

Imidazoquinoline Acts as Immune Adjuvant for Functional Alteration of Thymic Stromal Lymphopoietin-Mediated Allergic T Cell Response¹

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Atopic dermatitis is a major allergic disease that develops through dysregulation of Th2-mediated inflammation. Although dendritic cells (DCs) have been thought to play a critical role in the upstream phase of the allergic cascade, conventional drugs such as steroids and chemical mediator antagonists target the effector cells or factors in allergic inflammation. Recently, it has been demonstrated that interaction between thymic stromal lymphopoietin (TSLP) and human DCs plays an essential role in evoking inflammatory Th2 responses in allergy through OX40 ligand expression on DCs. In this study, we provide evidence that R848, an imidazoquinoline compound, which is a TLR ligand and a strong Th1 response-inducing reagent, is a potent adjuvant for the alteration of the Th2-inducing potency of human DCs activated by TSLP (TSLP-DCs). R848 inhibited the inflammatory Th2-inducing capacity of TSLP-DCs and redirected them to possessing an IL-10 and IFN- γ -producing regulatory Th1-inducing capacity. This functional alteration depended on both repression of OX40 ligand expression and induction of IL-12 production from DCs by the addition of R848. Additionally, R848 had the ability to inhibit the TSLP-mediated expansion and maintenance of the Th2 memory response. These findings suggest that imidazoquinoline may be a useful in the treatment of allergic diseases that are triggered by TSLP. *The Journal of Immunology*, 2008, 181: 5340–5349.

About 20% of the world's population suffers from allergic diseases, which include atopic dermatitis, asthma, allergic rhinitis, and food allergies. Allergic inflammation is the result of a complex immunological cascade leading to the dysregulated production of Th2 cell-derived cytokines such as IL-4, IL-5, and IL-13 (1–3), which in turn triggers IgE production, eosinophilia, and mucus production (4–6). It has recently been proposed that thymic stromal-derived lymphopoietin (TSLP),³ an IL-7-like cytokine, and myeloid dendritic cells (DCs) represent the early molecular and cellular components that trigger allergic inflammatory cascade in diseases such as atopic dermatitis and asthma (7–9). TSLP is expressed by keratinocytes of patients with atopic dermatitis, and TSLP expression is associated with Langerhans cell migration and activation in situ (10). Several recent reports show that the overexpression of TSLP in mice leads to the development of atopic dermatitis, confirming the link between TSLP and the development of atopic dermatitis (11–13). Furthermore, TSLP expression is increased in the airways of patients with asthma and is correlated with the Th2-attracting chemokines and dis-

ease severity (14–18). TSLP strongly activates human CD11c⁺ myeloid DCs without inducing Th1-polarizing cytokines IL-12, IL-23, or IL-27, and these TSLP-activated DCs (TSLP-DCs) instruct naive CD4⁺ T cells to differentiate into inflammatory Th2 cells (10, 19) that produce large amounts of TNF- α , but little or no IL-10.

OX40 ligand (OX40L) is preferentially expressed by TSLP-DCs and is a DC-derived positive signal that induces the inflammatory TNF- α ⁺IL-10[–] Th2 cells in the absence of Th1-polarizing cytokines (19). These findings have provided the functional and molecular basis for the concept that DCs play a central role in triggering the allergic inflammation. Moreover, the subtype of CD4⁺ Th2 memory cells expressing the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is important in the maintenance of Th2-mediated allergic skin diseases (20–22). A specific accumulation of CRTH2⁺CD4⁺ T cells surrounded by the mature CD11c⁺ DCs has been shown in the dermis of TSLP-expressing lesional skin of atopic dermatitis, and TSLP-DCs induce the expansion of CRTH2⁺CD4⁺ cells and the further polarization of the Th2 phenotypes through OX40L (20, 22). These findings suggest a critical role for TSLP in the generation and maintenance of Th2 responses by way of activating DCs at the inflammatory sites. Therefore, interference with or alteration of the function of the TSLP-DCs that induce and maintain inflammatory Th2 response may constitute a rational treatment strategy for allergic diseases.

Previously, it has been shown that imidazoquinoline compounds, imiquimod and its derivative R848, are specific ligands for TLR7 and TLR8 (23), which have potent antiviral and antitumor properties in animals (24–29). Human myeloid DCs express TLR8 (30) and therefore respond to imidazoquinolines (31), and the imidazoquinoline-activated human myeloid DCs produce inflammatory cytokines such as TNF- α , IL-6, and IL-12. We previously reported that R848-activated DCs show a strong ability to induce IFN- γ -producing Th1 cells by the DC-derived IL-12 (32). In the

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³ Abbreviations used in this paper: TSLP, thymic stromal lymphopoietin; CBA, cytometric beads array; DC, dendritic cell; OX40L, OX40 ligand; PGN, peptidoglycan; p-stat5, phosphorylated stat5.

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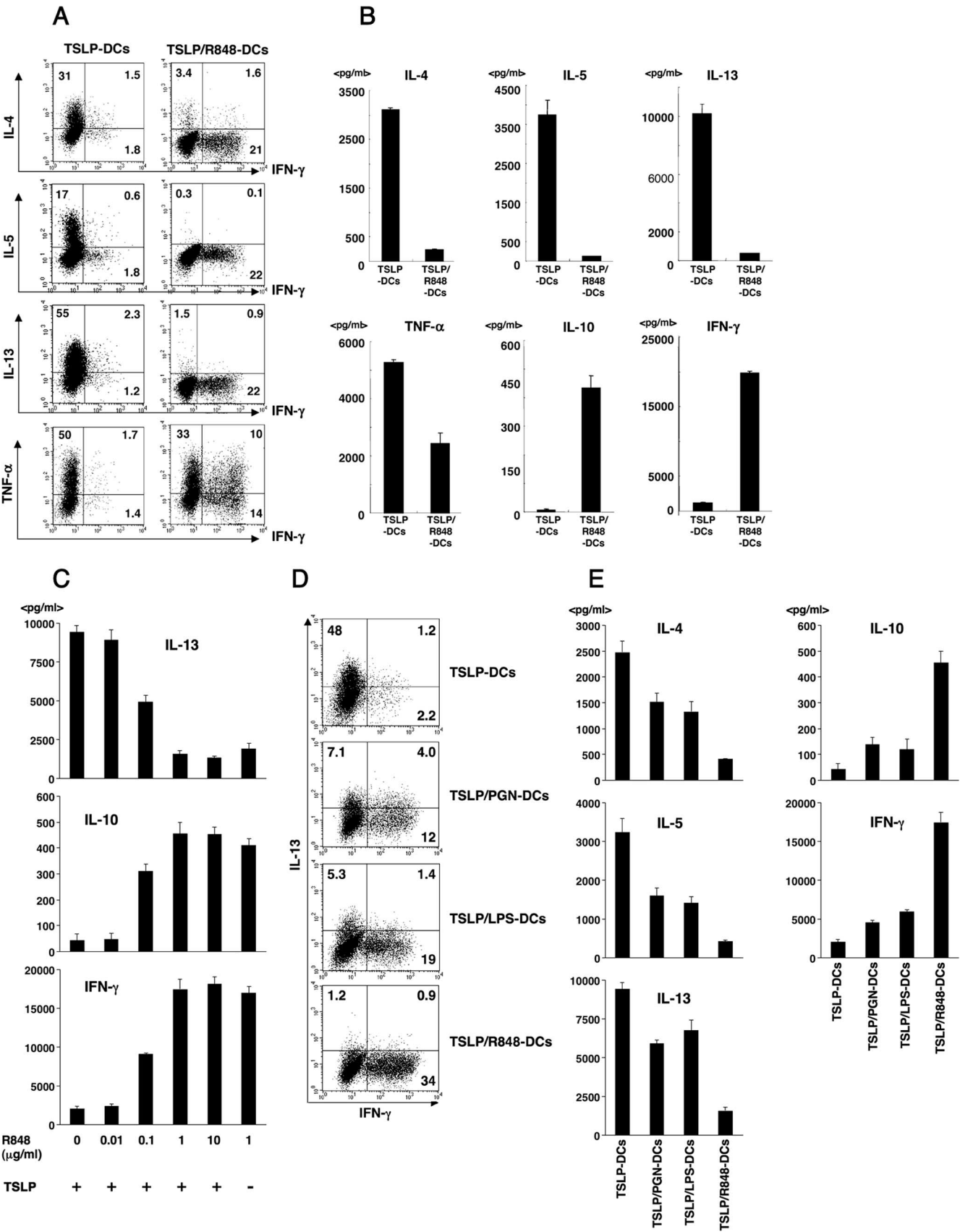


FIGURE 1. R848 treatment of DCs with TSLP suppresses DC-mediated induction of inflammatory Th2 response. *A* and *B*, Naive CD4⁺ T cells were cultured for 7 days with DCs pretreated with TSLP alone or TSLP plus 1 μ g/ml R848. Cytokine production by CD4⁺ T cells was analyzed intracellularly by (*A*) flow cytometry and measured in supernatants by (*B*) CBA. *C*, Naive CD4⁺ T cells were cultured for 7 days with DCs pretreated with TSLP plus different concentrations (0.01–10 μ g/ml) of R848. Cytokine production was measured in supernatants of cultured CD4⁺ T cells by CBA. *D* and *E*, Naive CD4⁺ T cells were cultured for 7 days with DCs pretreated with TSLP alone, TSLP plus 1 μ g/ml R848, TSLP plus PGN, or TSLP plus LPS. Cytokine

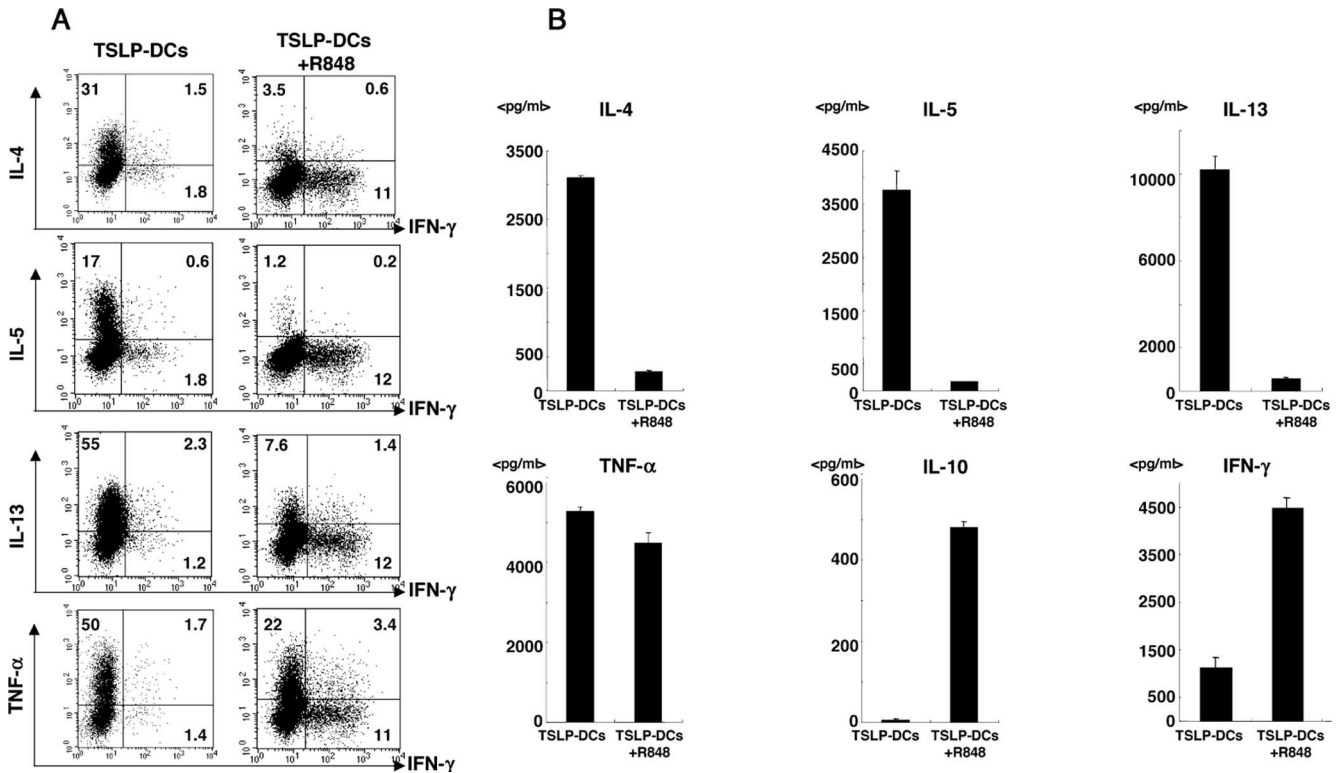


FIGURE 2. Subsequent addition of R848 into DC-T cell coculture suppresses induction of inflammatory Th2 response. DCs were pretreated with TSLP and were then cultured for 7 days with naive CD4⁺ T cells. R848 was added at the beginning of DC-T cell coculture. **A**, Cytokine production by CD4⁺ T cells was analyzed intracellularly by flow cytometry. Percentages of the respective cytokine-producing T cells are indicated. Similar results were observed in four independent experiments, and the results of a representative experiment are shown. **B**, Cytokine production was measured in supernatants after restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by CBA. CBA data are means $\pm$ SEM of four independent experiments.

field of clinical dermatology, imiquimod has been used as an effective adjuvant for the treatment of genital warts caused by human papillomavirus (33). Based on these findings, we hypothesized that imidazoquinoline could act as an immune adjuvant to alter the function of the TSLP-DCs that induce and maintain the pathogenic inflammatory Th2 cell responses.

In this study, we found that R848 forced DCs to produce IL-12 and inhibited the up-regulation of OX40L expression on DCs, even in the presence of TSLP. As a result, imidazoquinoline suppressed the capacity of TSLP-DCs to induce the inflammatory Th2 cell response. Our findings suggest this immune adjuvant as a candidate for the treatment of atopic dermatitis.

Materials and Methods

Isolation and culture of blood DCs

CD11c⁺ DCs were isolated from the buffy coat of blood from healthy adult volunteers (Japan Red Cross Society, Osaka Blood Center), as described (34). Briefly, the DC-enriched population (CD4⁺/CD3⁻/CD14⁻ cells) was obtained from PBMCs by negative and subsequent positive immunoselections (34). The CD11c⁺/lin⁻/BDCA4⁻/CD4⁺ cells (CD11c⁺ myeloid DCs) were sorted by a FACSAria (BD Biosciences) by using PE-labeled or allophycocyanin-labeled anti-CD11c (BD Biosciences), allophycocyanin-labeled or PE-labeled anti-CD304 (BDCA-4/neuropilin-1) (Miltenyi Biotec), a mixture of FITC-labeled mAbs against lineage markers CD3 (M2AB: Exalpha), CD14 (M5E2: BD Biosciences), CD15 (M5E2: BD Biosciences), CD16 (J5511: Exalpha), CD19 (HIB19: BD Biosciences), and CD56 (NCAM16.2: BD Biosciences), and PE-Cy5.5-labeled anti-CD4 (Caltag Laboratories) to reach >99% purity. The CD11c⁺ DCs were cul-

tured in RPMI 1640 medium and supplemented with 2% human AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin G, and streptomycin for our culture. Cells were seeded in flat-bottom 96-well plates in the presence of 15 ng/ml of TSLP (R&D Systems) and/or R848 (0.01–10 μ g/ml; InvivoGen), peptidoglycan (PGN) (*Bacillus subtilis*; 2 μ g/ml; InvivoGen), and LPS (*Escherichia coli* 0111:B4; 1 μ g/ml; InvivoGen) at 5×10^4 cells in 200 μ l of medium per well for 30 min or 24 h.

Naive, and CRTH2⁺CD4⁺ memory T cell subset purification

CD4⁺ T cells were enriched using CD4⁺ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. Enriched CD4⁺ T cells were stained with FITC-labeled lineage cocktail (CD8, CD14, CD16, CD19, CD56, BDCA2, TCR γ/δ , and glycophorin A) or PE-Cy5.5-labeled anti-CD4, allophycocyanin-labeled anti CD45RA (BD Biosciences), or biotin-labeled anti-CRTH2 (Miltenyi Biotec) followed by allophycocyanin-labeled anti-streptavidin (BD Biosciences). Stained cells were sorted by fractions of CD4⁺CD45RA⁺ naive T cells and CD4⁺CRTH2⁺ Th2 memory cells (purity >99%).

Analyses of DCs

To analyze the expression of costimulatory molecules, the culture DCs were stained with FITC-labeled anti-CD40 (5C3: BD Biosciences), CD80 (BB-1: Ancell), or CD86 (2331: BD Biosciences) and then analyzed by a FACSCalibur (BD Biosciences). The production of cytokines in the culture supernatants after 24 h was determined by cytometric beads array (CBA; kits for IL-12p70 and TNF- α , IL-6, and IL-10 were purchased from BD Biosciences). To evaluate surface OX40L expression, CD11c⁺ DCs freshly isolated or after being cultured at different time points were stained with PE-labeled anti-OX40L mAb (Ancell) or with an isotype-matched control mAb. To evaluate intracellular phosphorylated stat5, the DCs were stained with Alexa 488-labeled anti-phospho-stat5 (BD Biosciences) after

production by CD4⁺ T cells was analyzed intracellularly by flow cytometry (*D*) and measured in supernatants by CBA (*E*). Percentages of the respective cytokine-producing T cells are indicated in dot plots. Similar results were observed in four independent experiments and the results of a representative experiment are shown (*A* and *D*). CBA data are means $\pm$ SEM of four independent experiments (*B*, *C*, and *E*).

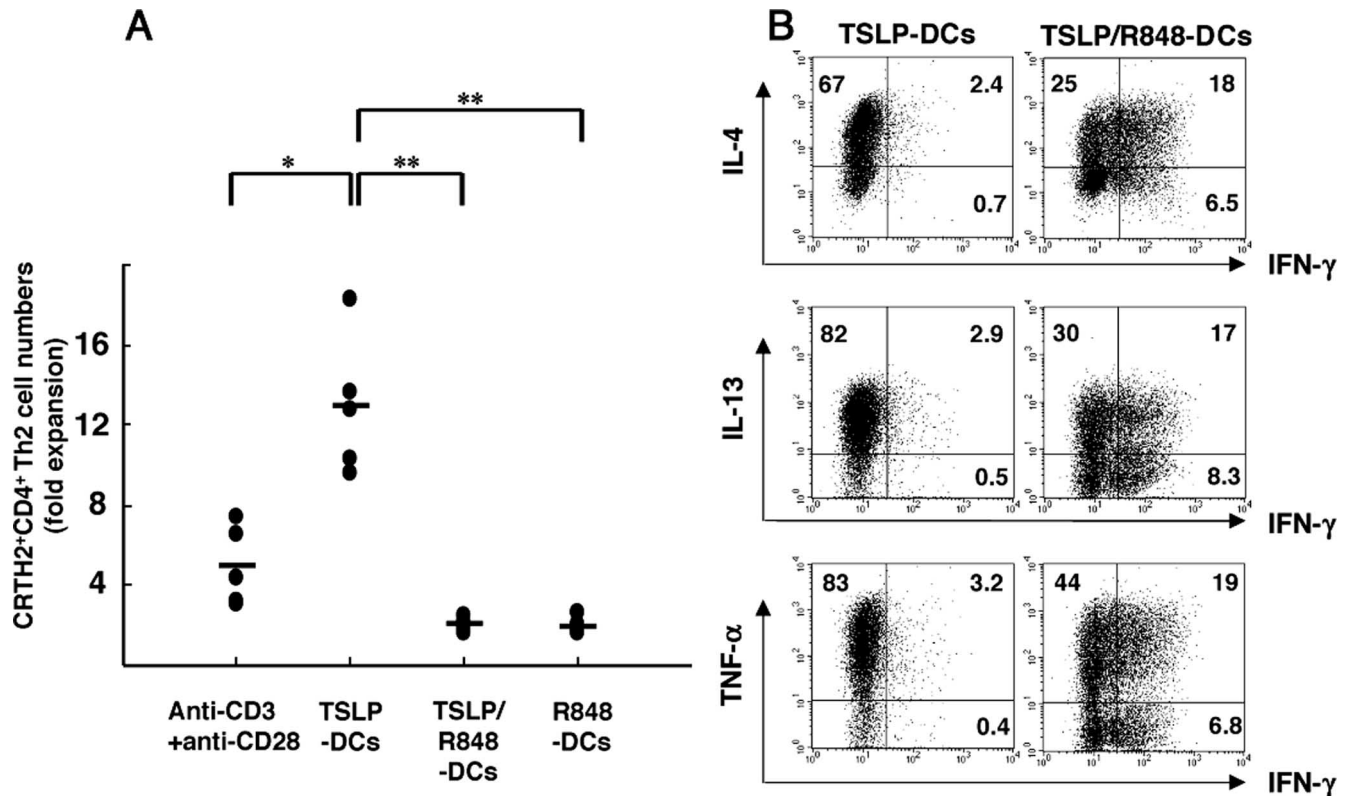


FIGURE 3. R848 inhibits maintenance of CRTH2⁺CD4⁺ Th2 memory cells induced by TSLP-DCs. **A**, CRTH2⁺CD4⁺ T cells were activated with immobilized anti-CD3 plus soluble anti-CD28, allogeneic TSLP-DCs, TSLP/R848-DCs, or R848-DCs. After a 7-day culture, viable T cells were counted with a trypan blue exclusion test. Horizontal bars indicate the median of five independent experiments. Statistical significance was determined using a paired Student's *t* test (*, *p* < 0.05 and **, *p* < 0.01). **B**, Cytokine production of the primed CRTH2⁺CD4⁺ T cells was analyzed intracellularly by flow cytometry. Percentages of the respective cytokine-producing T cells are indicated. Similar results were observed in four independent experiments, and the results of a representative experiment are shown.

fixing and permeabilization using a Cytofix/Cytoperm kit (BD Biosciences) and were then analyzed by a FACSCalibur.

DC-T cell coculture

After 24 h of culture under different conditions, CD11c⁺ DCs were collected and then cocultured with 2×10^4 freshly purified allogeneic naive CD4⁺ T cells (DC-to-T cell ratio, 1:4) and 1×10^4 freshly purified allogeneic CRTH2⁺CD4⁺ Th2 memory cells (DC-to-T cell ratio, 1:2) in 96-well round-bottom culture plates. We used the following reagents for culture conditions: anti-OX40L mAb (ik-5: 50 μg/ml), anti-IL-12 Ab (AF-219-NA, R&D Systems: 1 μg/ml), and recombinant soluble OX40L (200 ng/ml; R&D Systems). Mouse IgG2a and Goat IgG (R&D Systems) and PBS were used as controls.

Analyses of T cell cytokine production

After 7 days of DC-T cell coculture, the primed 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-13, TNF-α, and IFN-γ were measured by CBA (all kits from BD Biosciences), and IL-17 was measured by ELISA (R&D Systems). For intracellular cytokine production, the primed CD4⁺ T cells were restimulated with 50 ng/ml of PMA plus 2 μg/ml of ionomycin for 4 h. Brefeldin A (10 μg/ml) was added during the last 2 h. The cells were stained with the combination of PE-labeled mAbs to IL-4, IL-5, IL-13, or TNF-α, FITC-labeled anti-IFN-γ (all from BD Biosciences) using a Fix and Perm kit (Caltag Laboratories).

T cell expansion assay

We used freshly isolated CRTH2⁺CD4⁺ Th2 memory cells (purity >99%), and the 2×10^4 T cells were stimulated for 7 days with immobilized anti-CD3 (OKT3, 5 μg/ml) and soluble anti-CD28 (1 μg/ml) or with DCs precultured with TSLP and/or R848, 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.

T cell suppressive function assay

We used 1×10^4 freshly isolated CD4⁺ naive T cells, cultured T cells generated from naive T cells primed with TSLP-DCs (TSLP-T), and cultured CRTH2⁺CD4⁺ Th2 memory cells primed with TSLP-DCs (TSLP-T_M) as responders, and cultured T cells generated from autologous naive T cells primed with TSLP/R848-DCs (TSLP/R848-T) as suppressors. These cell types and their mixtures (at a suppressor-to-responder ratio of 1:2, or at different ratios) were then stimulated in round-bottom 96-well plates for 5 days by culturing with 5×10^3 irradiated allogeneic monocyte-derived DCs as stimulators, which were generated from isolated CD14⁺ monocytes by CD14 microbeads (Miltenyi Biotec) by 5 days of culturing with 200 ng/ml GM-CSF and 100 ng/ml IL-4 (R&D Systems). T cells and monocyte-derived DCs alone were used as controls. After 5 days, cells were pulsed with 1 μCi [³H]thymidine for 16 h before collecting, and cellular proliferation was assessed by [³H]thymidine incorporation. In some experiments, naive T cells prelabeled with CFSE (Molecular Probes) and TSLP/R848-T were separately cultured by semipermeable membrane in Transwells (96-well plates; Corning Costar), and a mixture of neutralizing anti-IL-10 Ab (200 ng/ml) plus IL-10 receptor (10 ng/ml) (R&D Systems) Abs was added.

Results

Treatment of DCs with R848 suppresses induction of TSLP-mediated inflammatory Th2 response

Because imidazoquinoline is a strong inducer of Th1 cells through DC activation (32), we investigated whether R848 can suppress the function of TSLP in the induction of the inflammatory Th2 response that triggers the immune cascade of allergy. Naive CD4⁺ T cells were cocultured for 7 days with allogeneic DCs pretreated

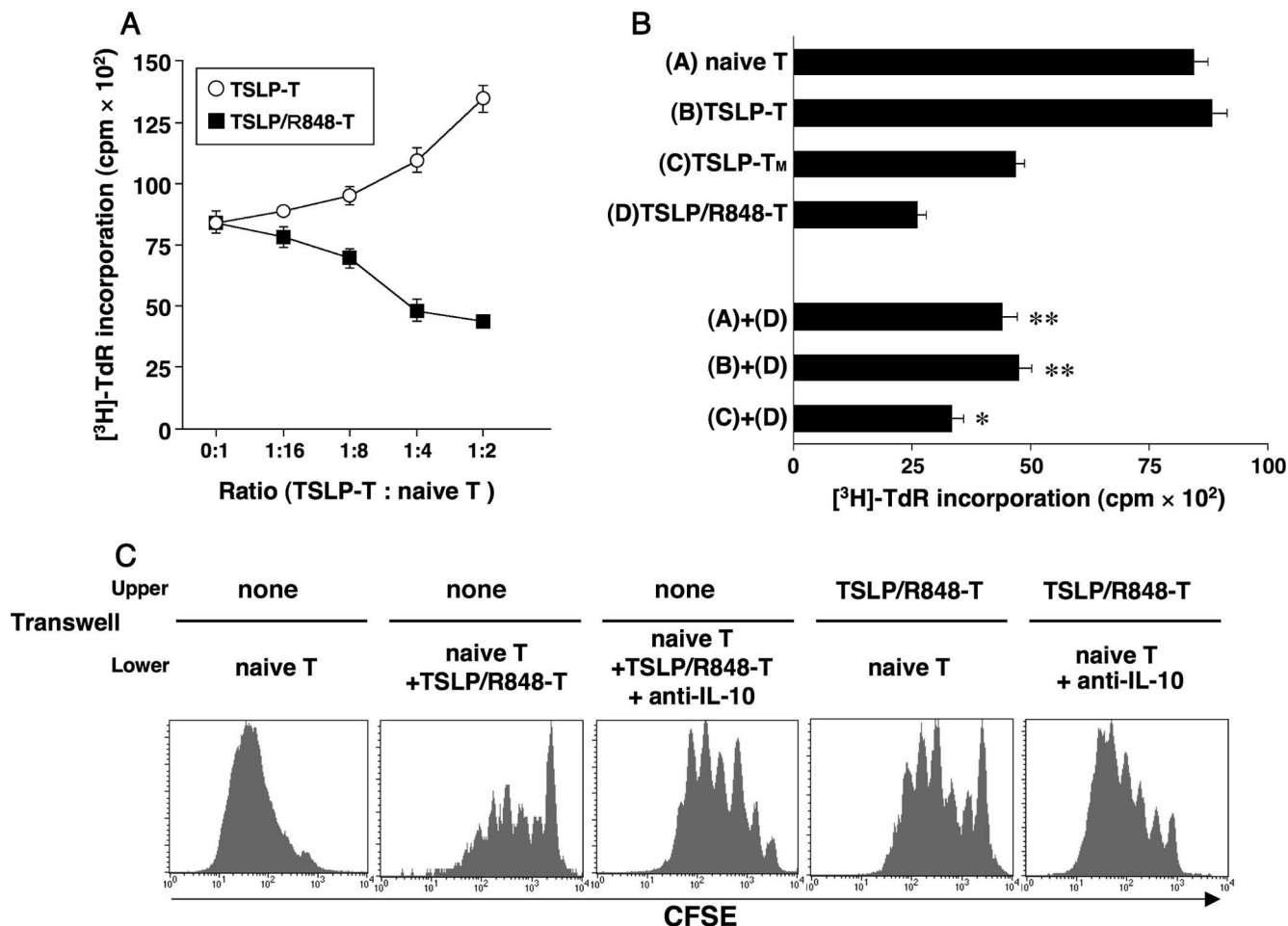


FIGURE 4. R848 treatment on TSLP-DCs equips T cells with regulatory function to suppress the Th2 cell proliferation. CD4⁺ naive T cells (T), inflammatory Th2 cells generated from naive CD4⁺ T cells by TSLP-DCs (TSLP-T), CCR2⁺CD4⁺ Th2 memory cells primed with TSLP-DCs (TSLP-T_M), and T cells generated from naive CD4⁺ T cells induced by TSLP/R848-DCs (TSLP/R848-T) were used for functional assay. Mixtures of T cell populations were cultured for 5 days with allogeneic monocyte-derived DCs as stimulator. **A**, Naive T cells were used as responders and TSLP-T or TSLP/R848-T were used as suppressors. Ratios of suppressor-to-naive T cells are shown. Proliferation was assessed by [³H]thymidine incorporation, and error bars represent the SEM of triplicate wells. **B**, CD4⁺ naive T cells (T), TSLP-T, and TSLP-T_M were used as responder, and TSLP/R848-T were used as suppressors. The ratio of responder-to-suppressor was 2:1. Proliferation was assessed by [³H]thymidine incorporation, and error bars represent the SEM of triplicate wells. Statistical significance was determined using paired Student's *t* test (*, *p* < 0.005 and **, *p* < 0.001). **C**, CD4⁺ naive T cells were used as responders and TSLP/R848-T were used as suppressors. CD4⁺ naive T cells were labeled with CFSE before culture, and cells after culturing were analyzed by flow cytometry, gated on CD4⁺CFSE⁺ cells. In some T cell cultures, responders and suppressors were separated into lower and upper wells by semipermeable membrane of the Transwells or cultured in the same well, and neutralizing anti-IL-10 plus anti-IL-10 receptor Abs were added. The ratio of responders-to-suppressors was 2:1. Similar results were observed in three independent experiments, and the results of a representative experiment are shown.

with TSLP alone (TSLP-DCs) or TSLP plus R848 (TSLP/R848-DCs), and the cytokine production by the primed CD4⁺ T cells was then examined. TSLP-DCs induced naive CD4⁺ T cells to differentiate into the T cells producing IL-4, IL-5, IL-13, and TNF- α , but less IFN- γ (Fig. 1A). This cytokine profile is consistent with an inflammatory Th2 cell response, as previously described (10). TSLP/R848-DCs were incapable of inducing these inflammatory Th2 cells. Confirming the cytokine production at the single-cell level, we found with the total cytokine production of the primed T cells following restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by CBA analysis that CD4⁺ T cells primed by TSLP-DCs produced large amounts of IL-4, IL-5, IL-13, and TNF- α but low amounts of IL-10 and IFN- γ , whereas the T cells primed by TSLP/R848-DCs produced significantly low levels of IL-4, IL-5, IL-13, and TNF- α and concomitantly increased levels of IL-10 and IFN- γ (Fig. 1B). This ability to counteract the TSLP-DC-mediated inflammatory Th2 cell response was in a R848 dose-

dependent manner with maximal effect at 1 μ g/ml R848 for pretreatment (Fig. 1C).

Human myeloid DCs express TLR2 and TLR4 as well as TLR8 and then can respond to PGN from Gram-positive bacteria and LPS from Gram-negative bacteria through TLR2 and TLR4, respectively, to produce IL-12 (30, 35). Therefore, to assess whether R848 is unique in the inhibition of the inflammatory Th2 cell response, we used PGN or LPS instead of R848 to pretreat the DCs. We found that pretreatment with PGN and LPS as well as R848 all inhibited the generation of Th2 cells producing IL-13 and promoted that of Th1 cells producing IFN- γ in intracellular cytokine findings (Fig. 1D). These responses were confirmed by CBA analysis showing that both TSLP/PGN-DCs and TSLP/LPS-DCs had the ability to counteract the inflammatory Th2 cell response, but to a lesser extent than TSLP/R848-DCs (Fig. 1E). Inflammatory cytokine IL-17, which is involved in the development of autoimmune diseases

(36), was undetectable in any supernatants of the primed T cells (data not shown). Although common TLR stimuli could suppress the TSLP-mediated inflammatory Th2 cell response, R848 is more potent than the TLR2 ligand or TLR4 ligand.

Subsequent addition of R848 into coculture of T cells and TSLP-DCs suppresses induction of inflammatory Th2 response

To assess whether R848 also has the ability to counteract the function of DCs that have already been activated by TSLP, DCs were pretreated with TSLP first and R848 was subsequently added into the coculture of naive CD4⁺ T cells and TSLP-DCs. The addition of R848 into the DC-T cell coculture also led to a significantly decreased production of IL-4, IL-5, IL-13, and TNF- α , but a concomitantly increased production of IL-10 and IFN- γ by T cells (Fig. 2). Thus, as was the case in DC pretreatment with R848, R848 functioned at the level of the interaction between DCs and T cells as an inhibitor of the inflammatory Th2 response.

R848 inhibits maintenance of CRTH2⁺CD4⁺ Th2 memory cells induced by TSLP-DCs

CRTH2⁺CD4⁺ Th2 memory cells play a critical role in the maintenance of Th2-mediated atopic dermatitis (20–22). Considering the therapeutic use of imidazoquinoline for atopic dermatitis, its effect on already activated Th2 memory cells needs to be clarified. Therefore, we next investigated whether R848 has the ability to inhibit the expansion of CRTH2⁺CD4⁺ Th2 memory cells and to convert the Th2 phenotype of CRTH2 cells maintained by TSLP-DCs. CRTH2⁺CD4⁺ Th2 memory cells were cocultured for 7 days with allogeneic TSLP-DCs, TSLP/R848-DCs, or R848-DCs, and cell expansion and cytokine production of the primed CRTH2⁺CD4⁺ Th2 memory cells were analyzed. We found that TSLP-DCs induced robust expansion of CRTH2⁺CD4⁺ Th2 memory cells, with a 12-fold increase in the total number of T cells compared with polyclonal stimulation with anti-CD3 and anti-CD28 Abs, whereas TSLP/R848-DCs or R848-DCs failed their expansions (Fig. 3A). Furthermore, we found with the single-cell cytokine staining that TSLP-DCs could maintain CRTH2⁺CD4⁺ Th2 memory cells to produce Th2 cytokines, IL-4, IL-13, and TNF- α but not IFN- γ , whereas TSLP/R848-DCs dramatically decreased the frequency with which Th2 cells produced IL-4, IL-13, and TNF- α but concomitantly induced >20% of IFN- γ -producing T cells from CRTH2⁺CD4⁺ Th2 memory cells (Fig. 3B). These findings suggest that R848 could suppress the Th2 functional attributes of CRTH2⁺CD4⁺ Th2 memory cells that are expanded and maintained by TSLP-DCs.

R848 treatment of TSLP-DCs redirects functional regulatory Th1 cells to suppress the Th2 cell responses

The next question was whether IL-10⁺IFN- γ ⁺ T cells induced by R848 treatment to TSLP-DCs have an immunosuppressive function, because IL-10⁺IFN- γ ⁺CD4⁺ type 1 regulatory T (Tr1) cells were originally isolated from patients with severe combined immunodeficiency who had undergone successful HLA-mismatched bone marrow transplantation (37, 38), and also IL-10⁺IFN- γ ⁺ Th cells generated from naive CD4⁺ T cells in humans (39, 40) and mice (41, 42) have a regulatory function. We here found that, in contrast to T cells primed by TSLP-DCs, which did not suppress the proliferation of naive T cells in response to allogeneic stimulation, T cells primed by TSLP/R848-DCs inhibited the bystander naive T cell proliferation in a dose-dependent manner (Fig. 4A). We next examined the ability of the T cells primed by TSLP/R848-DCs to exhibit the suppressive function against the induced inflammatory Th2 cells or CRTH2⁺CD4⁺ Th2 memory cells primed

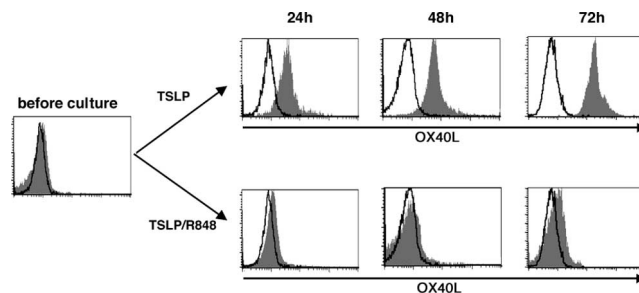


FIGURE 5. R848 inhibits up-regulation of OX40L on DCs induced by TSLP. OX40L expression on TSLP-DCs and TSLP/R848-DCs was analyzed by flow cytometry at different time points. The staining profiles of anti-OX40L mAb and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in four independent experiments, and the results of a representative experiment are shown.

by TSLP-DCs. Inflammatory Th2 cells induced by TSLP-DCs underwent strong proliferation and, to a lesser extent, the expanded CRTH2⁺CD4⁺ Th2 memory cells primed by TSLP-DCs did in response to allogeneic stimulation, whereas T cells primed by TSLP/R848-DCs were relatively anergic (Fig. 4B). Notably, the T cells induced by TSLP/R848-DCs had the ability to significantly suppress bystander proliferation of both inflammatory Th2 cells and CRTH2⁺CD4⁺ Th2 memory cells (Fig. 4B). To further examine whether T cell-derived IL-10 is involved in the immunosuppression, we used the Transwell system and anti-IL-10 Abs to block soluble IL-10 function. Anti-IL-10 Abs partially inhibited the suppressive function of T cells primed by TSLP/R848-DCs, as indicated by CFSE-labeling experiments (Fig. 4C). Furthermore, this T cell-mediated suppression was retained when T cells primed by TSLP/R848-DCs were separated from naive T cells by the semipermeable membrane of the Transwell, and almost completely blocked by the addition of anti-IL-10 with Transwell (Fig. 4C),

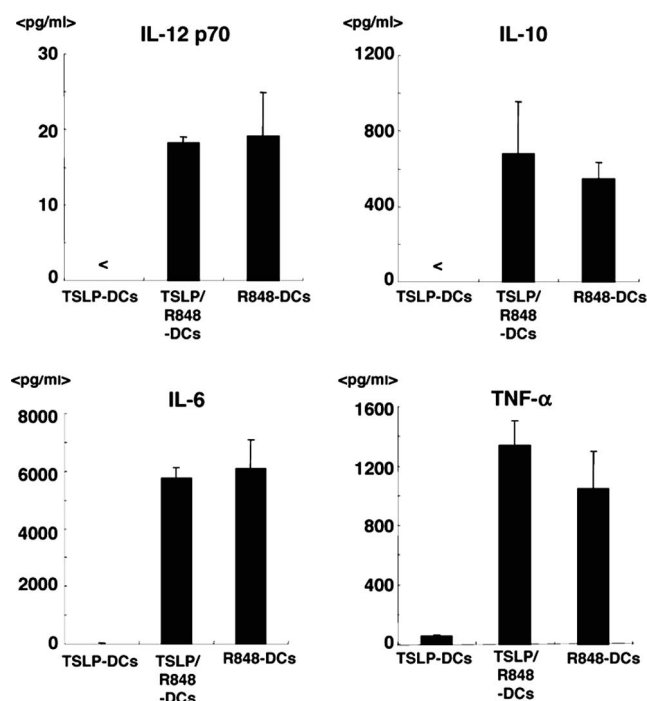


FIGURE 6. R848 activates DCs to produce IL-12 in the presence of TSLP. DCs were cultured with the indicated stimuli for 24 h. Culture supernatants were collected and the cytokine levels were analyzed by CBA. CBA data are means $\pm$ SEM of four independent experiments.

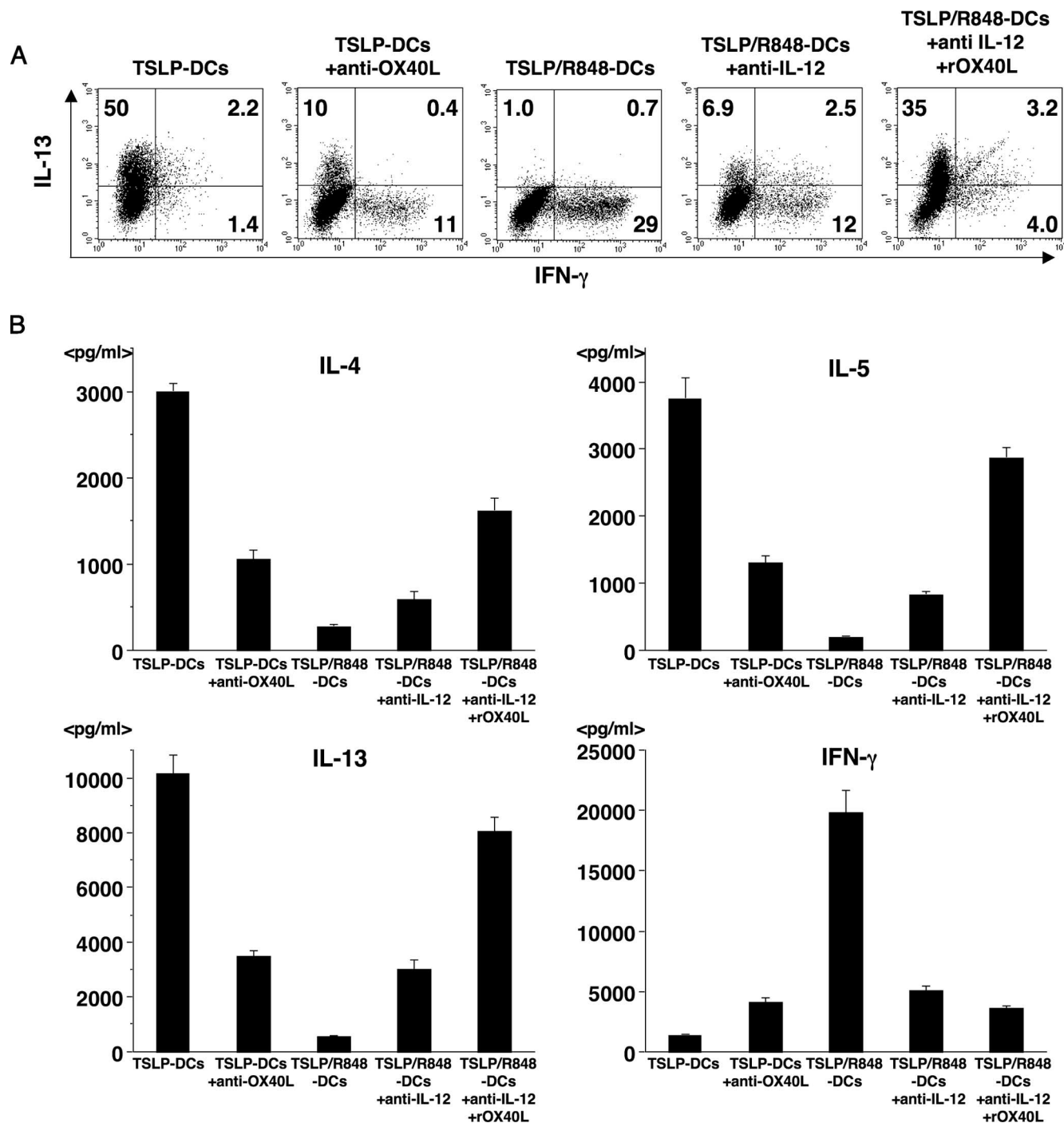


FIGURE 7. Ability of R848 to counteract Th2 responses depends on both IL-12 induction and OX40L repression. DCs were pretreated with TSLP alone or TSLP plus R848 and were then cultured for 7 days with naive CD4⁺ T cells. Anti-OX40L mAb, anti-IL-12 Ab, and/or soluble recombinant OX40L were added to the DC-T cell coculture. A, Cytokine production by CD4⁺ T cells was analyzed intracellularly by flow cytometry. Percentages of the respective cytokine-producing T cells are indicated. Similar results were observed in five independent experiments, and the results of a representative experiment are shown. B, Cytokine production was measured in supernatants after restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by CBA. CBA data are means $\pm$ SEM of four independent experiments.

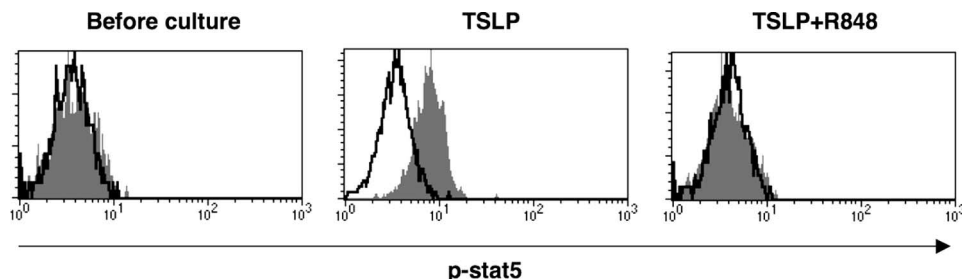
confirming that IL-10 secreted by T cells is involved in their suppressive function. These findings suggest that R848 redirects the TSLP-DC function from inducing inflammatory Th2 cells to functional IL-10-producing regulatory Th1 cells that have the ability to inhibit the bystander Th2 responses mediated by TSLP-DCs.

R848 inhibits up-regulation of OX40L on DCs induced by TSLP

TSLP has recently been reported to preferentially induce OX40L on DCs (19), which is responsible for the induction of inflamma-

tory Th2 responses. Additionally, the inflammatory Th2 cell induction is required for not only OX40L but also a default mechanism of absent IL-12 (19). Therefore, to determine the molecular mechanism by which R848 treatment of TSLP-DCs counteracts the induction of the inflammatory Th2 response, we first investigated whether R848 affects OX40L expression on DCs activated by TSLP. TSLP definitely induced OX40L expression on DCs, and the expression level was increased until 72 h, as was previously reported (19). We found not only that R848 alone did not induce

FIGURE 8. R848 inhibits phosphorylation of stat5 in DCs stimulated by TSLP. Staining with anti-p-stat5 mAb (shaded) and isotype-matched control (solid line) for freshly isolated DCs or DCs stimulated with the indicated stimuli for 30 min. Similar results were observed in two independent experiments, and the results of a representative experiment are shown.



DCs to express OX40L (data not shown) but also that the addition of R848 suppressed the TSLP-mediated OX40L up-regulation during a 72-h maturation process (Fig. 5). These findings suggest that R848 acts as a potent inhibitor of OX40L expression on DCs.

R848 activates DCs to produce IL-12 in the presence of TSLP

Because imidazoquinoline R848 has been shown to stimulate human myeloid DCs for IL-12 production (31, 32), we next investigated the ability of R848 to stimulate DCs even cultured with TSLP to secrete IL-12. TSLP showed a poor ability to induce DCs to secrete IL-12p70, other proinflammatory cytokines IL-6 and TNF- α , and antiinflammatory cytokine IL-10 (Fig. 6). Nevertheless, TSLP strongly activated DCs to express surface CD40, CD80, and CD86 (data not shown), as previously shown (10). However, we found that R848 stimulated DCs to produce large amounts of IL-12p70, IL-6, IL-10, and TNF- α even in the presence of TSLP (Fig. 6).

Ability of R848 to alter the DC-mediated inflammatory Th2 responses depends on both IL-12 induction and OX40L repression

The fact that R848 is a strong inducer of Th1-inducing cytokine IL-12 from DCs (32) (Fig. 6) and the finding that R848 suppressed the up-regulation of Th2-inducing molecule OX40L on TSLP-DCs (Fig. 5) suggest that R848 breaks the permissive condition for inducing the inflammatory Th2 response. To confirm the involvements of IL-12 and OX40L in the function of TSLP/R848-DCs that inhibit the induction of the inflammatory Th2 response, first we added anti-OX40L mAb to block the OX40L function on TSLP-DCs and then compared the response with that induced by TSLP/R848-DCs. The addition of anti-OX40L mAb into the coculture of T cells and TSLP-DCs resulted in reduced numbers of Th2 cells producing IL-13 and modestly increased numbers of IFN- γ -producing Th1 cells (Fig. 7A), as previously described (19), but to a lesser extent than TSLP/R848-DCs, which induced a striking IFN- γ -producing Th1 cell response. These were confirmed by CBA analysis (Fig. 7B). Next, we blocked the IL-12 effect from TSLP/R848-DCs by adding neutralizing anti-IL-12 Ab to the DC-T cell coculture and added OX40L stimulation to T cells using soluble recombinant OX40L (rOX40L) to investigate the restoration of the Th2 response. The addition of anti-IL-12 Ab alone inhibited the generation of IFN- γ -producing Th1 cells but slightly promoted the generation of Th2 cells producing IL-4, IL-5, and IL-13 (Fig. 7), confirming that IL-12 production from DCs is not sufficient to inhibit the generation of Th2 cells. The addition of rOX40L alone into the DC-T cell coculture did not induce Th2 cells producing IL-4, IL-5, or IL-13 (data not shown). When we added both rOX40L and anti-IL-12 Ab in the coculture, we found that the generation of Th2 cells was restored and the generation of IFN- γ -producing Th1 cells was almost completely inhibited (Fig. 7). These results suggest that the ability of R848 to counteract Th2

cell differentiation induced by TSLP-DCs depends on both IL-12 production and the repression of OX40L on DCs.

R848 inhibits TSLP-induced stat5 activation in DCs

TSLP is known to induce stat5 activation (34, 43, 44). Therefore, to investigate whether R848 can alter the signaling pathway triggered by TSLP, we measured phosphorylated stat5 (p-stat5) in DCs in response to TSLP and/or R848. p-stat5 was induced by TSLP at 30 min of culture. This rapid increase was completely blocked by simultaneous addition of R848 to DCs (Fig. 8). Thus, R848 alters the TSLP-mediated signaling pathway of human DCs.

Discussion

The use of drugs to treat allergies has historically been focused on the effector phase of the classical immune cascade of the allergy such as T cells, mast cells, and eosinophils (5, 6, 10). These drugs include corticosteroids, chemical mediator antagonists, anti-IgE Abs (45), and soluble IL-4 receptor α -chains (46), which basically target the effector cells or factors. Meanwhile, it has long remained an enigma how dysregulated Th2 responses are initialized in the upstream phase of allergy, although there is an increasing number of studies that imply that DCs play a critical role in the triggering phase of allergy (47–49). Recently, Liu et al. demonstrated that the interaction between epithelial cell-derived TSLP and human myeloid DCs triggers the immunological cascade that produces allergic immune responses through inflammatory Th2 cells (10, 50) and identified a specialized molecular pathway that links TSLP-DCs to the generation and maintenance of the inflammatory Th2 response through OX40L (19, 20). OX40L, this TNF superfamily on APCs, has been implicated in the development of allergic diseases and inflammatory disorders in both human studies (51, 52) and mouse disease models (53–57). Recently, it has been shown that OX40L is a critical trigger of the inflammatory Th2 response (19, 58, 59) and is responsible for Th2 memory cell expansion (20, 60). Additionally, OX40L is a potent inhibitor of IL-10 production in CD4⁺ T cell polarization, leading to an inflammatory response (61). Therefore, the effector molecule OX40L, as the interface between TSLP-DCs and inflammatory Th2 cells, has emerged as a novel target for the treatment of allergic diseases.

In this context, we herein show that R848 acts as an inhibitor of OX40L expression on DCs and consequently has the ability to counteract the TSLP-DC-mediated function of inducing inflammatory Th2 cells and of maintaining Th2 memory cell response. Moreover, R848 endowed Th cells with an IL-10-mediated regulatory function to suppress the bystander inflammatory Th2 responses. Notably, on the basis of our results, R848 appears to exhibit its function not only in the priming phase of DCs by TSLP but also in the later phase of priming T cells by TSLP-DCs. In addition to these specialized roles of R848 for DCs in allergic responses, there may be a great advantage; that is, because imidazoquinoline has already been used to topically treat viral-mediated

skin diseases by cream formulation (62–65), this imidazoquinoline drug could be used without systemic side effects when R848 is clinically applied for the treatment of atopic dermatitis.

The mechanism by which the functional alteration of TSLP-DCs is achieved by R848 was shown to involve mainly two molecular events: down-regulation of OX40L expression and IL-12 induction. The ability of OX40L to induce the Th2 cell response requires a microenvironment lacking IL-12 (19). This finding, together with our result showing that simultaneous addition of rOX40L plus anti-IL-12 Abs into the coculture of T cells and TSLP/R848-DCs restored the Th2 cell response, suggests that R848 acts as both OX40L inhibitor and IL-12 inducer to remove the Th2-permissive microenvironment created by TSLP-DCs. Furthermore, R848 stimulated DCs even in the presence of TSLP to directly produce considerable amounts of IL-10. As IL-10 is recognized to be an antiinflammatory cytokine that counteracts inflammation (66–69), the increase in T cell-derived IL-10 and DC-derived IL-10 production by R848 may possibly contribute to the alleviation or prevention of allergic inflammation.

Although the signaling pathway through which R848 exerts its functional alteration of TSLP-DCs is currently unknown, our finding that the TLR2 ligand and TLR4 ligand displayed a similar inhibitory function for the TSLP-mediated inflammatory Th2 cell response indicates that common signaling through the TLR adaptor protein MyD88 is involved in this function. However, R848 showed a more potent effect in converting the function of TSLP-DCs than did other TLR ligands. This might be associated with the fact that, of the TLR ligands, R848 is the most potent stimulator of human myeloid DCs to produce IL-12 (35). Furthermore, TSLP is known to activate stat5 in myeloid DCs (34). Our present study has identified that R848 inhibits the TSLP-induced phosphorylation of stat5 in DCs. Although it is still unclear how stat5 activation is linked with the specific features of both OX40L induction and defect of IL-12 in TSLP-DCs, R848 appears to at least alter intracellular signaling through TSLP. What the downstream molecules of stat5 in this pathway are and how signaling through TLRs intersect with the TSLP-stat5 pathway need to be studied in the future.

In conclusion, our study may provide insights into the possible treatment of allergic diseases such as atopic dermatitis that stem from TSLP. The imidazoquinoline derivative R848 works as an inhibitor of the DC-mediated inflammatory Th2 response regardless of TSLP function to induce allergic inflammation. On the basis of the new concept of targeting the triggering phase rather than the effector phase of the inflammatory allergic cascade, our results suggest that imidazoquinoline could, as a potent immune adjuvant, be used as a new drug for the treatment of atopic dermatitis.

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Disclosures

The authors have no financial conflicts of interest.

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