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Cutting Edge: Identification of the Thymic Stromal Lymphopoietin –Responsive Dendritic Cell Subset Critical for Initiation of Type 2 Contact Hypersensitivity

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J Immunol 2013; 191:4903-4907; Prepublished online 11 October 2013;

doi: 10.4049/jimmunol.1302175

<http://www.jimmunol.org/content/191/10/4903>

Supplementary Material

<http://www.jimmunol.org/content/suppl/2013/10/11/jimmunol.1302175.DC1.html>

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Cutting Edge: Identification of the Thymic Stromal Lymphopoietin–Responsive Dendritic Cell Subset Critical for Initiation of Type 2 Contact Hypersensitivity

Masayuki Kitajima and Steven F. Ziegler

The cytokine thymic stromal lymphopoietin (TSLP) has been implicated in the initiation and progression of allergic inflammation through its ability to activate dendritic cells (DCs). However, the identity of the DC subset that responds to TSLP is not known. In this study we use a CCL17 reporter strain to identify the TSLP-responsive DC subset. In vitro, TSLP induced CD11b^{high} DCs to express CCL17, to increase CCR7-mediated migration activity, and to drive Th2 differentiation of naive CD4 T cells. In vivo, following skin sensitization, we found that a subset of Ag-bearing CCL17⁺CD11b^{high} migratory DCs, but not Ag-bearing CCL17[−] migratory DCs, in skin lymph nodes were capable of driving Th2 differentiation and were dramatically reduced in TSLPR-deficient mice. Taken together, these results demonstrate that TSLP activated a subset of CD11b⁺ DCs in the skin to produce CCL17, upregulate CCR7, and migrate to the draining lymph node to initiate Th2 differentiation. *The Journal of Immunology*, 2013, 191: 4903–4907.

Thymic stromal lymphopoietin (TSLP) is a type I cytokine, most closely related to IL-7, that is expressed primarily by epithelial cells at barrier surfaces (1). Importantly, TSLP expression was markedly elevated in lesional skin atopic dermatitis (AD) patients and in the lungs of asthmatics (2, 3). Consistent with these observations, mice overexpressing TSLP or treated with TSLP developed Th2-type allergic inflammation (4, 5). These data demonstrate that TSLP is an initiation factor for allergic inflammation.

TSLP activated human DCs to induce costimulatory molecules (2), the Th2-associated chemokines CCL17 and CCL22, and drive Th2 cell differentiation in vitro (6). Consistent with these data, elevated CCL17 has been detected in AD patients, and CCL17 is associated with CCR7- and CXCR4-dependent migration of cutaneous DCs and induction of contact hypersensitivity (CHS) in mice (7–9). We showed that TSLP induced

CCL17 expression in bone marrow–derived FLT3 ligand (FLT3L)–induced DCs (FL-DCs) in a STAT5-dependent manner (5, 10), and STAT5 activation by TSLP in DCs is important for initiation of the Th2 response in mice (10).

Human AD shares many features with CHS (11). The type of CHS response is dependent on the dose and type of allergen and adjuvant, and both Langerhans cells and dermal DCs (DDCs) can act as APCs in many CHS responses (12). The hapten FITC combined with dibutyl phthalate exhibits increased ear swelling and serum IgE as Stat6-dependent Th2-dominant CHS in mice (13). We previously showed that TSLPR and STAT5 in DDCs are required for the development of Th2-type CHS (10, 14). In this study, we used CCL17-enhanced GFP (eGFP) reporter mice to detect TSLP-responding DCs. In vitro, TSLP induced CCL17-eGFP⁺CD11b^{high}CD24^{low} DCs with increased CCR7-mediated migration. A similar population appeared in draining lymph nodes (dLNs), in a TSLP-dependent manner, 24 h after sensitization. Both in vitro and in vivo, CCL17-eGFP⁺ DCs could promote Th2 differentiation and CD4 T cell proliferation. Taken as a whole, these data demonstrate that TSLP activated a subset of DDCs to migrate to dLNs and attract CD4 T cells for differentiation into Th2 cells.

Materials and Methods

Animals

Female BALB/c and CD45.1 congenic mice on a BALB/c background were purchased from Taconic Farms and The Jackson Laboratory. Tslpr-deficient (TSLPR^{−/−}) mice were backcrossed to BALB/c for 12 generations. OVA-specific TCRαβ (DO11.10) transgenic (Tg) mice were purchased from The Jackson Laboratory (15). CCL17-eGFP Tg mice on a BALB/c background were from Dr. Irmgard Förster (7). All mice for this study were maintained under pathogen-free conditions. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines at the Benaroya Research Institute.

Flow cytometry analysis

Cell preparations and flow cytometric analysis were described previously (10). The Abs were used anti-CD11c, anti-CD11b, anti-MHC class II (MHC II), anti-CD24, anti-B220, anti-CD86, anti-OX40L, anti-CD274, anti-CCR7, anti-

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Received for publication August 23, 2013. Accepted for publication September 16, 2013.

This work was supported by National Institutes of Health Grants R01 AI068731, R01 AR055695, R01 AR056113, and U19 AI071130 and by the Food Allergy Initiative (to S.F.Z.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AD, atopic dermatitis; CHS, contact hypersensitivity; DC, dendritic cell; DDC, dermal DC; dLN, draining lymph node; eGFP, enhanced GFP; FL-DC, FLT3 ligand–induced DC; FLT3L, FLT3 ligand; LC, Langerhans cell; LN, lymph node; mDC, migratory DC; MHC II, MHC class II; pDC, plasmacytoid DC; Tg, transgenic; TRITC, tetramethylrhodamine isothiocyanate; TSLP, thymic stromal lymphopoietin.

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CD4, anti-CD45.1, anti-CD207, anti-IL-4, anti-IFN- γ , anti-phosphorylated STAT5 mAb, and streptavidin (eBioscience, BioLegend, or BD Biosciences).

Bone marrow DC cultures

A modified method was used for bone marrow DC cultures as previous described (5). Cells were purified using a MACS CD11c purification kit (Miltenyi Biotec) or BD FACSAria II cell sorter. Cells were stimulated with medium alone or medium plus indicated dose of TSLP (gift from Amgen), 10 ng/ml IL-7, or 10 ng/ml GM-CSF (eBioscience) for 24 h.

In vitro migration assay

In vitro migration assays were performed using 5- μ m pore size Transwell plates (Costar) and CCL21 (PeproTech). The assay was performed for 4 h, and the numbers of DCs and CCL17-eGFP expression that migrated to the lower well were determined using microsphere beads (Dynospheres) by BD FACSCalibur (BD Biosciences). The numbers were divided by the input samples and are represented as percentage migration.

In vitro proliferation assay and CD4 cell differentiation cultures

A modified method was used for bone marrow DC cultures as previous described (10). Supernatants were collected and ELISA was performed as described (10).

In vivo CD4 differentiation by FL-DC transfer

Sorted DCs were pulsed with OVA₃₂₃₋₃₃₉ at 1 μ M for 1 h. After washing, DCs (3×10^4 cells/mouse) were transferred i.v. Splenic CD4⁺ T cells (2×10^6 cells/mouse) from DO11.10 Tg mice were transferred i.v. 1 d before DC transfer. Five days after DC transfer, cells from skin LN were isolated. For intracellular cytokine staining, cells were stimulated with PMA and ionomycin for 4 h in the presence of monensin. For measuring cytokine production, skin LN cells (4×10^6 cells/ml) were stimulated with OVA₃₂₃₋₃₃₉ at 1 μ M for 3 d. Supernatants were collected and ELISA was performed as described (10).

Tetramethylrhodamine isothiocyanate sensitization and isolation of DCs from ear and skin-draining LNs

Hapten tetramethylrhodamine isothiocyanate (TRITC) sensitization and isolation of DCs from skin and skin LNs were performed as previously described (10, 14). Cells were purified using a MACS CD11c purification kit or a BD FACSAria II cell sorter.

Statistical analysis

The significance between two groups was determined by a two-tailed Student *t* test. A *p* value <0.05 was considered statistically significant.

Results and Discussion

TSLP induced CCL17-eGFP⁺ CD11b^{high} DCs in bone marrow-derived FL-DCs

FLT3L induces differentiation of mouse bone marrow cells into heterogeneous DC subtypes (FL-CD11b DCs, CD11c⁺B220[−]CD11b^{high}; FL-CD24 DCs, CD11c⁺B220[−]CD24^{high}; and FL-plasmacytoid DCs [pDCs], CD11c⁺B220⁺CD11b[−]), which showed equivalent properties to splenic CD8[−] DCs, CD8⁺ DCs, and pDCs (16). We have shown that TSLP induced phosphorylation of STAT5 and upregulation of costimulatory molecules in FL-CD11b DCs (5, 10). To identify the subset capable of expressing CCL17 following TSLP exposure, we generated FL-DCs from CCL17-eGFP bone marrow and cultured the cells in TSLP. TSLP induced CCL17-eGFP expression in CD11b^{high} DCs in a dose-dependent manner, whereas the FL-CD24 DC and FL-pDC subset failed to express CCL17 (Fig. 1A and data not shown). Consistent with these data, TSLPR-deficient FL-CD11b DCs did not express CCL17 following TSLP treatment, whereas IL-7- and GM-CSF-mediated CCL17-eGFP expression was equivalent between TSLPR-sufficient and -deficient CCL17-eGFP Tg CD11b DCs (Fig. 1B and data not shown).

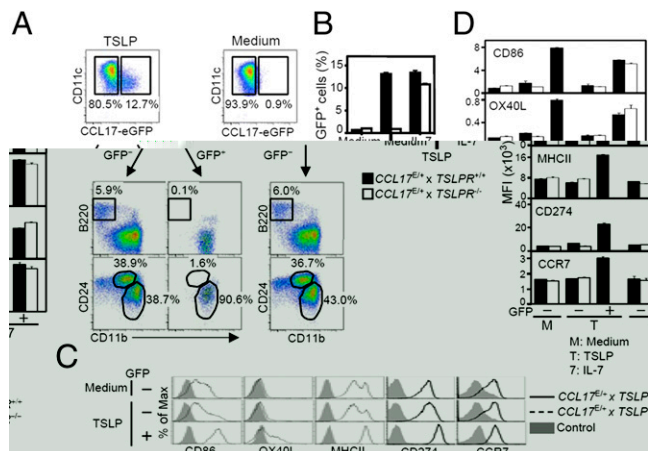


FIGURE 1. TSLP induced activated CCL17-eGFP⁺ FL-CD11b DCs. Bone marrow cells from *CCL17^{+/+} × TSLPR^{+/+}* and *CCL17^{+/+} × TSLPR^{−/−}* mice were differentiated by FLT3L. (A and B) *CCL17^{+/+}* FL-DCs were cultured with or without TSLP for 24 h. Expression of CCL17-eGFP and DC subset makers on CD11c⁺ FL-DCs were analyzed. (C and D) Sorted *CCL17^{+/+} × TSLPR^{+/+}* and *CCL17^{+/+} × TSLPR^{−/−}* FL-CD11b^{high} DCs were cultured with or without TSLP or with IL-7. Mean fluorescence intensities (MFIs) were determined by flow cytometry. Three independent experiments were performed with similar results.

Next, we examined expression of surface molecules on TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs. CCL17-eGFP⁺ CD11b DCs had high-level expression of the costimulatory molecules CD86, OX40L, CD274, and MHC II and chemokine receptor CCR7 compared with medium- and TSLP-treated CCL17-eGFP[−] FL-CD11b DCs (Fig. 1C, 1D). Additionally, TSLP-treated TSLPR-deficient DCs were equal to medium-treated DCs. Thus, these results indicate that TSLP-induced CD11b⁺ CCL17-eGFP⁺ DCs represent the DC subset activated by TSLP.

TSLP-induced CCL17-eGFP⁺ DCs increased migration activity and induction of Th2 differentiation

CCR7 is important for the migration of skin DCs into skin-draining LNs under both steady-state and inflammatory conditions (17). Also, CCL17 has also been found to be important for cutaneous DC migration to skin LNs (9). Interestingly, we found that CCR7 expression was elevated on TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs compared with control CCL17-eGFP[−] FL-CD11b DCs (Fig. 1). Using a Transwell migration assay we found that TSLP-treated FL-CD11b DCs had enhanced CCL21-mediated migration compared with untreated FL-CD11b DCs (Fig. 2A). TSLP-treated TSLPR-deficient FL-CD11b DCs failed to show increased migration, whereas migration was equivalent between GM-CSF-treated FL-CD11b DCs from TSLPR-sufficient and -deficient FL-CD11b DCs (Fig. 2A). Consistent with this activity, the proportion of migrated CCL17⁺ DCs was increased in TSLP-induced FL-DCs (Fig. 2B).

Consistently, migration by CCL21 of CCL17-eGFP⁺ FL-CD11b DCs from TSLP-treated cultures was increased compared with CCL17-eGFP[−] FL-CD11b DCs from the same cultures. The CCL17[−] cells showed equivalent migration as did untreated CCL17-eGFP[−] CD11b DCs (Fig. 2C, Supplemental Fig. 1A). Thus, these results indicate that TSLP-induced CCL17⁺ FL-CD11b DCs display increased CCR7-mediated migration activity compared with their CCL17[−] counterparts.

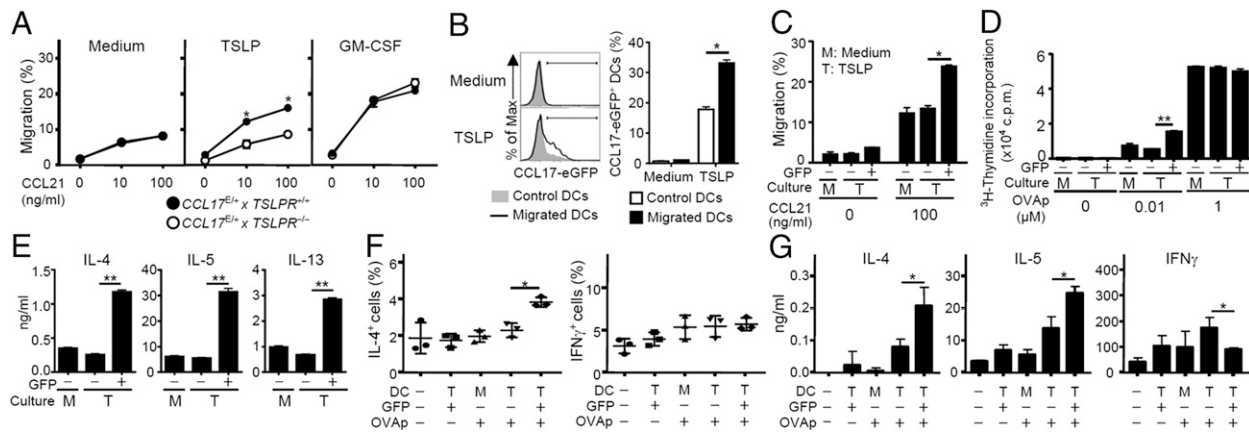


FIGURE 2. TSLP induced functional CCL17-eGFP⁺ FL-CD11b DCs for Th2 response. Sorted FL-CD11b DCs were cultured with or without TSLP or with GM-CSF. Then, cells were cultured in Transwell plates with CCL21 at the indicated dose for 4 h. Cells from the lower well were analyzed. **(A)** Percentage of migration was calculated. **(B)** CCL17-eGFP expression in control and migrated FL-CD11b DCs was analyzed by flow cytometry. Migrated DCs represented DCs from the lower well. DCs cultured with conventional wells were used as control DCs. **(C–E)** Medium-, TSLP-induced CCL17-eGFP⁺, and CCL17-eGFP[−] FL-CD11b DCs were sorted. **(C)** Migration activity was measured. DCs were pulsed with the indicated concentration **(D)** or 0.01 μM **(E)** antigenic OVA peptide for 1 h, cultured with DO11.10 Tg CD4 cells. **(D)** Forty-eight hours later, cells were pulsed with [³H]thymidine for an additional 16 h and proliferation was measured. **(E)** Five days later, cells were restimulated for 48 h, and cytokine concentrations in the culture supernatant were measured by ELISA. **(F and G)** DCs were pulsed with 1 μM antigenic OVA peptide, and then DCs were transferred into BALB/c mice i.v. 1 d after CD45.1⁺ DO11.10 Tg CD4 cell transfer. Five days after DC transfer, skin LN cells were isolated: **(F)** cells were stimulated with PMA plus ionomycin for 4 h, then stained with Abs. **(G)** Skin LN cells were cultured in the presence of 1 μM antigenic OVA peptide for 72 h, and then cytokine concentrations in the culture supernatant were measured by ELISA. Three mice were used. Results are representative of two to three independent experiments. **p* < 0.05, ***p* < 0.01 (Student *t* test).

Next, we investigated the CD4 response initiated by TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs. TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs were purified, pulsed with the indicated dose of antigenic peptide, and cultured with DO11.10 CD4 T cells for 2 d, after which proliferation was measured. DO11.10 cells, cocultured with TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs, displayed increased CD4 proliferation at a low dose of peptide (0.01 μM), whereas proliferation was equivalent at higher Ag doses (1 μM) (Fig. 2D). To examine CD4 T cell differentiation by these DCs, sorted DCs and DO11.10 T cells were cultured with 0.01 μM peptide for 5 d, and then an equal number of cells was restimulated with plate-coating anti-CD3 and anti-CD28 Abs for 48 h. Levels of IL-4, IL-5, and IL-13 in the supernatant were substantially increased in cultures containing CCL17-eGFP⁺ FL-CD11b DCs compared with those with CCL17-eGFP[−] FL-CD11b DCs, whereas the level of IFN-γ was not increased (Fig. 2E and data not shown). TSLPR-deficient DO11.10 CD4 cells responded equivalently to TSLPR-sufficient DO11.10 CD4 cells, suggesting that the T cells do not require TSLP responsiveness (data not shown). Furthermore, to confirm that TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs promoted Th2 differentiation in vivo, we transferred OVA peptide-pulsed CCL17-eGFP⁺ or CCL17-eGFP[−] FL-CD11b DCs into host mice 1 d after transfer of DO11.10 cells. Consistent with in vitro data, IL-4⁺ DO11 CD4 cells in skin LNs were increased in mice that received CCL17-eGFP⁺ FL-CD11b DCs (Fig. 2F). Additionally, when skin LN cells from the transferred mice were cultured with OVA peptide for 72 h, the concentration of IL-4 and IL-5 in cultures from CCL17-eGFP⁺ FL-CD11b DC transferred mice was higher than those from mice that received unpulsed CCL17-eGFP⁺ FL-CD11b DCs or OVA-pulsed CCL17-eGFP[−] FL-CD11b DCs (Fig. 2G). Taken together, these results demonstrate that TSLP-induced CCL17-eGFP⁺ FL-CD11b DCs are capable of promoting the Th2 differentiation of naive CD4 T cells.

Ag-bearing CCL17-eGFP⁺ migratory DCs were reduced in TSLPR-deficient mice

We have previously shown that the type 2 CHS response to FITC was TSLP-dependent (14). Whereas DCs appeared to be the TSLP target in the skin, displaying an Ag uptake and migration deficit, the precise nature of the APC population responding to TSLP in this model was not clear (10, 18). In a similar CHS model, using TRITC as the hapten, it was found that CCL17-expressing DDCs were critical for the inflammatory response (7, 9). Taken as a whole, these data suggest that TSLP induction of CCL17 by DDCs is a critical aspect of the CHS response. To test this directly we TRITC-sensitized CCL17-eGFP^{+/−} mice, which either could or could not respond to TSLP, and examined Ag-bearing CCL17⁺ cells (as measured by eGFP expression) in both skin and dLNs 24 h later. At that time there was a significant increase in the both the number and frequency of TRITC⁺CCL17⁺MHC II^{high} in both skin and LNs in TSLPR-sufficient mice that was not seen in TSLPR-deficient mice (Fig. 3). DCs in skin LNs can be separated into CD11c⁺MHC II^{high} migratory DCs (mDCs) and CD11c⁺MHC II^{low} resident DCs, with the TRITC⁺CCL17⁺ cells into LNs following sensitization having the mDC phenotype (Supplemental Fig. 1B). Interestingly, the number of TRITC⁺CCL17[−] DCs in the LNs was unchanged regardless of TSLPR status, whereas the number and frequency of TRITC⁺ mDCs in the LNs that expressed CCL17 1 d after TRITC sensitization was significantly increased (Fig. 3C, 3D). These results indicate that allergen-mediated TSLP production in skin induces CCL17 expression by skin-resident DCs. These DCs then migrate to the LN that drains the site of sensitization.

TSLP induced migration of Ag-bearing CCL17-eGFP⁺ CD11b^{high} mDCs

Previous studies using this model of CHS have shown that three subsets of DDCs (CD207[−]CD11b^{high}, CD207[−]

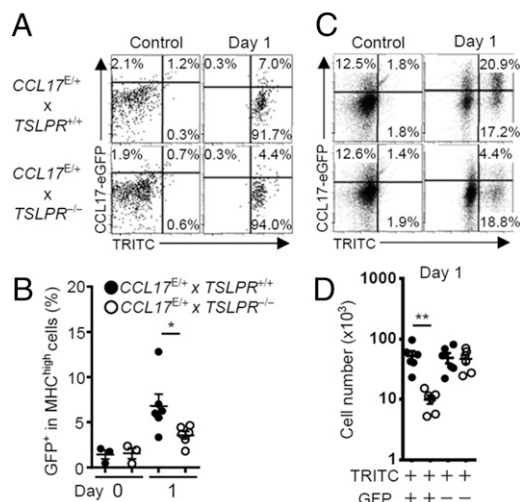


FIGURE 3. Ag-bearing CCL17-eGFP⁺ CD11b mDCs were reduced in TSLPR-deficient mice. Mice were sensitized on ear or shaved abdomen with 0.1% TRITC/dibutyl phthalate on day 0. Untreated mice were used as control. **(A and B)** Ear skin cells were gated on MHC II^{high} cells as skin DCs, and profiles of TRITC and CCL17-eGFP are shown. **(C)** LN cells were gated on mDCs (CD11c⁺MHC II^{high}), and profiles of TRITC and CCL17-eGFP are shown. **(D)** Numbers of DCs in LNs were calculated from flow cytometric data and total cell numbers, which were counted by the trypan blue exclusion method.

CD11b⁺ to low, and CD207⁺) migrate to the dLNs, peaking 24–48 h after sensitization, whereas epidermal Langerhans cells (LCs) peaked 3–4 d later (19). To identify the TSLP-responsive DC subset in skin-draining LNs, we examined CCL17 expression in Ag-bearing DCs 24 h after TRITC sensitization of WT CCL17-eGFP mice. TRITC⁺CCL17-eGFP⁺ mDCs were predominantly CD11b^{high} (>80%; Fig. 4A, Supplemental Fig. 2). Alternatively, TRITC⁺CCL17-eGFP[−] mDCs were a mixture of all three subsets 1 d after sensitization. To determine whether TSLP can act directly on CD11b^{high} DCs, we assessed phosphorylation of STAT5 in skin LN mDCs following TSLP treatment (Fig. 4B). TSLP induced pSTAT5 in skin LN CD11b^{high} mDCs of TSLPR-sufficient, but not TSLPR-deficient, mice. These results indicate that TSLP acts directly on dermal CD11b^{high} mDCs to induce CCL17 expression and migration to LNs.

TSLP-mediated Ag-bearing CCL17⁺CD11b^{high} mDCs induced Th2 differentiation

In vitro, TSLP-activated DCs have been shown to promote Th2 differentiation of naive CD4 T cells (2). In vivo, Ag-bearing DCs from TSLPR-deficient mice, isolated 24 h after FITC sensitization, displayed a reduced capacity for promoting CD4 proliferation and Th2 differentiation (14). To examine the function of TSLP-induced TRITC⁺CCL17⁺ mDCs, we isolated TRITC⁺CCL17⁺ and TRITC⁺CCL17[−] mDCs from skin LNs 24 h after TRITC sensitization. The cells were pulsed with the indicated dose of antigenic peptide and cultured with CD4 T cells from DO11.10 TCR Tg mice, and proliferation and cytokine production were measured as in Fig. 2. DO11.10 CD4 T cells in cultures with TRITC⁺CCL17⁺ mDCs had increased proliferation compared with those cultured with TRITC⁺CCL17[−] mDCs at moderate doses of the peptide (0.01 and 0.1 μM), whereas proliferation induced by high dose of the peptide (1 μM) was equivalent (Fig. 4C). We

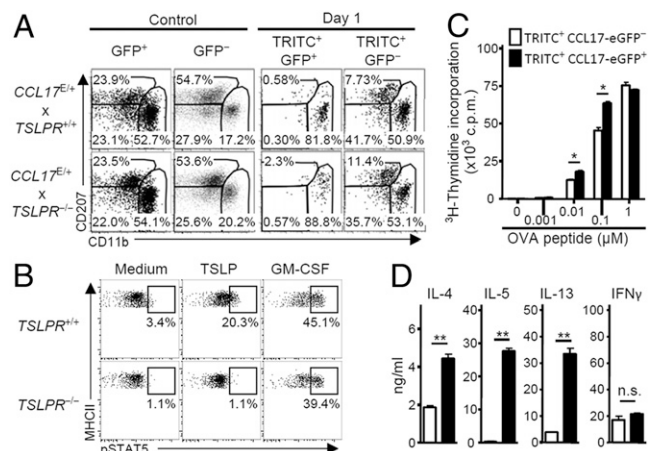


FIGURE 4. TSLP-mediated Ag-bearing CCL17-eGFP⁺ CD11b mDCs induced Th2 differentiation. **(A)** Skin LN cells after sensitization were stained, and profiles of CD207 and CD11b are shown. **(B)** Isolated skin LN CD11c⁺ cells were stimulated with medium, TSLP, or GM-CSF for 45 min and then pSTAT5 was analyzed. Skin LN cells were pooled from three to four mice. Results are representative of three independent experiments. **(C and D)** Skin LN cells were isolated 24 h after sensitization. TRITC⁺CCL17-eGFP[−] mDCs and TRITC⁺CCL17-eGFP⁺ mDCs were sorted, DCs were pulsed with **(C)** indicated concentration or **(D)** 0.1 μM peptide, and then cells were cultured with DO11.10 Tg CD4 cells. Skin LN cells were pooled from five to six mice. Proliferation and cytokine concentrations in supernatants were measured as in Fig. 2D and 2E. Results are representative of two to three independent experiments. **p* < 0.05, ***p* < 0.01 (Student *t* test).

next determined whether these mDCs were capable of driving CD4 T cell differentiation. Levels of IL-4, IL-5, and IL-13 in the supernatant were markedly increased in the cultures containing TRITC⁺CCL17⁺ DCs, whereas the level of IFN-γ was unaffected (Fig. 4D). As supporting reports, Kumamoto et al. (20) reported that MGL2⁺CD11b⁺ DDCs are sufficient to initiation of CHS response, and McLachlan et al. (21) reported that quantitative major CD11b^{high} DDCs, but not LCs, are important for induction of Ag-specific effector T cells and regulatory T cells simultaneously using ear injection and conditional depletion of LCs. Additionally, CD207⁺ DDCs may contribute to optimal Th1-type DNFB-CHS response, and LCs may have a regulatory role for attenuating DNFB-CHS response using conditional depletion of LCs (22–25). Thus, these results indicate that Ag-bearing, TSLP-induced, CCL17⁺ mDCs in skin-draining LNs can induce CD4 proliferation and Th2 differentiation efficiently, similar to the FL-CD11b DCs.

Whereas a connection between epithelial-derived TSLP and the initiation of type 2 inflammation is quite clear, the nature of the cells involved in this process are not. In this study, we demonstrate that TSLP-induced migration of CCL17-producing DCs is critical for initiation of Th2-type CHS. Consistent with previous work with human DCs (6), TSLP-activated bone marrow-derived DCs expressed high levels of CD86 and OX40L, as well as increased CCR7-mediated migration activity, and were capable of driving Th2 differentiation (Figs. 1, 2). Additionally, in a model of CHS, TSLP mediated the migration of Ag-bearing CD11b⁺ mDC into skin LNs (Figs. 3, 4). These results suggest that CCL17 expression defines the DC subset that is TSLP responsive both in vitro and in vivo.

Acknowledgments

We are grateful to Dr. Daniel J Campbell for helpful comments in the preparation of the manuscript. We also thank Sylvia McCarty for assistance with preparing the manuscript.

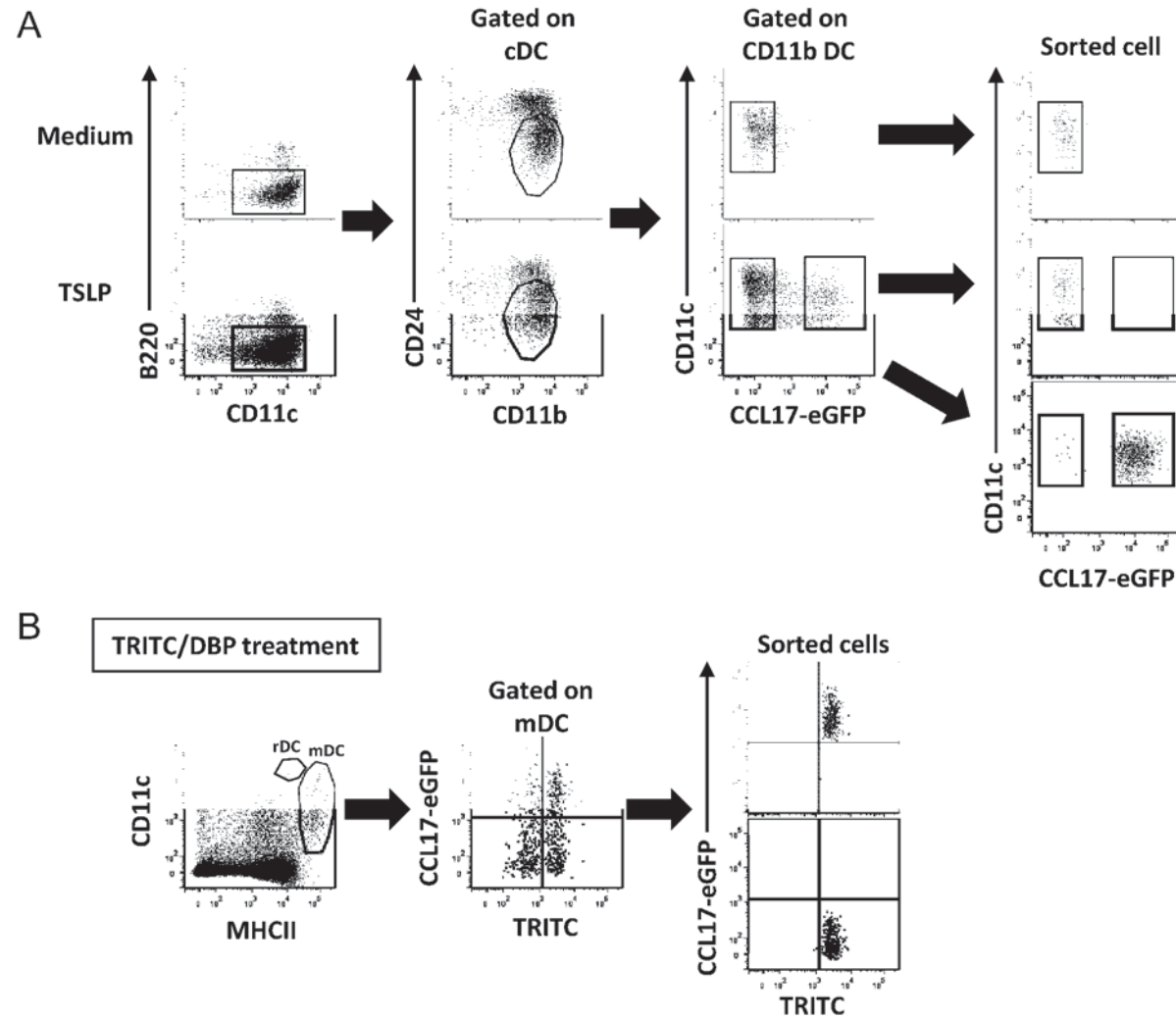
Disclosures

The authors have no financial conflicts of interest.

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S Fig. 1



S Fig. 2

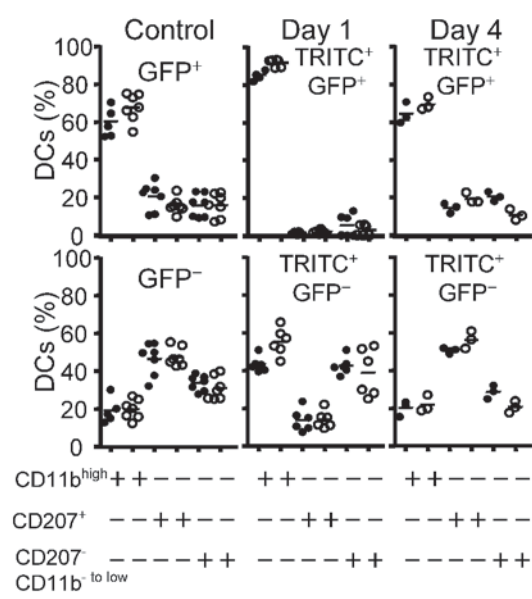


Figure S1 Sorting strategies for CCL17-eGFP⁺ DCs

(A) Plots show the sorting strategy for CCL17-eGFP⁺ and -eGFP⁻ FL-CD11b DCs using FACS Aria cell sorter. (B) Plots show the sorting strategy for TRITC⁺ CCL17-eGFP⁺ and -eGFP⁻ FL-CD11b DCs in skin LN cells 1 day after sensitization.

Figure S2 Characterization of migrated DC subset in skin LNs between TSLPR-sufficient and -deficient mice.

As in figure 4A, *CCL17^{E/+} x TSLPR^{+/+}* and *CCL17^{E/+} x TSLPR^{-/-}* mice were sensitized on day 0. Skin LN cells 0, 1, 4 days after sensitization were stained with anti-CD207 mAb and anti-CD11b mAb. Cells were gated on CD11b^{high}, CD207⁺, and CD207⁻ CD11b^{- to low} DCs.