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ORIGINAL ARTICLE

IL-33 Promotes the Induction and Maintenance of Th2 Immune Responses by Enhancing the Function of OX40 Ligand

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ABSTRACT

Background: In Th2 immune responses, TSLP is a key player by induction of OX40-ligand (OX40L) on dendritic cells (DCs), which is the trigger to induce Th2 cell-mediated allergic cascade. Thus, TSLP-DC-OX40L axis might be the principal pathway in the inflammatory cascades in atopic dermatitis and asthma. IL-33, which is produced by epithelial cells, has been implicated in the Th2 immune responses and pathogenesis of the allergic disorders. However, the role of IL-33 in the Th2-polarizing TSLP-DC-OX40L axis still remains largely elusive. We focused on the ability of IL-33 to promote OX40L-mediated Th2 responses.

Methods: Purified human naïve or memory $CD4^+$ T cells were stimulated with recombinant OX40L or TSLP-treated DCs (TSLP-DCs) in the presence of IL-33, and the cytokine production by the primed T cells was examined. We also performed immunohistochemical analyses for the expression of IL-33 in specimens of lymph node and skin from the patients with atopic dermatitis.

Results: IL-33 remarkably enhanced TSLP-DCs-driven or OX40L-driven Th2 responses from naïve T cells and the Th2 functional attributes of $CRTH2^+$ $CD4^+$ Th2 memory cells by the increased production of IL-5, IL-9, and IL-13. In addition, IL-33 was expressed in the nuclei of epithelial cells in not only skin lesion but also lymph nodes of the patient with atopic dermatitis, suggesting a specialized role in adaptive T cell-priming phase.

Conclusions: IL-33 works as a positive regulator of TSLP-DC-OX40L axis that initiates and maintains the Th2 cell-mediated inflammatory responses, and therefore, it would be a new therapeutic target for the treatment of allergic disorders.

KEY WORDS

atopic dermatitis, dendritic cells, epithelial cells, Th2 cytokines, Th2 responses

INTRODUCTION

The prevalence of allergic diseases, such as atopic dermatitis, asthma, and allergic rhinitis, has increased.[1] These allergic disorders are characterized by inflammatory processes in which the T helper type 2 (Th2) cells are crucial for the initiation and maintenance of allergic immune responses.[2] Th2 cell-derived cytokines such as IL-4, IL-5, and IL-13, induce the activation of immunological effector system assembled by B cells, eosinophils, and mast cells, leading to increased IgE concentrations, mast-cell degranulation, mucus hypersecretion, and eosinophil-mediated inflammation.[3] Recently, it has been clarified an integral role of dendritic cells (DCs) in the cellular cascade that initiate Th2 responses in allergy.[4] Furthermore, thymic stromal lymphopoietin (TSLP),[5] an epithelial cell-derived cytokine, is found to equip DCs with a programming role in Th2 polarization in allergic inflammation.[6] TSLP is highly expressed by keratinocytes in the skin of patients with atopic dermatitis[6] and by airway epithelial lesion of patients

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with asthma.[7] Also in mice, overexpression of TSLP develops atopic dermatitis-like skin lesion with infiltration of Th2 cells and elevated serum IgE, and triggers bronchial allergic inflammation.[8] TSLP receptor-deficient mice failed to induce antigen-specific inflammatory Th2 cell response and asthma in response to inhaled antigen.[9] Thus, TSLP appears to be the first epithelial cell-derived cytokine that directly triggers DC-mediated Th2 allergic inflammation, which is the critical cascade downstream in allergic disease pathogenesis.[10] The human CD11c$^+$ myeloid DCs activated by TSLP prime naïve Th cells to differentiate into inflammatory Th2 cells that produce IL-4, Il-5, IL-13 and high levels of TNF-α while little IL-10 and interferon-γ.[6] In this process, OX40 ligand (OX40L), which is preferentially induced on human DCs by TSLP, has been identified as a principal mediator that induces the generation of inflammatory Th2 cells.[11] Thus, the interacting axis of OX40L on DCs and OX40 on T cells is the essential element in inducing aberrant inflammatory Th2 cells that cause allergic disorders.

In addition, memory Th2 cells are the principal cells responsible for the maintenance of chronic allergic inflammation and the relapse of allergic inflammation upon re-exposure to allergens.[12,13] TSLP-activated DCs (TSLP-DCs) also play a role of homeostatic expansion of allergen-specific Th2 memory cells and further polarization of the Th2 phenotypes through OX40L expression,[14] contributing to the maintenance of chronic allergic inflammation. With regard to this point, another epithelial cell-derived cytokine IL-25 plays an enhancing role of function and expansion of Th2 memory cells induced by TSLP-DCs.[15] Thus, the OX40L on DCs and epithelial cell-derived cytokines might be the early and principal molecular components that trigger and maintain the allergic inflammatory cascades in diseases such as atopic dermatitis and asthma.[16,17]

IL-33, identified as an epithelial cell-derived cytokine in the IL-1 family, is found to be synthesized as a 30-kDa precursor molecule and cleaved by caspase-1 upon inflammasome activation into an 18-kDa mature/bioactive form.[18] IL-33 is mainly expressed in a variety of non-hematopoietic cells such as fibroblasts, adipocytes, smooth muscle, endothelial, and bronchial epithelial cells.[18-20] The formerly orphaned IL-1 receptor-related protein, ST2, is found to be the IL-33 receptor,[21] and IL-33/ST2 axis activates immune cells involved in allergic inflammation such as Th2 cells,[18,22] NKT cells,[23] eosinophils,[24] basophils,[25] mast cells,[26] and group 2 innate lymphoid cells.[27] Indeed, increased IL-33 expression is found in inflamed skin tissue of atopic dermatitis patients[28] and in the serum of asthmatic patients,[29] and IL-33 has been implicated in the pathogenesis of asthma, atopic dermatitis, and anaphylactic shock.[30] IL-33 functions as selective Th2 chemoattractant and enhances the IL-5 and IL-13 expression in mouse Th2-polarized cells in vitro, leading to severe pathological changes in mucosal organs with epithelial hyperplasia and eosinophil and neutrophil infiltrations in the colonic mucosa.[18] Furthermore, IL-33 is found to activate mouse bone marrow-derived myeloid DCs to prime naïve T cells to produce IL-5 and IL-13[31,32] and to directly stimulate mouse naïve CD4$^+$ cells to differentiate into Th2 cells producing IL-5 and IL-13,[33] indicating a contribution to Th2-oriented allergic inflammation. Of interest is that IL-33 has been described as a chromatin-associated transcriptional regulating factor.[34] Thus, IL-33 has dual roles; an intracellular nuclear factor and a Th2-inducing cytokine. Given the functional involvement of IL-33 in the Th2 immune responses and allergic inflammation, we hypothesized that human IL-33 plays a possible role in the key Th2-polarizing cascade, the generation and maintenance of OX40L-dependent pathway. Here we have investigated how IL-33 participates in OX40L-mediated Th2 immune responses, and how it mediates the progress in allergic inflammatory cascades. In this study, we have shown that IL-33 is a positive regulator of TSLP-DC-OX40L axis that potentiates the differentiation from human naïve Th cells to Th2 cells and expansion of memory Th2 cells, and it dramatically enhances the production of allergy-associated cytokines, IL-5, IL-9 and IL-13.

METHODS

MEDIA AND REAGENTS

RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin and heat-inactivated 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) was used for cell cultures throughout the experiments. We used 15 ng/ml TSLP (R&D Systems, Minneapolis, MN, USA), 1 μg/ml R848 (InvivoGen, San Diego, CA, USA), recombinant IL-4 (R&D Systems) and 10 μg/ml recomibinant IL-33 (R&D Systems). Recombinant OX40L/CD32-transfected L fibroblasts as OX40L stimulation and CD32-transfected L fibroblasts as control were used for T cell stimulation, as described previously.[35]

ISOLATION AND CULTURE OF BLOOD DCs

Human peripheral blood DC subsets (myeloid DCs and pDCs) were isolated from PBMCs from healthy adult donors, as described previously.[36] The CD11c$^+$ DCs were cultured in flat-bottom 96-well plates in the presence of TSLP, R848, or IL-33 at 5×10^4 cells in 200 μl of medium per well for 24 h.

PURIFICATION OF NAÏVE AND CRTH2$^+$CD4$^+$ MEMORY T CELL SUBSET

CD4$^+$ T cells were enriched using CD4$^+$ T cell isolation kit II (Miltenyi Biotec, Teterow, Germany) according to the manufacturer's instructions. Enriched CD4$^+$ T cells were stained with FITC-labeled lineage cocktail (CD8, CD14, CD16, CD19, CD56, BDCA2,

TCR-α/β, and glycophorin A) or PE-Cy5.5-labeled anti-CD4, allophycocyanin-labeled anti CD45RA (BD Biosciences, Franklin Lakes, New Jersey, USA), or biotin-labeled anti-CRTH2 (Miltenyi Biotec) followed by allophycocyanin-labeled anti-streptavidin (BD Biosciences). Stained cells were sorted by fractions of $CD4^+CD45RA^+$ naïve T cells and $CD4^+CRTH2^+$ Th2 memory cells (purity >99%).

ANALYSES OF DCs

To analyze the expression of surface molecules, DCs were stained with FITC-labeled CD86 mAb (BD Biosciences) and PE-labeled anti-OX40L mAb (Ancell, Bayport, MN, USA), and then analyzed by a FACSCalibur (BD Biosciences). Isotype matched mAbs (R&D Systems) were used as negative controls.

T CELL STIMULATION

2×10^4 freshly purified naïve $CD4^+$ T cells (DC-to-T cell ratio, 1 : 4) were cocultured with allogeneic $CD11c^+$ DCs precultured with different conditions for 24 h, and 1×10^4 freshly purified $CRTH2^+CD4^+$ Th2 memory cells (DC-to-T cell ratio, 1 : 2) were cocultured with autologous $CD11c^+$ DCs in 96-well round-bottom culture plates for 7 days. In some experiments, T cells were cultured for 5 days with irradiated OX40L/CD32-transfected L fibroblasts (2.5×10^4/well) as a platform to immobilize the stimulating anti-CD3 (OKT3, 0.2 μg/ml) and anti-CD28 (0.2 μg/ml) mAbs. CD32-transfected L fibroblasts were used as control L fibroblasts (parental L fibroblasts).

SURFACE MARKER ANALYSES OF T CELLS

Th cells were stained with PE-labeled ST2 (anti-IL-33 receptor) mAb (Medical & Biological Laboratories, Nagoya, Japan) or anti-CD124 (IL-4 receptor α) mAb to determine their expressions. Isotype matched PE-labeled unrelated mAbs (R&D Systems and Beckman Coulter, Brea, CA, USA) were used as negative controls.

ANALYSES OF T CELL CYTOKINE PRODUCTION

The cultured $CD4^+$ T cells were collected and washed. For detection of cytokine production in the culture supernatants, the T cells were restimulated with immobilized anti-CD3 (OKT3, 5 μg/ml) and soluble anti-CD28 (1 μg/ml) at a concentration of 10^6 cells/ml for 24 h. The levels of IL-4, IL-5, IL-10, IL-9, IL-13, TNF-α, and IFN-γ were measured by ELISA (kits from R&D Systems or eBioscience, San Diego, CA, USA).

T CELL EXPANSION ASSAY

Freshly isolated 2×10^4 $CRTH2^+CD4^+$ Th2 memory cells were stimulated for 5 days with CD32-transfected or CD32/OX40L-transfected L fibroblasts precoated with anti-CD3 (OKT3, 0.2 μg/ml) and anti-CD28 (0.2 μg/ml) mAbs, as described above. The cultured T cells were collected and resuspended in an EDTA-containing medium to dissociate the clusters. Viable cells were counted by trypan blue exclusion of the dead cells.

COLLECTION OF HUMAN SAMPLES

This study was approved by the Institutional Review Board of Kansai Medical University (PBMCs from healthy adult donors; IRB No. 0640 and specimens from patients with atopic dermatitis; IRB No. 0793) and the research was in compliance with the Helsinki declaration. This study was approved by the review board for human research at Kansai Medical University. Skin specimens were taken by 6-mm punch biopsies from normal skin of healthy subject and lesional skin of atopic dermatitis defined according to standard clinical criteria. IRB No. 0640 includes blood collection and IRB No. 0793 includes skin biopsy from our collaborators (as healthy volunteers) for this research. In these collections, informed consent acquisition was performed by our collaborators with free intention. When developed lymph node swelling was observed, lymph node specimens from the patients with atopic dermatitis were also taken by biopsies.

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed on frozen sections of human skin specimen. After fixation in acetone, specimens were stained with monoclonal anti-IL-33 mAb (R&D Systems) or isotype control, and the avidin-biotin-peroxidase complex method using a labelled streptavidin-biotin kit (Dako, Glostrup, Denmark) before visualization of the immunoreactions. After immunodetection, the sections were counterstained with hematoxylin.

RESULTS

IL-33 DID NOT ACTIVATE HUMAN MYELOID DCs TO INDUCE THE EXPRESSION OF OX40L

It has been reported that IL-33, like TSLP, is produced from the epithelial tissue, particularly from the skin in lesion with atopic dermatitis,[37] and activate mouse bone marrow-derived myeloid DCs to express OX40L and CCL17.[31] Therefore, we first examined whether IL-33 can activate human $CD11c^+$ myeloid DCs to induce the up-regulation of OX40L and CD86 on cell surface. In line with the previous studies,[6,11] TSLP showed the strong ability to induce DC activation by the substantial upregulation of OX40L, CD86, but TLR ligand R848 only induced CD86 upregulation (Fig. 1). We here found that IL-33 could barely induce the increase in the expression of OX40L and CD86 on human blood myeloid DCs (Fig. 1). Addition of IL-33 together with TSLP did not induce further upregulation of these molecule expression compared with TSLP alone. This result indicates a less

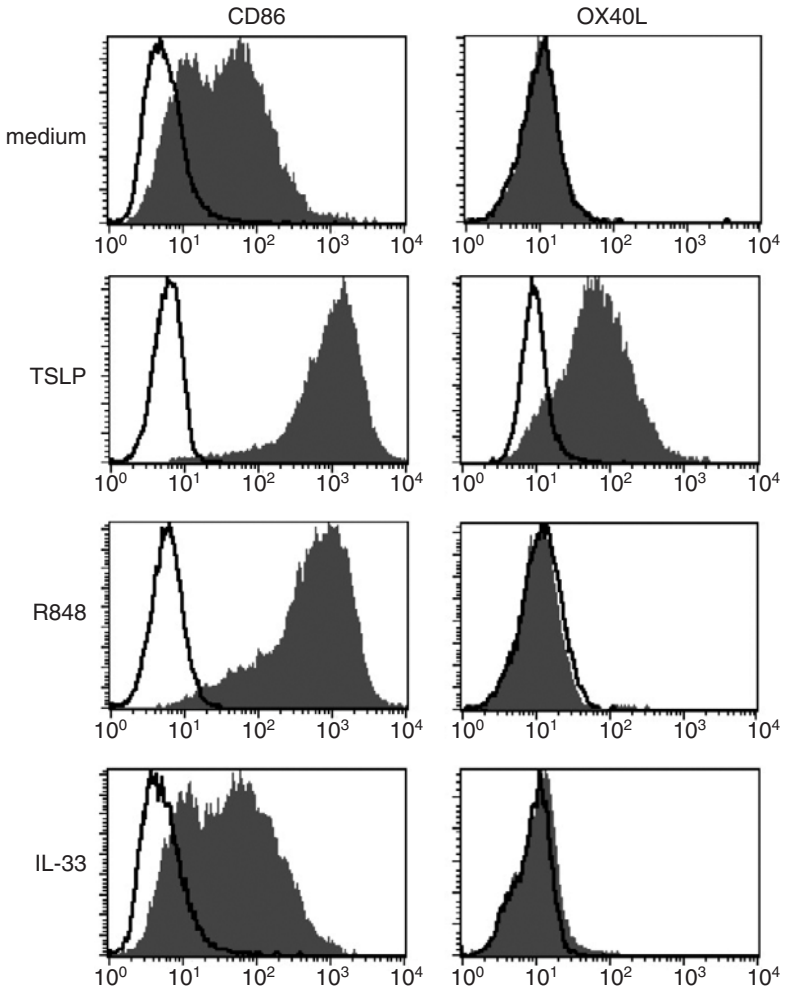

Fig. 1 Surface marker analyses on DCs after the culture with IL-33. Human blood myeloid CD11c$^+$ DCs were isolated from the peripheral blood, and were cultured with medium alone, TSLP, R848, or IL-33 (50 ng/ml) for 24 hrs. The DCs were then stained with mAb against CD86 or OX40L (filled histograms), and analyzed by a FACSCalibur. The DCs stained with isotype-matched controls were represented as open histograms. Similar results were observed in four independent experiments and the results of a representative experiment are shown.

ability of IL-33 to activate human myeloid DCs, unlike mouse DCs, at least with respect to the expression of OX40L and CD86. Thus, unlike TSLP, which directly activates human DC subset to mediate Th2 response, IL-33 could not induce the strong DC activation in humans in vitro.

IL-33 ENHANCES TSLP-DC-MEDIATED Th2 CELL RESPONSE IN OX40L AXIS

We next assessed the direct effect of IL-33 on the CD4$^+$ T cells for Th2-polarizing axis. IL-33 itself had an ability to induce Th2 cells that produce IL-5, IL-9, and IL-13 (Fig. 2A) and had no effects of IL-4, IL-10, TNFα, nor IFNγ production, when naïve T cells were stimulated with anti-CD3 and CD28 mAbs, this being dose-dependent fashion of IL-33 (Fig.2B). As has been reported, Th2 polarization was synergistically enhanced by IL-4 and OX40L.[11] Thus, we next examined whether IL-33 and DC-derived OX40L could work synergistically in driving Th2 cell responses. Naïve CD4$^+$ T cells were cocultured for 7 days with allogeneic DCs pretreated with TSLP (TSLP-DCs), and the cytokine production by the primed CD4$^+$ T cells was then examined. Addition of IL-33 into the coculture of TSLP-DCs and naïve T cells induced a marked increase in the productions of IL-5, IL-9, and IL-13, as shown in Figure 3. This was the case when naïve T cells were stimulated with OX40L-transfected L fibroblasts with anti-CD3/CD28 mAbs (instead of TSLP-DCs). Increased productions of IL-4, IL-5, IL-9, IL-13,

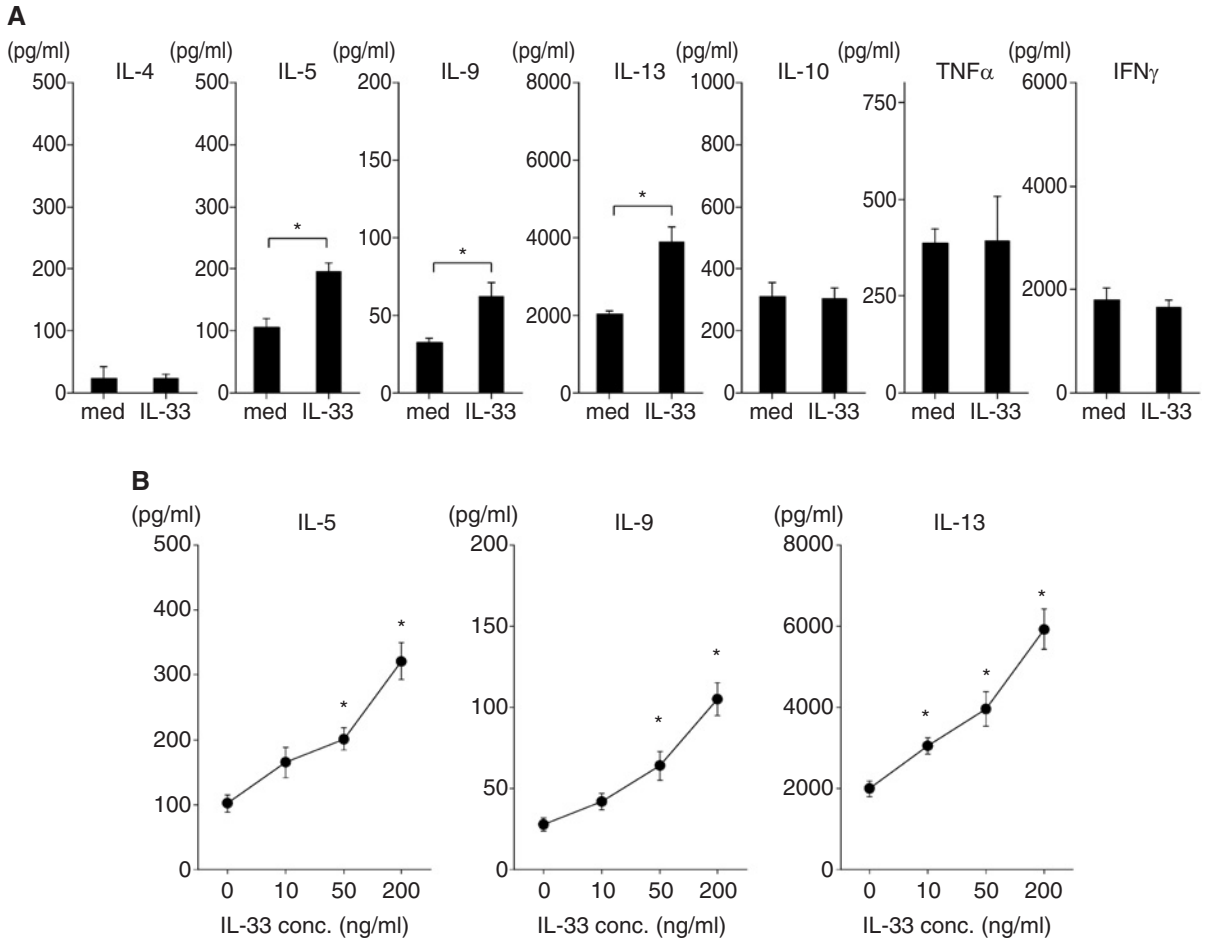

Fig. 2 Effect of IL-33 on naïve Th cell differentiation. Freshly purified naïve CD4+ T cells were stimulated with immobilized anti-CD3/CD28 mAbs on CD32 transfected-L fibroblasts in the presence or absence of IL-33 (**A**; 50 ng/ml and **B**; different doses). Five days after the initiation of the culture, the supernatants were collected and the amounts of cytokines indicated in the figures were determined by ELISA. Data are means ± s.e.m. of five independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$).

and TNFα with decreased productions of IL-10 and IFNγ were observed when T cells were stimulated with OX40L-transfected L cells (Fig. 4A), whereas the Th2 polarization was further promoted by the addition of IL-33, as indicated in the dramatically elevated production of IL-5, IL-9, and IL-13 without affection of IL-4, IL-10, TNFα, nor IFNγ (Fig. 4A). Therefore, IL-33 can collaboratively promote the Th2 differentiation with OX40L.

To identify the mechanism underlying this process, we analyzed surface expression of IL-33 (ST2) on T cells stimulated with OX40L-transfected L fibroblasts. Freshly isolated naïve T cells and activated T cells cultured with anti-CD3/CD28 mAbs expressed little IL-33R (Fig. 4B). Interestingly, recombinant OX40L upregulated their surface expression of IL-33R (Fig. 4B), indicating that DC-derived OX40L creates a susceptible condition of developing Th2 cells to IL-33.

IL-33 ENHANCES IL-4-MEDIATED Th2 CELL RESPONSE

IL-4 is another critical environmental Th2 polarizing signal.[38-40] Next, we examined synergistic function of IL-33 and IL-4 in the Th2 polarization. Naïve T cells were stimulated with IL-4 and anti-CD3/CD28 mAbs for 7 days. Unlike OX40L, IL-4 contributes to the generation of conventional Th2 cells that produce IL-5, IL-9, IL-13, and IL-10 but not TNF-α production (Fig. 5A). When IL-33 was added to the culture, the productions of IL-5, IL-9, and IL-13 were remarkably elevated without affection of IL-10, IFN-γ, nor TNF-α (Fig. 5A), as tested in the culture with TSLP-DCs (Fig. 3) and with OX40L-transfected L fibroblasts (Fig. 4A). Thus, IL-33 also enhances the Th2 response elicited by IL-4.

Next, we cultured naïve T cells with IL-33 to determine whether IL-33 can enhance the expression of IL-

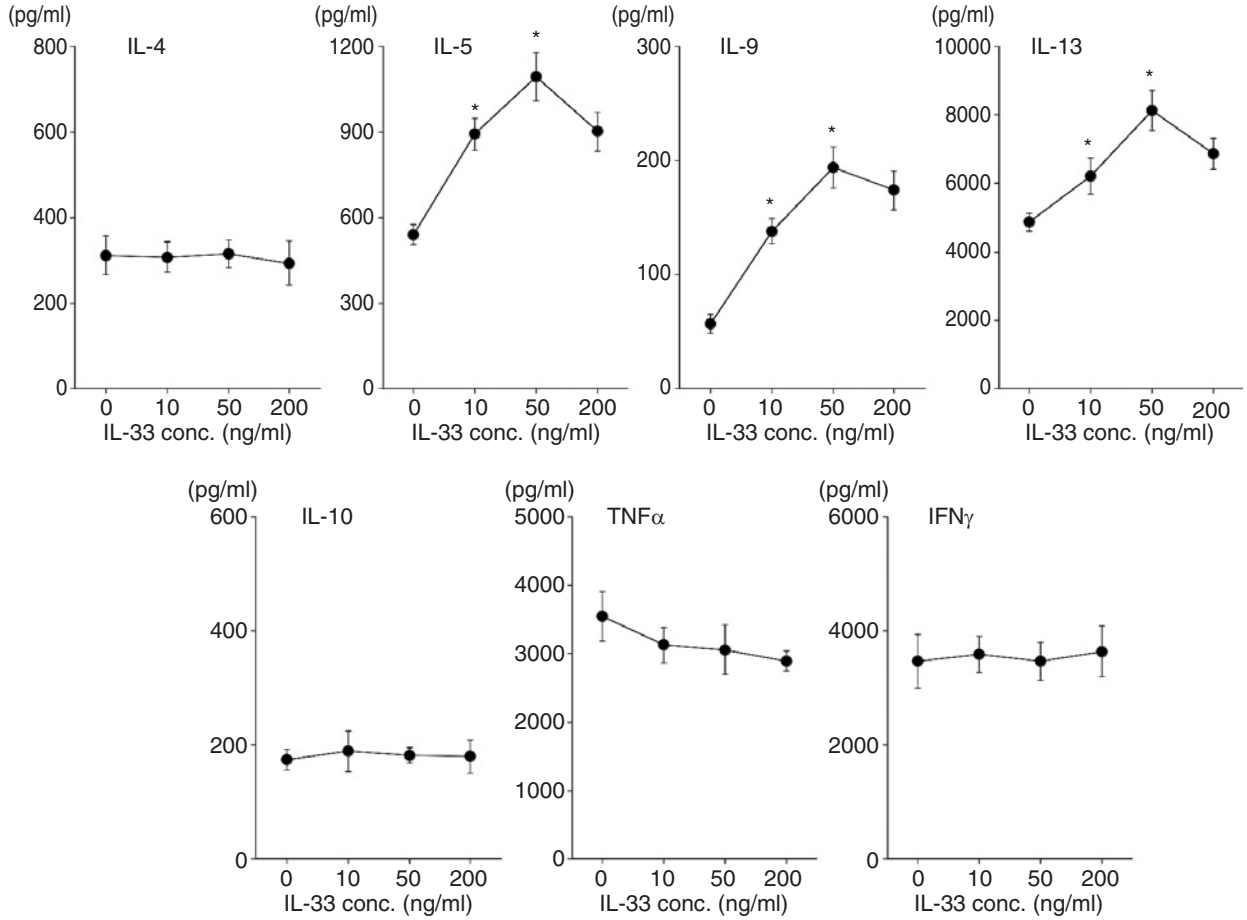

Fig. 3 Effect of IL-33 on TSLP-DC-mediated Th2 cell responses. The blood myeloid CD11c$^+$ DCs were cultured with 15 ng/ml of TSLP (TSLP-DCs). Freshly isolated naïve CD4$^+$ T cells were cocultured with allogeneic TSLP-DCs (DC-to-T cell ratio, 1 : 4) in the presence of graded doses of IL-33 (10-200 ng/ml) for 7 days. Cytokine production by CD4$^+$ T cells was measured in supernatants by ELISA. Data are means ± s.e.m. of five independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$).

4R (CD124) and whether IL-4 can enhance the IL-33R (ST2) on T cells. Although activated T cells cultured with anti-CD3/CD28 mAbs expressed detectable surface IL-4R, IL-33 further increased their expression of IL-4R (Fig. 5B). Meanwhile, stimulation of T cells with IL-4 in the presence of anti-CD3/CD28 mAbs enhanced the expression of IL-33R (Fig. 4B). Addition of IL-33 into IL-4 showed less effect on their expression. Thus, the mutual positive effect between IL-33 and IL-4 on the T cell expression of IL-4R and IL-33R might enhance the Th2 transition.

IL-33 ENHANCES MEMORY Th2 CELL RESPONSE

CRTH2$^+$ memory T cells continuously stimulated by allergens play a critical role for the maintenance of the allergic condition and OX40L on TSLP-DCs induces expansion of CRTH2$^+$ memory T cells.[14,41] Therefore, we next examined the effect of IL-33 on the activation of CRTH2$^+$ memory Th2 cells. Isolated CRTH2$^+$ memory T cells were stimulated with anti-CD3/CD28 mAbs on the OX40L-transfected L fibroblasts or the parental control L cells in the presence or absence of IL-33. We here found that OX40L stimulation enhanced the expansion of CRTH2$^+$ memory T cells and this effect was further amplified when added with IL-33 (Fig. 6A). Next, we analyzed surface expression of IL-33R on CRTH2$^+$ memory T cells. Freshly isolated CRTH2$^+$ memory T cells expressed detectable IL-33R and their expression was slightly upregulated after culture with anti-CD3/CD28 mAbs (Fig. 6B). OX40L stimulation further increased their expression of IL-33R (Fig. 6B), leading to a susceptible condition to IL-33. In either case stimulated with autologous TSLP-DCs or with OX40L-transfected L fibroblasts plus anti-CD3/CD28 mAbs, the increased productions of IL-5 and IL-13 from CRTH2$^+$ CD4$^+$ Th2 memory cells were observed when IL-33 was added in the culture (Fig. 6C). Thus, IL-33 can activate CRTH2$^+$ memory T cells to further produce Th2 cy-

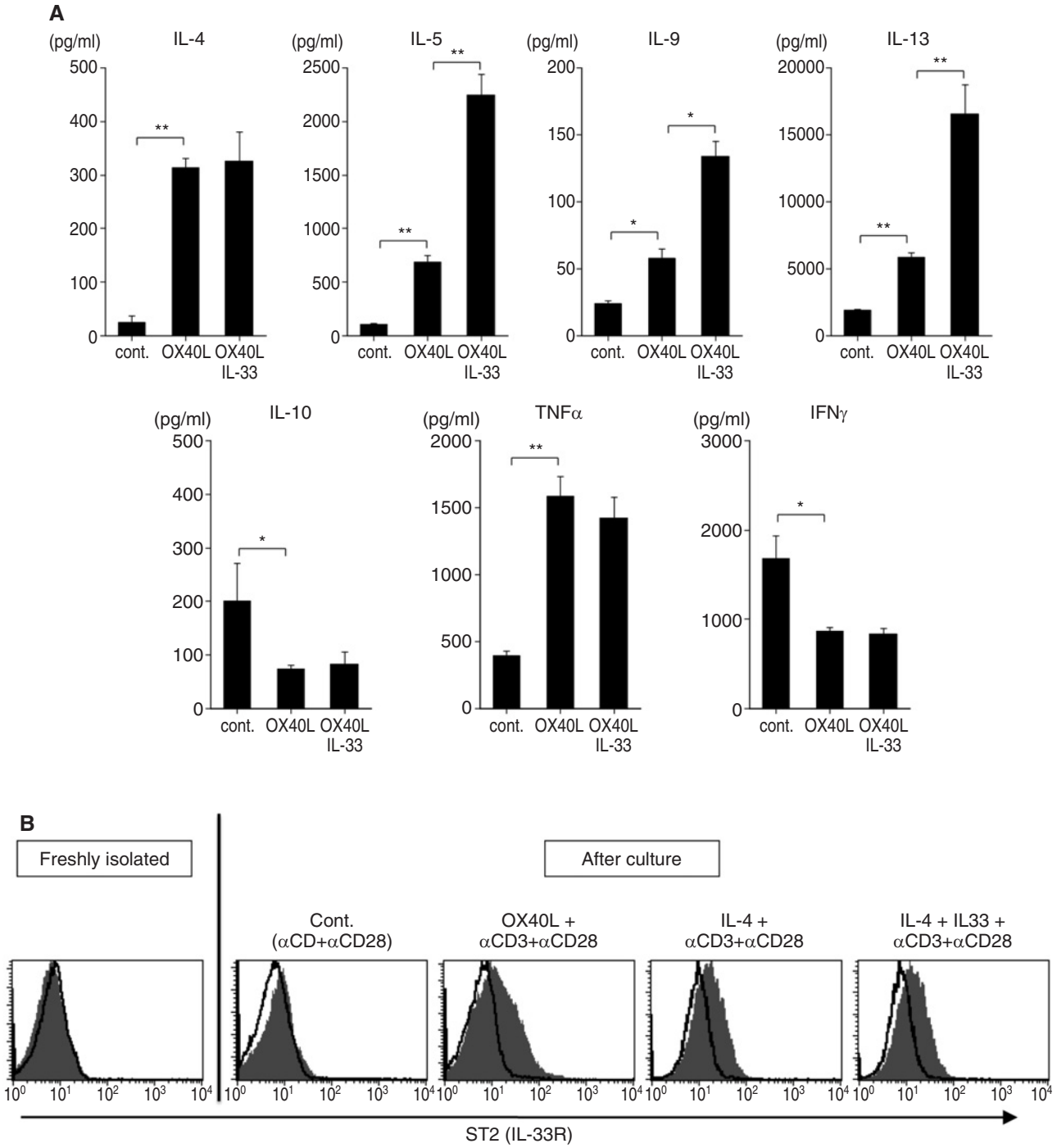

Fig. 4 Effect of IL-33 on OX40L-mediated Th2 cell responses. **A**: Naïve Th cells were cultured on OX40L-transfected L fibroblasts with immobilized anti-CD3/CD28 mAbs in the presence or absence of IL-33 (50 ng/ml). Naïve Th cells cultured with CD32-transfected L fibroblasts (parental L cells) plus anti-CD3/CD28 mAbs were served as controls. Five days after the initiation of the culture, the supernatants were collected and the amounts of cytokines indicated in the figures were determined by ELISA. Data are means ± s.e.m. of five independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). **B**: Naïve Th cells were stimulated with OX40L-transfected L fibroblasts or parental L cells with immobilized anti-CD3/CD28 mAbs in the presence or absence of IL-33 and/or IL-4 for 5 days. Before and after culture, the cells were stained with mAb against ST2 (IL-33R). The staining profiles of ST2 and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in three independent experiments and the results of a representative experiment are shown.

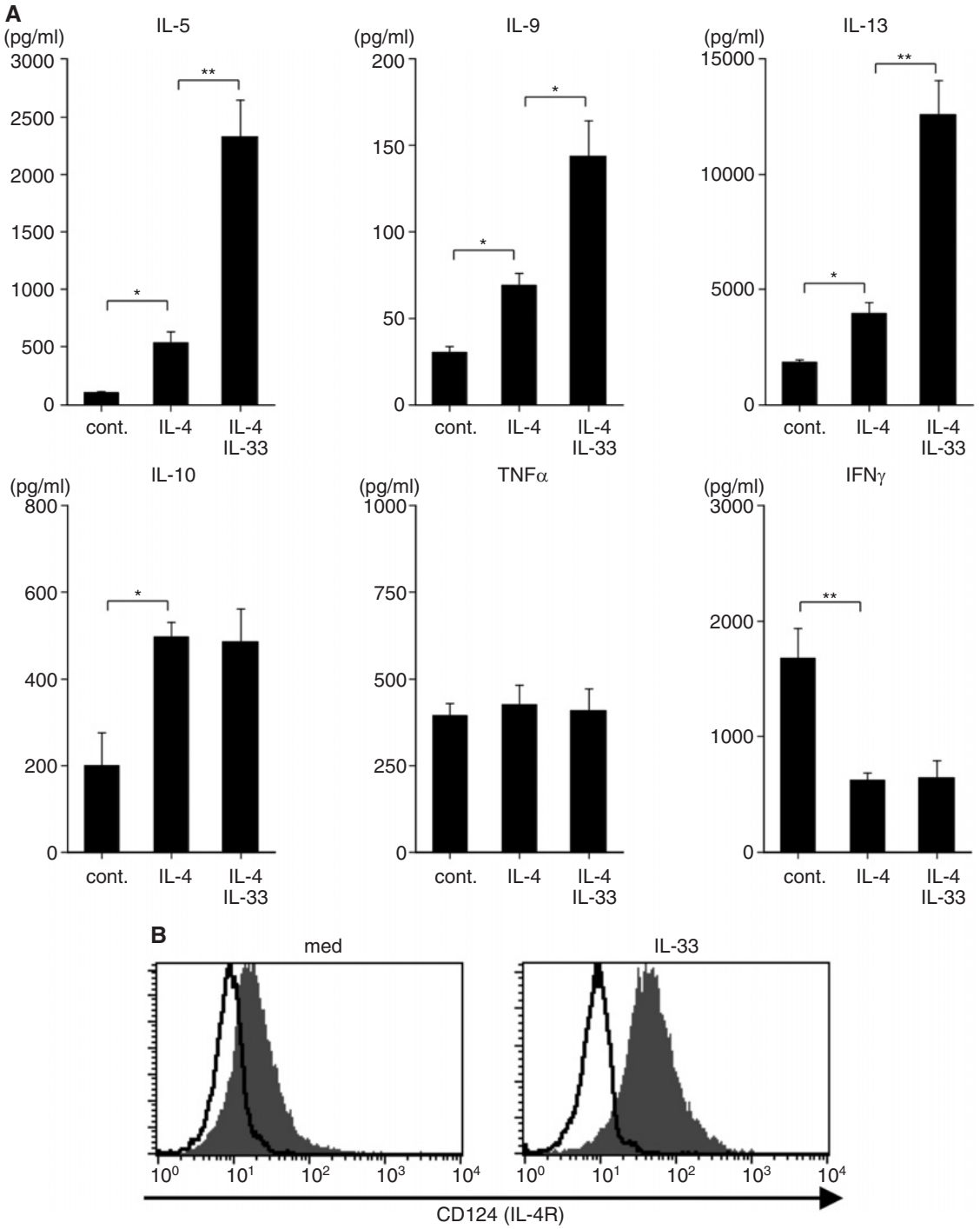

Fig. 5 Effect of IL-33 on IL-4-mediated Th2 cell responses. **A**: Naïve Th cells were stimulated with immobilized anti-CD3/CD28 mAbs (CD32-transfected L fibroblasts (parental L cells) were used as a platform) and IL-4 (25 ng/ml) in the presence or absence of IL-33 (50 ng/ml). Five days after the initiation of the culture, the supernatants were collected and the amounts of cytokines indicated in the figures were determined by ELISA. Data are means ± s.e.m. of five independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). **B**: Naïve Th cells were stimulated on CD32-transfected L fibroblasts with immobilized anti-CD3/CD28 mAbs in the presence or absence of IL-33 (50 ng/ml) for five days. The cells were stained with mAb against IL-4R (CD124). The staining profiles of IL-4R and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in three independent experiments and the results of a representative experiment are shown.

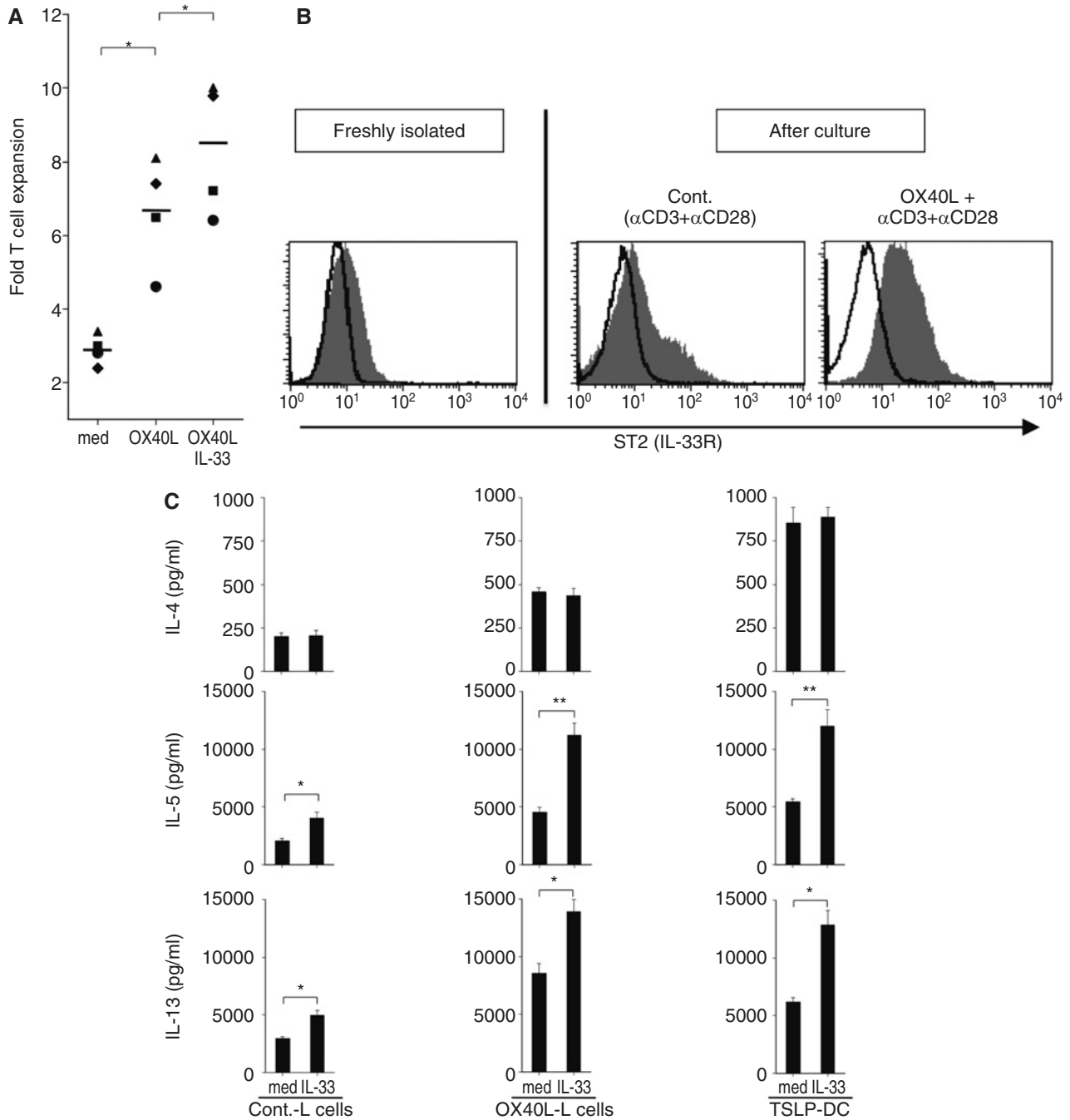

Fig. 6 Effect of IL-33 on memory Th2 cell responses. Memory Th2 cells, isolated as $CD4^+CRTH2^+$cells, were stimulated with autologous TSLP-DCs (DC-to-T cell ratio, 1 : 2) for 7 days or with OX40L-transfected L fibroblasts plus immobilized anti-CD3/CD28 mAbs for 5 days in the presence or absence of IL-33 (50 ng/ml). **A**: After culture with OX40L-transfected L fibroblasts (OX40L) or parental L cells (Cont.), viable T cells were counted with a trypan-blue exclusion test. Horizontal bars indicate the median of four independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$). **B**: Before and after culture with OX40L-transfected L fibroblasts (OX40L) or parental L cells (Cont.), the cells were stained with mAb against ST2 (IL-33R). The staining profiles of ST2 and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in three independent experiments and the results of a representative experiment are shown. **C**: After culture with TSLP-DCs, OX40L-transfected L fibroblasts or parental control L cells, the supernatants were collected and the amounts of cytokines indicated in the figures (vertical axis) were determined by ELISA. Data are means ± s.e.m. of five independent experiments. Statistical significance was determined using paired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

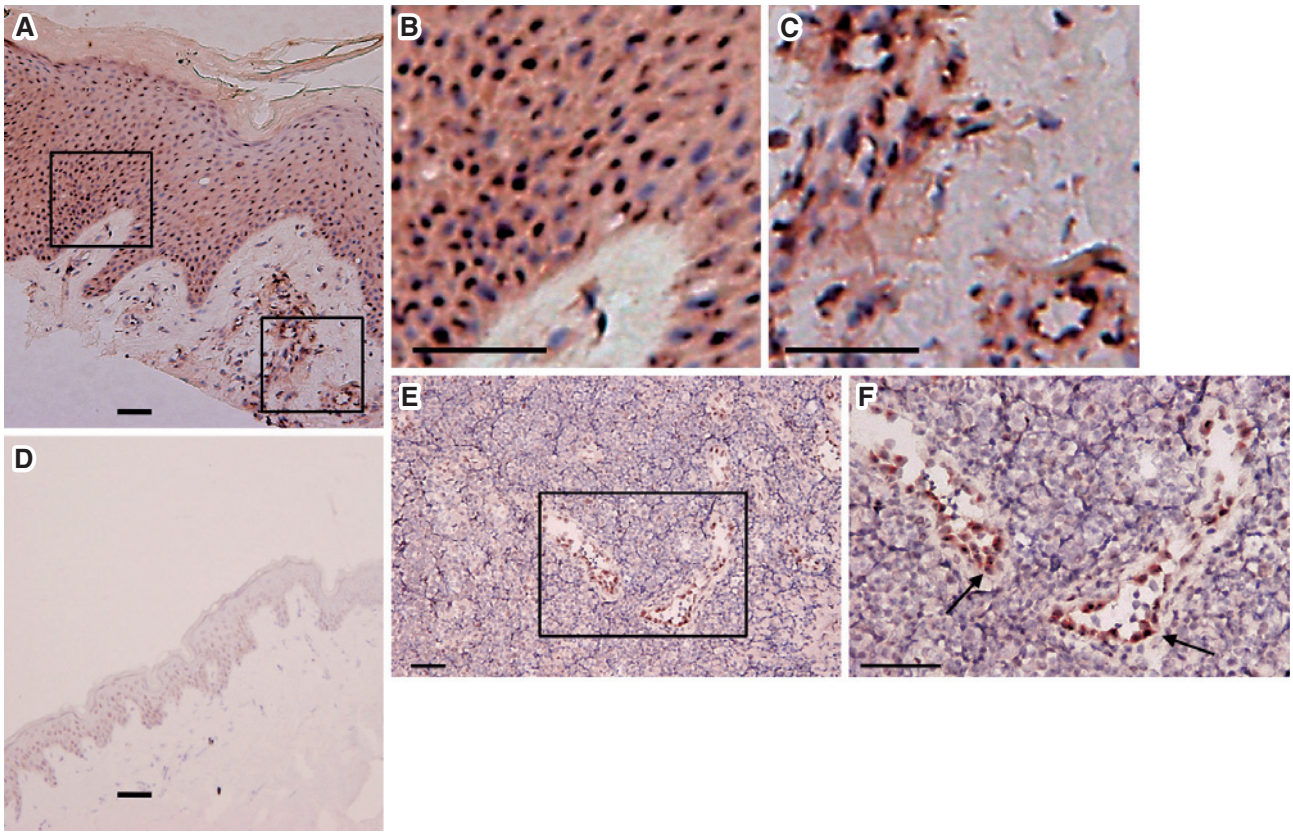

Fig. 7 Expression of IL-33 in the lesional skin and lymph node in the patients with atopic dermatitis. Immunohistochemical staining was performed on frozen sections of human specimen. **A**-**C**: Staining with anti-IL-33 mAb of biopsied skin specimen from the patients with atopic dermatitis. **B** shows keratinocytes (left open square in **A**) and **C** shows endothelial cells (right open square in **A**) of lesional skin. Representative data from two patients are shown. **D**: Staining with anti-IL-33 mAb of biopsied skin specimen from the normal volunteers. Representative data from two donors are shown. **E** and **F**: Staining with anti-IL-33 mAb of biopsied lymph nodes from the patients with atopic dermatitis. **F** shows crypt epithelial cells in the lymph nodes (open square in **E**). Representative data from two patients are shown. Original magnification × 20 in **A**, **D**, and **E**; × 50 in **B**, **C** and **F**. Arrows in **F** indicate crypt epithelial cells that are positive for IL-33. Scale bar, 10 μm.

tokines and it also assists their survival to maintain the allergic state.

IL-33 EXPRESSION IN SKIN AND INFLAMED LYMPHOID TISSUE OF ATOPIC DERMATITIS

The relevance of IL-33 expression in allergic diseases was confirmed by several immunohistochemical studies in the inflamed skin tissue of atopic dermatitis,[28,37] in the serum of asthmatic patients,[29] and in the lungs of mice with experimental allergic asthma.[42] Because of the present data showing the direct effects of IL-33 on Th2 polarization, we hypothesized that IL-33 could be expressed in lymphoid tissues where DCs prime naïve and memory T cells. Therefore, we performed immunohistochemical analyses to examine the expression of IL-33 in inflamed lymphoid tissues as well as skin specimen from the patients with atopic dermatitis.

Immunohistochemical staining revealed that most of nuclei in hypertrophic keratinocyte layer were more or less positive for IL-33 in the patients with atopic dermatitis (Fig. 7A, B). In atopic dermatitis patients but not the normal volunteer (Fig. 7D), IL-33 expression was upregulated in the entire skin. In addition, strong nuclear expression of IL-33 in endothelial cells of lesional skin from the patients with atopic dermatitis (Fig. 7C). More importantly, the strong nuclear expression of IL-33 was also detected at the crypt epithelial cells in the lymph nodes from the patients with atopic dermatitis (Fig. 7E, F). This finding implies a potential role of IL-33 in T cell priming in allergic inflammation. Thus, epithelial cell-derived IL-33 can positively regulate the OX40L-driven Th2-polarizing axis, leading to reinforcement of allergic responses in the regional area.

DISCUSSION

IL-33 is involved in the allergy-associated responses in which the activity of neutrophils and monocytes/macrophages have been influenced to increase cy-

tokine productions.[43] Furthermore, it is noted that IL-33 can directly act on both eosinophils to enhance their survival, adhesion, and CD11b expression because of their abundant expression of ST2[44] and basophils to induce the enhanced secretion of proinflammatory cytokines including IL-4, IL-5, IL-13, and CD11b expression along with their migratory activity.[23,25] It has recently been reported that IL-33 can support the expansion of newly identified innate immune effector nuocytes that mediate Th2 immune response by producing IL-4, IL-5 and IL-13[45] and that IL-33 expressed on skin may play an important role in pathogenesis of atopic dermatitis through activation of the innate lymphoid cells.[46] Together with these roles in the innate immunity, IL-33 also plays some roles in acquired type 2 immune responses through activating DCs and promoting Th2 cell differentiation.[31-33] Given these findings that IL-33 has various effects on different cell types, IL-33 could be a key cytokine that enhances and coordinates both innate and acquired allergic immune responses.

In addition to these functions of IL-33, we here found that IL-33 can participate in the Th2-polarizing DC-mediated OX40L effect as an important pathway to induce allergic response. IL-33 enhances IL-5 and IL-13 production in the developing Th2 cells mediated by OX40L not only in Th2 differentiation from naïve T cells but also on maintenance of memory Th2 cells. Unlike in the case of mouse DCs, which can respond to IL-33 to undergo maturation with their upregulated expression of OX40L,[31] direct responsiveness of human myeloid DCs to IL-33 was quite weak. This difference in the responsiveness of IL-33 to DCs between humans and mice still remains unclear and further studies will be required to determine how human DCs become to strongly respond to IL-33. Although OX40L cause allergic inflammation by concomitant promotion of inflammatory cytokine TNF-α production with development of Th2 cells,[11,35] our results suggest that IL-33 might contribute to the Th2 immune response by promoting the functional attributes of producing IL-5 and IL-13 on the OX40L-driven Th2 cell differentiation and maintenance without affecting TNF-α.

IL-5 has been reported to support the terminal differentiation and proliferation of eosinophilic precursors and to recruit eosinophils to the inflammatory site,[47] and thus, the recruited eosinophils produce cytotoxic substances such as major basic protein, eosinophilic cationic protein, and eosinophilic peroxidase, leading to tissue damage. In addition to this, IL-13, another upregulated cytokine by IL-33, could play a critical role in the development of bronchial asthma by airway hypersensitivity[48] and contribute to asthmatic airway obstruction by increase in the mucin secretion in the epithelial cells and mucus metaplasia,[49,50] which has been confirmed by the findings that IL-13 affects both ciliated and secretory cell differentiation in animal models of asthma[51] and that it also induces mucin secretion through TGF-β2 upregulation.[52] Thus, in the context of Th2 cytokine-associated allergic response, IL-33 can be indirectly involved in the enhancement of inflammatory responses and aggravation of pathogenic condition through the increases in IL-5 and IL-13 production.

Of note is that in our results, IL-33 enhanced the production of IL-9 in the developing Th2 cells induced by either TSLP-DCs or OX40L. Th9 cells are closely associated with Th2 cells, as Th2 cells co-express both IL-4 and IL-9 in the early phase of Th2 differentiation, and the Th2 cytokine IL-4 provides one of the key signals for Th9 induction.[53] In addition, OX40L leads to a remarkable increase in Th9 differentiation from naïve $CD4^+$ T cells.[54] Recently, it has been reported that IL-33 induces IL-9 production in Th2 cells and basophils,[55] and IL-9 has been previously implicated in the pathogenesis of asthma and other allergic diseases in human studies.[56-58] Thus, IL-33 is also involved in the Th2-mediated allergic inflammation in line with the production of IL-9, which possesses various effects on different cell types in which it promotes mast cell growth and production of proinflammatry cytokines IL-1β and IL-6 and also IL-5 and IL-13.[59] Furthermore, IL-9 is suggested to be a key molecule that affects differentiation of Th17 cells along with TGF-β1 through activation of STAT3.[60] Therefore, upregulation of IL-9 production induced by IL-33 leads to an increase in inflammatory cells and worsening of regional allergic state.

Based on our finding showing the expression of IL-33 in the crypt epithelial cells in the lymph nodes, together with the fact that IL-33 is constitutively expressed in high endothelial venules and fibroblastic reticular cells in normal lymphoid tissues,[19] it has been speculated that IL-33 expression in the epithelial and endothelial cells in lymphoid tissues can participate in the allergic adaptive responses by enhancing the DC-driven Th2 differentiation from naïve T cells through OX40L at the T cell priming phase. By contrast, considering the function of IL-33 as chemoattractant for Th2 cells and the distribution of infiltrated $CRTH2^+$ memory Th2 cells co-localized with DCs in the dermis of lesional skin of atopic dermatitis,[14] skin-derived IL-33 expression might be related to the aggravation of chronic atopic dermatitis by its ability to maintain memory Th2 cells after the recruitment to the skin lesional area.

Our study now identifies the nature of the responses elicited by IL-33, which might pathophysiologically amplifies both OX40L-driven inflammatory Th2 immune responses and IL-4-mediated classical Th2 cell differentiation. Although IL-4 is the critical Th2-polarizing factor,[39] human DCs activated by TSLP or other stimuli have no capacity to produce IL-4.[6,11] Therefore, IL-4 is not the DC-derived original trigger of Th2 responses. Recent studies have sug-

gested that basophils are most likely the key source of IL-4 in induction of Th2 immunity.[61] Thus, basophil-IL-4 axis, in addition to DC-OX40L axis, might be another pathway in driving Th2-type immune response. With respect to this context, IL-33 is found to target basophils to induce activation and production of IL-4.[62] Therefore, our results suggest that IL-33 links DC-OX40L axis and basophil-IL-4 axis to tailor the Th2 type immune responses.

In conclusion, we identified a specialized role of IL-33, which works as a positive regulator of DC-OX40L axis that promotes the differentiation from naïve Th cells into Th2 cells and maintain memory Th2 cells. Based on our and other findings,[30,63] IL-33 would be recognized as an important epithelial cytokine system to contribute to the Th2 immune responses besides IL-25 and TSLP. Therefore, the regulation of IL-33 production is one of the therapeutic plans that ameliorate allergic states. Thus, our results provide a new insight into the biological action of IL-33 underlying the Th2-mediated allergic response and would pave the way for the development of therapeutic strategy to ameliorate allergic diseases such as atopic dermatitis or asthma.

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