



IgE isotype switch and IgE production are enhanced in IL-21-deficient but not IFN- γ -deficient mice in a Th2-biased response

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Abstract

IgE plays a critical role in the pathogenesis of allergy and asthma. Therefore, suppression of IgE production would provide therapeutic benefits to patients suffering from these diseases. We have reported that the production of IgE is regulated differently in the spleen vs. the draining lymph nodes (LN). IgE isotype switch and IgE producing B cell expansion occur in the draining LN after antigen (Ag) immunization, but do not happen in the spleen. In addition, a population of pre-existing IgE⁺ cells is observed in the spleen of normal or sham immunized mice, but is not present in the draining LN. To further understand the regulation of IgE production in different lymphoid organs, and the potential inhibitory factors of IgE isotype switch in the spleen, the involvement of IL-21 and IFN- γ in regulating IgE production was investigated by using the IL-21 and the IFN- γ deficient mice. We found that in the absence of IL-21 IgE isotype switch and IgE⁺ cell clonal expansion were dramatically enhanced in the spleen and IgE isotype switch was partially increased in the draining LN. In addition, IgE production of the pre-existing CD19⁺CD5⁺B220^{low} IgE⁺ cells in the spleen was also increased in the absence of IL-21 under physiological conditions. In contrast, using the IFN- γ deficient mice, we did not observe a negative impact of IFN- γ on either IgE isotype switch or IgE production. Our data suggest that IL-21 appears to be a critical cytokine to keep low IgE levels under physiological and pathological conditions.

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1. Introduction

IgE plays a pivotal role in allergic disorders. After cross-linking with their corresponding antigens, the receptor bound IgE triggers de-granulation of mast cells and basophiles. These cells then release a variety of inflammatory mediators that cause immediate-type hypersensitivity reactions in allergies and asthma [1]. It has been well documented that serum IgE levels, including both Antigen (Ag) specific and by-stander total IgE (Ag unidentified), are usually much higher in patients with allergic disorders compared to those of normal individuals [2–4]. Therefore, it is important to understand the pathological sources of the

elevated serum IgE. Our recent study indicated that spleen and draining LN function differently in IgE production [5]. After Ag immunization, IgE isotype switch only happened in the draining LN, but not in the spleen [5]. As a result, Ag specific IgE was mainly generated from the draining LN, while total IgE was produced from both the spleen and draining LN [5]. However, the mediators that might inhibit IgE isotype switch in the spleen are not well understood.

IL-21 is a newly described multifunctional cytokine produced by activated CD4⁺ T cells. It has been reported that IL-21 co-stimulates T and B cell proliferation and regulates natural killer cell activation and expansion [6,7]. Structurally, IL-21 is a typical four-helix-bundle cytokine with significant homology to IL-2, IL-4, and IL-5. It signals via a class I receptor that is selectively expressed on lymphoid cells [6]. Recently IL-21 has been reported to inhibit IgE but enhance IgG1 and IgG3 isotype switch and production in

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both human and mouse systems [8–10]. IFN- γ , a Th1 cytokine, has also been reported to directly inhibit IgE but favor IgG1 class switch in B cells [11]. In addition, in vivo studies indicated that increased levels of IFN- γ clearly suppressed Ag-specific IgE but not IgG1 levels [12,13]. Therefore, both IL-21 and IFN- γ could function as the suppressive mediators of IgE production in the spleen.

In this study, the involvement of IL-21 and IFN- γ in controlling IgE production in a Th2 biased immune response was investigated using the IL-21 and the IFN- γ deficient mice immunized with a standard ovalbumin (OVA)/alum protocol. Our results indicated that, in this study system, IL-21 but not IFN- γ appeared to play a major role in controlling IgE production.

2. Materials and methods

2.1. Animals

IL-21 genetically deficient mice were generated by homologous recombination in embryonic stem (ES) cells. A gene-targeting vector was constructed in which IL-21 exons 1 and 2 were replaced with IRES-LacZ/M C1neo cassette. 129sv-derived ES cells were electroporated with the IL-21 targeting vector and selected in G418. ES cell clones carrying a targeted IL-21 gene were identified by genomic Southern blot analysis. Targeted ES were microinjected into C57BL/6. Resulting male chimeras were bred with C57BL/6 female mice. The offspring were analyzed for germ-line transmission of the mutant IL-21 allele by PCR and Southern blot analyses. Mice heterozygous for IL-21 (C57BL/6J/129) mutant were intercrossed to generate IL-21 homozygous mice. IFN- γ deficient and wild-type control mice (C57BL/6J background) were purchased from Jackson laboratory.

Female, 6–8 weeks, IL-21 or IFN- γ deficient mice, or wild-type littermate control mice 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 μ g 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 15, five 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

Staining for intracellular IgE was performed as previously reported [5]. Briefly, the cells isolated from the 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 Company) for 1 h on ice. The cells were then incubated with FITC-labeled anti-IgE mAb and PE-labeled anti-CD19 mAb (B.D. Pharmingen) for 30 min on ice. The cell content of IgE was then analyzed on a FACSCalibur (BD Biosciences) using CellQuest Software. The background staining for intracellular IgE was determined using an isotype control mAb (BD Biosciences). The specificity of the IgE staining has been confirmed using an excess amount of soluble mouse IgE.

2.4. Visualization of intracellular IgE with the ImageStream

The ImageStream is a multispectral imaging flow cytometer that collects high-resolution brightfield, darkfield and fluorescence imagery of cells in flow. This technology combines the quantitative power of large sample sizes common to flow cytometry with the high information content present in microscopic images. The novel architecture of the instrument makes it ideally suited for experiments requiring photometric and morphometric analysis such as quantifying apoptosis using nuclear fragmentation [14], measuring translocation of cytoplasmic proteins [15] or visualizing the cytoplasmic proteins in rare cell subsets.

Primary mouse splenocytes were harvested and stained as described, and images of 50,000 cells were collected on the ImageStream. The instrument was configured for maximum fluorescence sensitivity using 200 mw of 488 nm laser excitation, and a camera setting of 32 stages for channel 1 (SSC- side scatter) and 512 stages for channels 2–6. A minimum channel 1 area of 50 pixels was set during acquisition to eliminate debris from the data file. Single color fluorescence controls were used to create a spectral compensation matrix and post acquisition compensation was applied using the IDEAS image analysis software. A FITC intensity vs. PE intensity plot was used to identify the 0.3% population of IgE FITC positive, CD19 PE negative cells. There were approximately 80 of these cells and their images were qualitatively assessed for both surface and cytoplasmic IgE FITC fluorescence.

2.5. Serum total IgE and antigen-specific IgE measurement

The serum levels of IgE were measured as previously described [5]. Briefly, total IgE levels were measured using ELISA (B.D. Pharmingen). To measure OVA specific IgE, plates were coated overnight at 4 °C with 20 μ g/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). Rat anti-IL-13 mAb was generated in house using standard hybridoma technology. The IC₅₀ of this antibody to mouse IL-13 (5 ng/ml) is 100 ng/ml in an IL-13 induced B9 (ATCC) cell proliferation assay (data not shown). Normal rat IgG, purchased from Jackson ImmunoResearch, was used as the 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 (CO₂ asphyxiation), and their serum, spleen and the mesenteric LN were collected and analyzed as described above.

2.7. Quantitative measurement of IL-21 and IFN- γ mRNA expression in the mouse spleen

Total RNA was isolated from frozen mouse spleen using total RNA isolation kits (Qiagen Inc., Valencia, CA). Total RNA was treated with DNase kits (Applied Biosystem, Foster City, CA) and RNA quality was analyzed with Agilent 2100 Bioanalyzer (Agilent). 2 μ g of mRNA was reverse-transcribed in a 100 μ l reaction in a PCR tube using reverse Transcription (RT) systems (Applied Biosystem, Foster City, CA). The quantitative PCR was performed using TaqMan ABI PRISM 7900HT Sequence Detection System (Applied Biosystem, Foster City, CA). GAPDH was used as endogenous reference. The gene expression data were analyzed using 2^{− $\Delta\Delta$ Ct} method described previously [16]. The sequences of primers and probe for mouse IL-21 were as following: sense- TCAAAC CCTGGAAACAATAAGACATT, anti-sense- TGGGTG TCCTTTTCTCATACGAAT, and probe-6FAM-CACAT AGCTAAATGCCCTTC-MGB. Primers and probe for mouse IFN- γ were purchased from Applied Biosystems. In all cases, TaqMan Universal PCR Master Mix (Applied Biosystem, Foster City, CA) was used.

2.8. Statistics

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

3. Results

3.1. IgE isotype switch was not detected in the mouse spleen after OVA immunization and the pre-existing IgE positive cells were CD19[−]CD5⁺B220^{low}

Our previous results indicated that there were pre-existing IgE⁺ cells in the mouse spleen, and OVA immunization neither induced the antigen-driven clonal expansion of

these cells nor new IgE isotype switch in the spleen [5]. In this study, using the same animal model, we further characterized the pre-existing IgE⁺ cells in the spleen and found that they were CD19 negative. In addition, OVA immunization did not increase the percentages of these cells (Fig. 1A). As shown in Fig. 1B, both surface and cytoplasmic IgE were detected in these pre-existing IgE⁺ cells with ImageStream technology. In addition, no IgE isotype switch or IgE⁺CD19⁺ cells were observed in the spleen after OVA immunization (Fig. 1A). In marked contrast, OVA immunization induced significant IgE isotype switch in the mesenteric draining lymph nodes and the IgE⁺ cells in the draining lymph nodes were CD19⁺ (Fig. 1A). As shown in Fig. 2A, the pre-existing IgE⁺ cells in the spleen were CD5⁺CD86⁺CD80[−]B220^{low} and none of these cell surface marker expression was dramatically affected by Ag immunization. Interestingly, OVA immunization appeared to down-regulate B220 expression on these cells (Fig. 2B). In addition, these cells were also SSC^{low} (side scatter^{low}, data not shown) and CD138⁺ (Fig. 2B).

OVA immunization enhanced IgE content of the IgE⁺CD19[−]CD5⁺B220^{low} cells in the spleen, although the percentage of these cells remained unchanged (Fig. 3 and [5]). The enhancement of IgE content in these cells was strongly inhibited by the neutralization of IL-4 but not of IL-13 (Fig. 3) and [5]. Moreover, neutralization of IL-4 or IL-13 did not affect the percentages of IgE⁺CD19[−]CD5⁺B220^{low} cells in the spleen (Fig. 3). These results indicate that the enhancement of IgE content (which may indicate IgE producing capacity) of the IgE⁺CD19[−]CD5⁺B220^{low} cells is the major contribution of

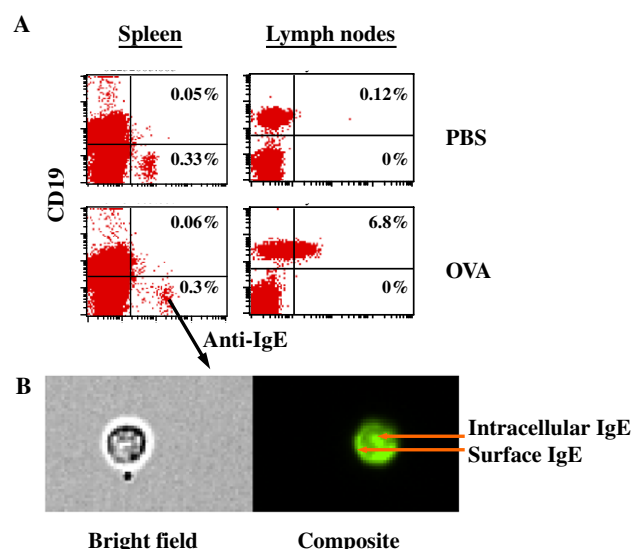


Fig. 1. IgE⁺ cells in the spleen were CD19 negative and IgE isotype switch occurred in the draining lymphoid nodes but not in the spleen after OVA immunization. Wild-type mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleen and the mesenteric lymph nodes were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE. The percentages of the IgE⁺ cells were analyzed using FACS (A); both cell surface and cytoplasmic IgE were demonstrated in a single CD19[−]IgE⁺ cell using the ImageStream technology (B).

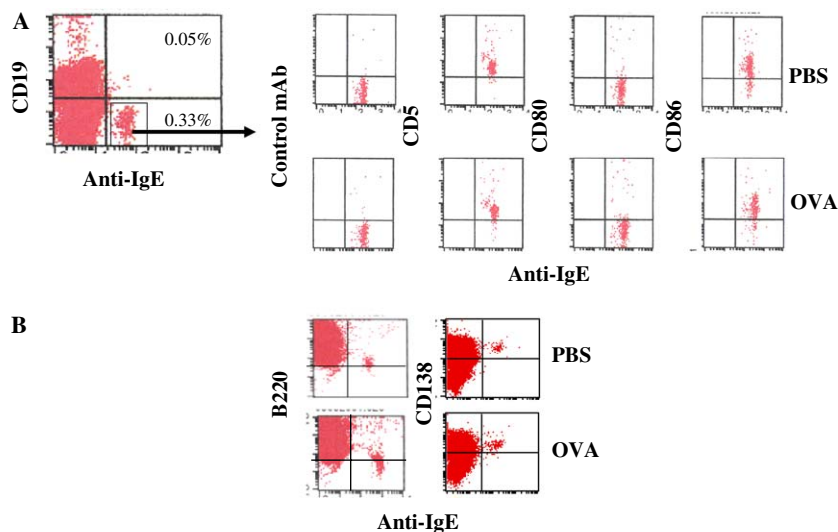


Fig. 2. IgE⁺CD19⁻ cells in the spleen were also CD5⁺CD86⁺CD80⁻B220^{low} and CD138⁺. Wild-type mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE (A). The cells were also stained for CD5, CD80 or CD86 (A) and B220 or CD138 (B) as indicated. The data represent two independent experiments.

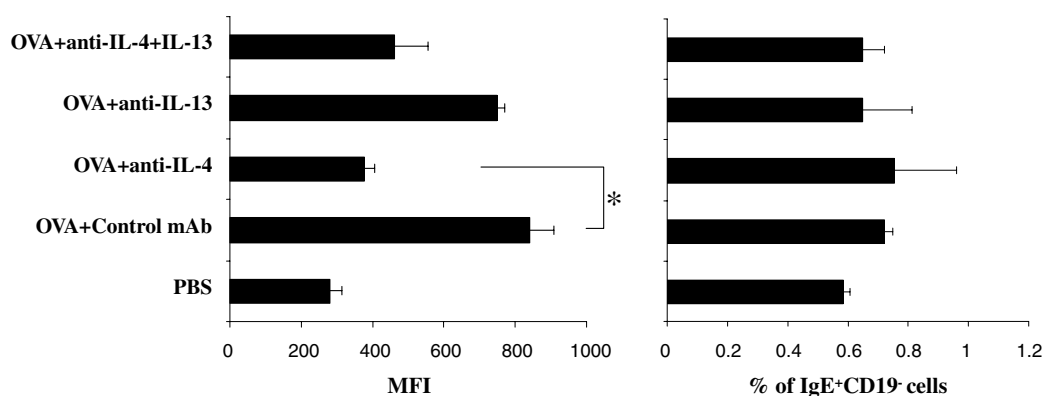


Fig. 3. The enhancement of the intracellular IgE contents of the IgE⁺CD19⁻ cells in the spleen was IL-4 dependent. Wild-type mice were given an intravenous injection of 0.5 mg/mouse monoclonal antibody (mAb) to IL-4, IL-13 or in combination on days 1, 4, 8, 10, 12 after OVA immunization. Normal rat IgG was used as a control. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized and stained for CD19 and IgE. The mean fluorescence intensity (MFI) and percentages of the CD19⁻IgE⁺ population were analyzed using FACS. The MFI of intracellular IgE staining was used as an indicator for IgE producing capacity. Each bar represents the mean $\pm$ SD, $n = 5$, $*p < 0.05$.

the increased IgE production in the spleen after Ag immunization, which is regulated by IL-4.

3.2. Lack of endogenous IFN- γ did not affect IgE⁺ cell number nor IgE production

Since IFN- γ has been reported as a negative regulator of IgE [12,13], and IFN- γ was constitutively expressed in the spleens of normal mice and was further enhanced after OVA immunization (Fig. 4), we investigated the potential impact of IFN- γ on IgE isotype switch and production in the spleen and the draining LN using the IFN- γ deficient mice. IFN- γ deficient and wild-type littermate mice were immunized with OVA/alum once a week for 2 weeks. The spleen and the mesenteric draining LN were harvested on day 15 after the first immunization as

described in Section 2. Single cells from the lymphoid organs were isolated and stained for their intracellular IgE content. As shown in Fig. 5, OVA immunization did not induce IgE⁺CD19⁺ cells or affect the percentage of the pre-existing IgE⁺CD19⁻CD5⁺B220^{low} cells in the spleen of the wild-type mice, but enhanced IgE content of the IgE⁺ cells. These results are consistent with our previous report [5]. Similar results were observed in the spleen of the IFN- γ deficient mice. In addition, no change of IgE isotype switch was observed in the draining lymph nodes of IFN- γ deficient mice comparing with that of the wild-type mice (data not shown). Furthermore, the total IgE levels in the sera were similar between the IFN- γ deficient mice and the wild-type mice (Fig. 6). Taken together, these results indicated that the deficiency of IFN- γ did not show a detectable impact on IgE isotype switch nor

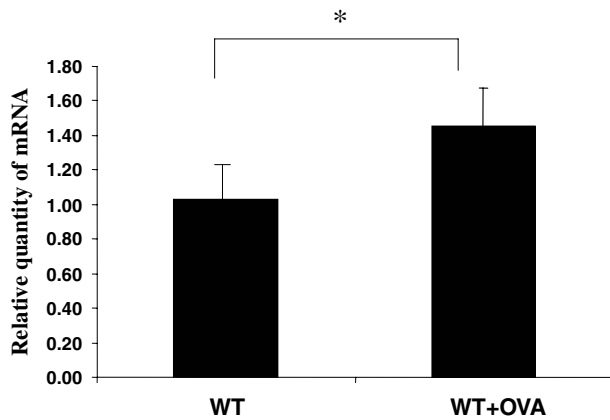


Fig. 4. IFN- γ was constitutively expressed in the wild-type mice and OVA immunization further enhanced IFN- γ expression. The spleens from the wild-type mice were collected before or on day 15 post-immunization and total RNA was isolated. IFN- γ expression was detected with quantitative RT-PCR as described in Section 2. Each bar represents the mean $\pm$ SD, $n = 5$, $*p < 0.05$.

on IgE production in the OVA induced mouse Th2 response.

3.3. Lack of endogenous IL-21 dramatically enhanced IgE isotype switch in the spleen and partially increased IgE isotype switch in the draining lymph nodes

Recently IL-21 has been reported to inhibit IgE but enhance IgG1 and IgG3 isotype switch and production in both human and mouse systems [8–10]. The IL-21 deficient mice were used to further investigate the potential impact

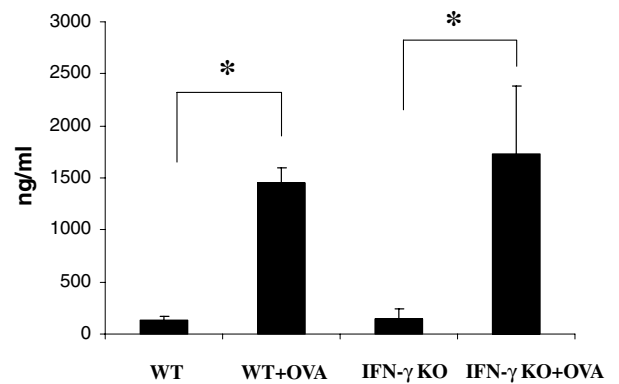


Fig. 6. The lack of IFN- γ did not affect serum total IgE levels. The IFN- γ deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Serum samples were collected on day 15 post immunization. The total IgE levels in the serum were measured using ELISA. Results were expressed as mean $\pm$ SD, $n = 5$, $*p < 0.05$.

of IL-21 on IgE production in different lymphoid organs. Mice lacking IL-21 developed normally to term and were fertile. The mutant animals had a normal life span and did not spontaneously develop any diseases (unpublished observation). In addition, there was no defect in IFN- γ expression in the IL-21 deficient mice (data not shown).

The IL-21 deficient and the wild-type littermate mice were immunized with OVA/alum once a week for two weeks. The spleen and mesenteric LN were harvested on day 15 after the first immunization as described in Section 2. Single cells from the spleen and the draining LN were isolated and stained for intracellular IgE content. As expected, OVA immunization did not induce IgE isotype

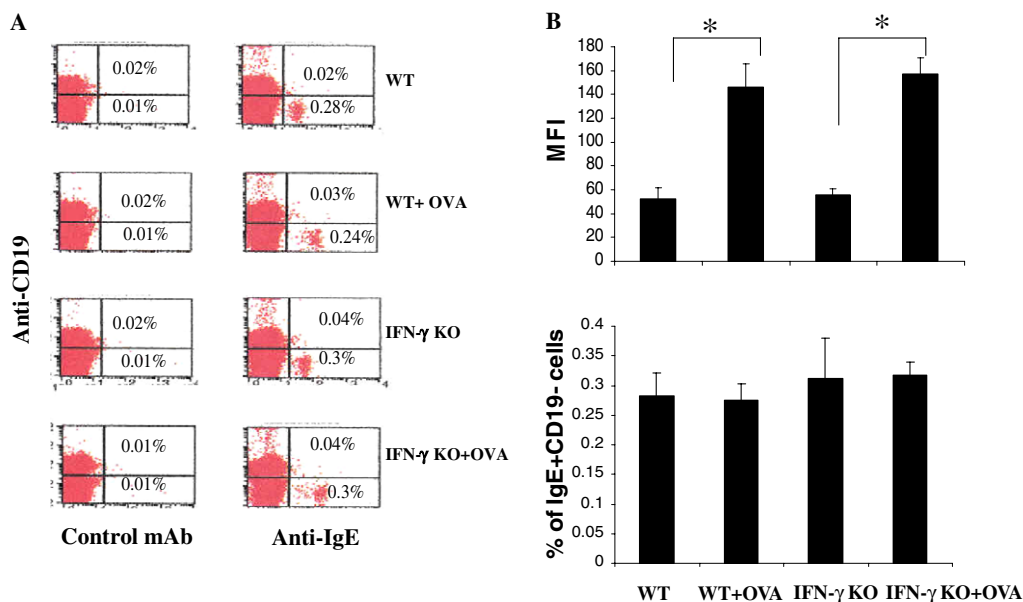


Fig. 5. The lack of IFN- γ affected neither IgE isotype switch nor IgE producing capacity of the CD19⁺IgE⁺ cells in the spleen. The IFN- γ deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE. The mean fluorescence intensity (MFI) and the percentage of the CD19⁺IgE⁺ were measured. The MFI was used as an indicator of IgE producing capacity. Panel A shows a set of representative plots. Panel B shows the summary results of five mice in each group. Each bar represents mean $\pm$ SD, $n = 5$, $*p < 0.05$.

switch in the spleen of wild-type littermate mice, but increased the intracellular IgE content in the pre-existing $\text{IgE}^+\text{CD19}^-\text{CD5}^+\text{B220}^{\text{low}}$ cells, (Fig. 7A). However, in marked contrast, the baseline numbers of the $\text{IgE}^+\text{CD19}^+$ cells were significantly elevated in the spleen of the IL-21 deficient mice and a significant further enhancement was observed after OVA/alum immunization (Fig. 7A and B). These results demonstrated that the lack of IL-21 significantly increased the percentages of the $\text{IgE}^+\text{CD19}^+$ cells in the spleen, therefore, indicating that IL-21 might be a

major inhibitory cytokine on IgE isotype switch in the spleen.

As shown in Fig. 7A, there were two distinct IgE^+ cell populations in the spleen of the IL-21 deficient mice, $\text{IgE}^+\text{CD19}^+$ and $\text{IgE}^+\text{CD19}^-\text{CD5}^+\text{B220}^{\text{low}}$ cells. The lack of IL-21 not only enhanced IgE isotype switch in the spleen, but also increased the baseline IgE contents of the pre-existing $\text{IgE}^+\text{CD19}^-\text{CD5}^+\text{B220}^{\text{low}}$ cells (Fig. 8A and B). However, the percentages of the pre-existing $\text{IgE}^+\text{CD19}^-\text{CD5}^+\text{B220}^{\text{low}}$ cells were not affected by the lack of IL-21 (Fig. 8A and C).

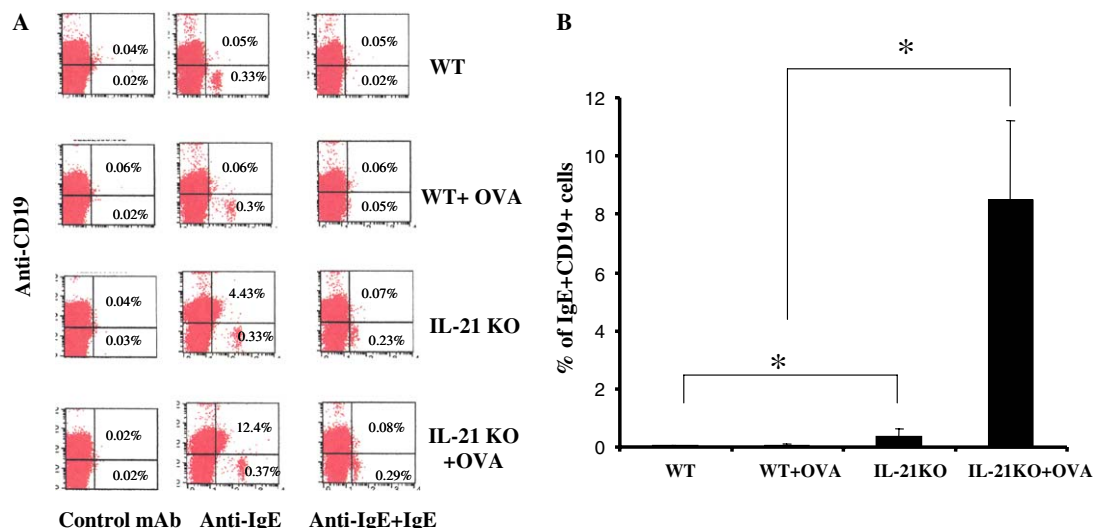


Fig. 7. The lack of IL-21 dramatically enhanced the IgE isotype switch in the spleen. The IL-21 deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE. The percentages of the $\text{CD19}^+\text{IgE}^+$ and $\text{CD19}^-\text{IgE}^+$ cells were analyzed using FACS. The specificity of the IgE staining was confirmed by pre-incubating the antibody with excess amounts of IgE (A). Panel A shows a set of representative plots. Panel B shows the summary results of five mice in each group. Each bar represents the mean $\pm$ SD, $n = 5$, $*p < 0.05$ comparing to the corresponding littermate wild-type control groups.

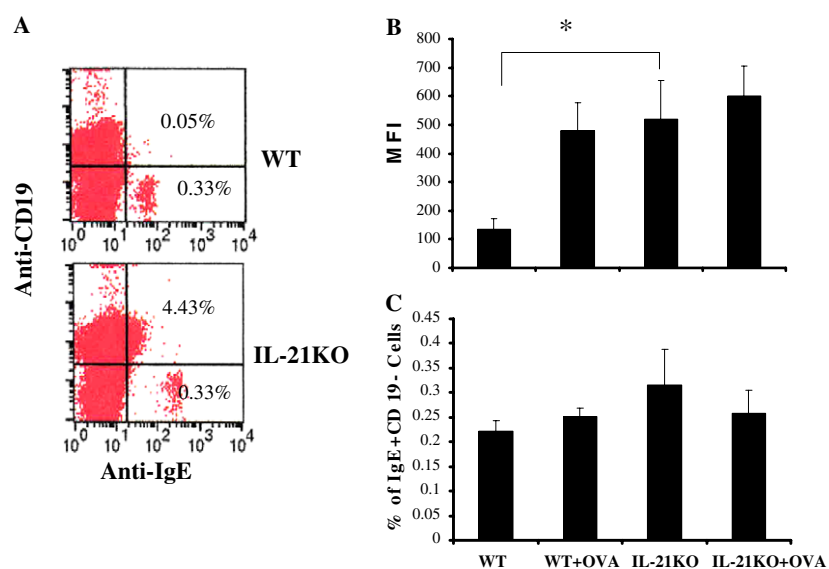


Fig. 8. The lack of IL-21 increased the intracellular IgE contents of the $\text{CD19}^-\text{IgE}^+$ cells in the spleen, but did not affect the percentage of these cells. The IL-21 deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Spleens were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE. The MFI of the intracellular IgE staining and the percentage of the $\text{CD19}^-\text{IgE}^+$ were measured. Panel A shows a set of representative plots. Panel (B and C) shows the summary results of five mice in each group. Each bar represents the mean $\pm$ SD, $n = 5$, $*p < 0.05$.

In contrast to the spleen, the numbers of the $\text{CD19}^+\text{IgE}^+$ cells were minimal in the mesenteric draining LN of the wild-type mice, and OVA/alum immunization dramatically increased the numbers of these cells (Fig. 9). However, in the IL-21 deficient mice, the baseline numbers of the $\text{IgE}^+\text{CD19}^+$ cells were significantly elevated, and a further increase was observed after OVA immunization (Fig. 9). No $\text{IgE}^+\text{CD19}^-\text{CD5}^+\text{B220}^{\text{low}}$ cells were observed in the mesenteric draining LN. These results demonstrated that the lack of IL-21 caused a moderate increased of IgE isotype switch, indicating that IL-21 probably also partially inhibited IgE isotype switch in the draining LN.

3.4. Both the OVA specific and total IgE levels were elevated in the serum of IL-21 deficient mice

OVA specific and the total IgE levels were measured using ELISA. As shown in Fig. 10, total and OVA specific

IgE levels in the serum were very low in the wild-type littermate mice. OVA/alum immunization increased both total and the OVA specific IgE levels. In the IL-21 deficient mice, the baseline total IgE levels were significantly elevated compared to those in the wild-type mice, and OVA/alum immunization dramatically increased both total and OVA specific IgE levels. These results again indicated that IL-21 probably is a major mediator suppressing IgE production in both physiological and pathological conditions.

4. Discussion

Our previous study indicated that the spleen and the draining LN (mesenteric lymph node) functioned differently in IgE production in a murine Th2-biased response induced with OVA/alum immunization [5]. Unlike the draining lymph nodes, no IgE isotype switch was observed in the spleen after OVA immunization. However, a popula-

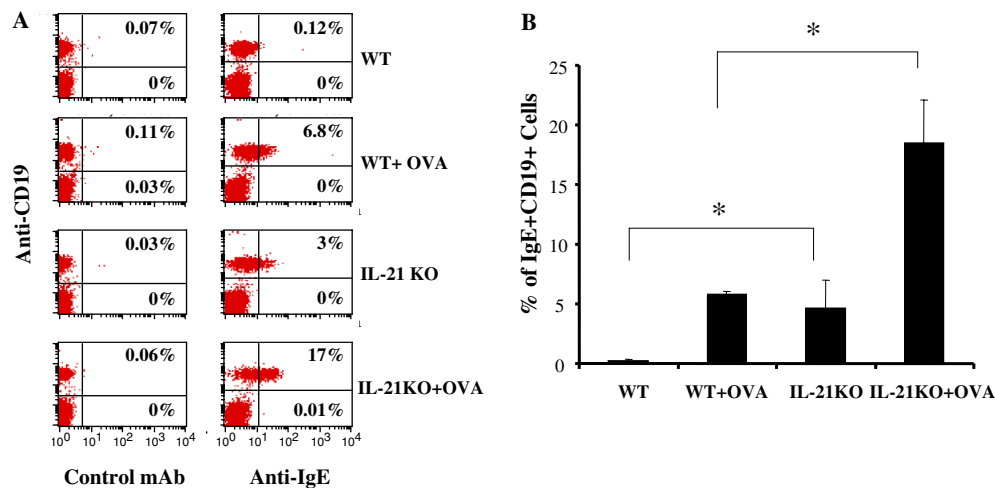


Fig. 9. The lack of IL-21 partially increased the IgE isotype switch in the draining lymph nodes. The IL-21 deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. The mesenteric lymph nodes were collected on day 15 and single cell suspensions were prepared. The cells were then fixed, permeabilized, and stained for CD19 and IgE. The percentages of the $\text{CD19}^+\text{IgE}^+$ cells were analyzed using FACS. Panel A shows a set of representative plots. Panel B shows the summary results of five mice in each group. Each bar represents the mean $\pm$ SD, $n = 5$, $*p < 0.05$.

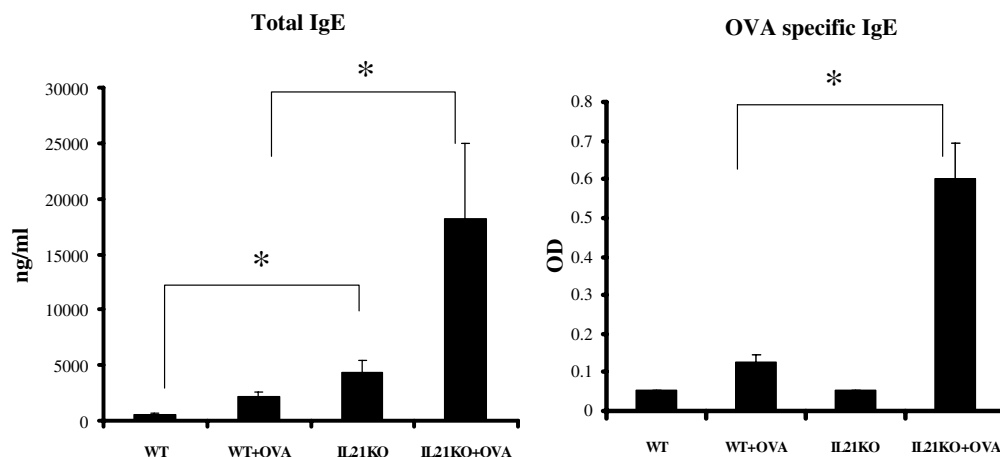


Fig. 10. The lack of IL-21 significantly increased the serum total IgE and OVA specific IgE levels. The IL-21 deficient and the wild-type littermate control mice were immunized with OVA/alum or PBS/alum on day 1 and day 8. Serum samples were collected on day 15 post-immunization. The OVA specific IgE and total IgE levels in the serum were measured using ELISA. Results were expressed as mean $\pm$ SD, $n = 5$, $*p < 0.05$.

tion of pre-existing IgE⁺ cells (IgE⁺CD19⁺CD5⁺B220^{low}) were found in normal or sham (PBS/alum) immunized mice [5]. OVA/alum immunization did not induce clonal expansion of these cells in the spleen, but enhanced the IgE intracellular contents of these cells at single cell levels. Antigen-specific IgE is produced mainly in the draining LN, but not in the spleen, and the spleen appears to only contribute to by-stander total IgE production [5], which is IL-4 dependent [5] and Fig. 2.

The mediators that might be involved in inhibiting IgE isotype switch in the spleen were not fully understood. Indeed, IgE isotype switch and IgE production are highly controlled processes regulated by cytokines and cellular interactions [17,18]. Numerous cytokines are involved in this processes, and among them, IL-21 and IFN- γ have been reported to inhibit IgE isotype switch and production [8–10]. Using IL-21 and IFN- γ deficient mice immunized with OVA/alum, we found that IL-21, but not IFN- γ , appeared to play a critical role in regulating IgE isotype switch and antigen-specific IgE production in the mouse spleen.

The potential lack of IFN- γ involvement in controlling IgE production in this Th2-biased response was supported by the similar observation regarding to IgE⁺ cells or IgE production between the wild-type and the IFN- γ deficient mice. In spite of the fact that IFN- γ was constitutively expressed in the littermate control mice and OVA immunization further enhanced IFN- γ expression. Additional studies using neutralizing anti-IFN- γ antibody would further confirm the contribution of IFN- γ to IgE production.

In contract to the observation in the IFN- γ deficient mice, OVA/alum immunization dramatically enhanced the expansion of the IgE⁺CD19⁺ cells in the spleens of the IL-21 deficient mice. It is unlikely that the effect of IL-21 deficiency on IgE⁺CD19⁺ cells was due to the lack of IFN- γ since IFN- γ expression was normal in the IL-21 deficient mice (data not shown). Moreover, the results in this study also indicated that IL-21 not only completely inhibited IgE isotype switch in the spleen, but also partially inhibited IgE isotype switch in the draining LN. Although OVA immunization was able to induce IgE isotype switch in the draining LN of wild-type mice, this effect was dramatically enhanced in the draining LN of the IL-21 deficient mice. Consistent with what was observed at IgE⁺ single cell level, both the antigen specific and total IgE levels in the serum of IL-21 deficient mice were significantly higher than those of the littermate wild-type mice. In addition, elevated baseline numbers of the IgE⁺CD19⁺ cells in the spleen and draining LN, as well as elevated total IgE levels in the sera, were observed in non-immunized IL-21 deficient mice. Taken together, these results strongly indicated that IL-21 probably was a critical cytokine inhibiting IgE production in both physiological and Th2 skewed conditions.

The pre-existing IgE⁺CD19⁺CD5⁺B220^{low} cells were only found in the spleen but not in the draining LN. The

cells could be from B cell lineage or could be cell expressing high affinity IgE receptors, such as basophils. Both cell surface and intracellular IgE staining were used to characterize these IgE⁺ cells. The results indicated that these cells were IgE producing cells since the mean fluorescent intensity of intracellular IgE staining was much stronger than those with the surface staining. Moreover, both surface and cytoplasmic IgE were demonstrated in these cells with the ImageStream technology, which further confirmed that these cells did produce IgE. In addition, these cells were also SSC^{low} (side scatter^{low}) and were CD138⁺. Therefore, it is highly unlikely that these cells belong to the basophil population, which is SSC^{intermediate} and CD138⁺ [19,20]. It is possible that the IgE⁺CD19⁺CD5⁺B220^{low} cells are derived from the B1 cells in the early stage of animal development and produce IgE isotype natural antibody. It is well known that B1 cells produce the full range of Ig isotype and most of the natural antibodies in sera but participate in relatively few antigen-specific responses [21–23]. Recently, Oliveria et al. demonstrated that the spleen was the major source for IgE⁺ B1 cells in the experimental murine schistosomiasis model [24]. However, further investigation is needed to better understand this IgE producing population and its potential pathogenesis in allergic conditions.

Interestingly, the number of the IgE⁺CD19⁺CD5⁺B220^{low} cells in the spleen of the IL-21 deficient mice did not increase, but the intracellular IgE content of these cells was dramatically enhanced based on fluorescent intensity of the IgE staining. These results suggest that IL-21 might inhibit the natural IgE antibody production of these cells in physiological conditions. In addition, the data in this study and a previous study [5] also indicate the intracellular IgE content (which may indicate the IgE producing capacity of these cells) was dramatically enhanced by IL-4 after antigen immunization. Thus, it appears that the relative balance between IL-21 and IL-4 might play an important role in modulating IgE production. In physiological conditions, IL-21 might be the dominant cytokine that keeps IgE at very low levels. In contrast, after antigen sensitization or in certain pathological conditions, IL-4 could become the dominant cytokine that might overcome the inhibitory effect of IL-21 and enhance the IgE production. Indeed, our data indicated that OVA immunization enhanced IL-4 production, but did not affect IL-21 expression (data not shown).

In summary, in this study, we demonstrated that the lack of IFN- γ has no effect on either IgE isotype switch or IgE production in the OVA induced Th2-biased response. In contrast, the lack of IL-21 dramatically enhanced IgE isotype switch and antigen driven IgE⁺ cell clonal expansion as well as the intracellular IgE contents of the pre-existing IgE⁺CD19⁺CD5⁺B220^{low} cells in the spleen. In addition, IgE isotype switch in the draining LN was also increased in the absence of IL-21. These results suggested that IL-21 probably is the key cytokine negatively regulating IgE production in both physiological and pathological conditions.

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