it does produce Ag-specific IgG1. OVA/alum immunization induced strong clonal expansion and isotype switch to IgG1 producing B cells. These results clearly point out differences in the regulation of IgE and IgG1

generation in the spleen. Indeed, several studies have demonstrated that different regulatory mechanisms were involved in IgE and IgG1 production. These studies indicated that allergen-specific IgE, but not IgG1 was inhibited by CD8⁺ cells that produced high levels of IFN- γ [4,35]. IFN- γ directly inhibits the B cell Ig class switch to IgE but favor the class switch to IgG1 [36] and in vivo studies indicated that increased levels of IFN- γ clearly suppressed Ag-specific IgE, but not IgG1 levels [34,35]. These results are consistent with our findings that high levels of IFN- γ were detected in the spleen but not in the draining LNs (data not shown), which might explain, at least partially, the observation that OVA-specific IgE was not produced in the spleen.

In summary, our findings indicate that the spleen and the draining LN function differently in IgE production. The spleen is mainly responsible for polyclonal IgE production, which is IL-4 dependent. Whereas, the draining LN contributes to both antigen specific as well as polyclonal IgE production and this process is also IL-4 dependent. This phenomenon might be unique to IgE since both spleen and the draining LN can generate Ag-specific IgG1.

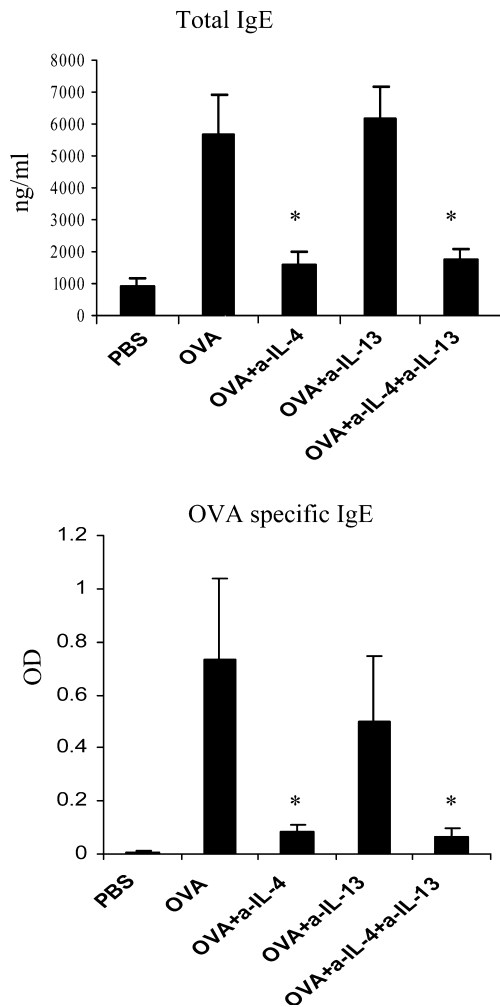


Fig. 9. Neutralization of IL-4 dramatically reduced both OVA-specific and total IgE levels in serum. BALB/C mice were treated with intravenous injections of 0.5 mg/mouse of anti-IL-4, or anti-IL-13, or in combination on days 1, 4, 8, 10, and 12 after OVA immunization. Serum samples were collected on day 15 post-immunization. The OVA-specific and total IgE levels in the serum were detected using ELISA. Results were expressed as means $\pm$ SD, $n = 4$. * $p < 0.05$ compared to control Ab-treated groups.

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