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CD326^{lo}CD103^{lo}CD11b^{lo} Dermal Dendritic Cells Are Activated by Thymic Stromal Lymphopoietin during Contact Sensitization in Mice

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The cytokine thymic stromal lymphopoietin (TSLP) is produced by epithelia exposed to the contact sensitizer dibutyl phthalate (DBP), and it is critical for the induction of Th2 immune responses by DBP-FITC. TSLP is thought to act on dendritic cells (DC), but the precise DC subsets involved in the response to TSLP remain to be fully characterized. In this study we show that a subset of CD326^{lo}CD103^{lo}CD11b^{lo} dermal DC, which we termed “triple-negative (TN) DC,” is highly responsive to TSLP. In DBP-FITC-treated mice, TN DC upregulated expression of CD86 and rapidly migrated to the draining lymph node to become the most abundant skin-derived DC subset at 24 and 48 h after sensitization. None of these responses was observed in TSLPR-deficient mice. In contrast, TN DC numbers were not increased after treatment with the allergen house dust mite or the bacteria *Escherichia coli* and bacillus Calmette–Guérin, which increased other DC subsets. In vivo, treatment with rTSLP preferentially increased the numbers of TN DC in lymph nodes. In vitro, TN DC responded to rTSLP treatment with a higher level of STAT5 phosphorylation compared with other skin-derived DC subsets. The TN DC subset shared the morphology, phenotype, and developmental requirements of conventional DC, depending on FLT3 expression for their optimal development from bone marrow precursors, and CCR7 for migration to the draining lymph node. Thus, TN DC represent a dermal DC subset that should be considered in future studies of TSLP-dependent contact sensitization and skin immune responses. *The Journal of Immunology*, 2014, 193: 2504–2511.

Thymic stromal lymphopoietin (TSLP) is an epithelial innate cytokine primarily expressed in the intestinal tract, as well as in the skin and lung during inflammation and TLR ligation (1). TSLP binds to a receptor complex composed of an IL-7R α chain and a TSLP-specific TSLPR chain (2, 3). TSLP has been associated with a wide range of immune responses, including allergic responses (4–6), but also CD8⁺ T cell responses such as those induced by influenza viruses (7).

TSLP is highly expressed in contact dermatitis and has been shown to play a key role in the Th2 response to dibutyl phthalate (DBP) (8), a plasticizer with adjuvant properties used during the sensitization stage of FITC-induced contact hypersensitivity (9). Compared to TSLPR-sufficient mice, TSLPR-deficient mice exposed to DBP-FITC generate significantly reduced Th2-type responses as measured by skin thickening, Th2 cytokine production in skin-draining lymph node (dLN), and serum IgE levels (8).

Conversely, inducible expression of TSLP in skin (10) or local treatment with rTSLP (11) resulted in increased Th2 cytokines and skin lesions resembling atopic dermatitis. Despite the critical role of TSLP in the response to DBP-FITC, additional factors elicited during sensitization are also important to the phenotype of the resulting T cell response (12).

Although TSLPR is expressed on many cell types, the role of TSLP in the response to DBP-FITC appears to be principally mediated via its effects on dendritic cells (DC). In the absence of TSLPR, or after treatment with anti-TSLP, the number of FITC⁺ DC in skin-dLN was significantly reduced (8). Additionally, induction of Th2 responses to DBP-FITC required intact TSLPR signaling in DC (13), whereas TSLPR-deficient and TSLPR-sufficient CD4⁺ T cells could both expand in response to DBP-FITC sensitization and produced similar amounts of IL-4 (14), indicating that TSLPR signaling in CD4⁺ T cells is not essential for the response to DBP-FITC. The DC subsets involved in the Th2 response to DBP-FITC have been investigated and identified by different studies as CD11b⁺CCL17⁺ (15) or CD207⁺CD103⁺MGL2/CD301b⁺ (16–18), with the former subset also shown to respond to TSLP (15).

We reasoned that if TSLP is critical for the response to DBP, the use of TSLPR-deficient mice should provide valuable information on the populations of skin DC that are involved in the sensitization phase of the response to DBP-FITC. Using this strategy, we identified a subset of CD326^{lo}CD11b^{lo}CD103^{lo}, or triple-negative (TN), dermal DC that was preferentially activated after treatment with DBP-FITC and was highly responsive to TSLP in vivo and in vitro. We also show that TN DC develop from bone marrow (BM) precursors in an FLT3-dependent fashion, and they require CCR7 to migrate to the dLN upon maturation. On the basis of these findings, we suggest that TN DC are a subset of conventional

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Abbreviations used in this article: BCG, bacillus Calmette–Guérin; BM, bone marrow; DBP, dibutyl phthalate; DC, dendritic cell; dLN, draining LN; HDM, house dust mite; i.d., intradermal(ly); LC, Langerhans cell; LN, lymph node; MHCII, MHC class II; TN, triple-negative; TSLP, thymic stromal lymphopoietin; WT, wild-type.

DC that are readily responsive to TSLP, and whose contribution to TSLP-dependent immune responses is likely to provide important information on the biology of this cytokine.

Materials and Methods

Mice

Specific pathogen-free C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME), CD45-congenic B6.SJL-*Ptprca*^a mice from the Animal Resources Centre (Perth, NSW, Australia), TSLPR-deficient mice (19), and Langerin reporter *Lang-EGFP* mice (20) were bred and housed at the Malaghan Institute of Medical Research Animal Facility. FLT3-deficient mice (21) and CCR7-deficient mice purchased from The Jackson Laboratory (22) were maintained in specific pathogen-free conditions at the Centenary Institute Animal Facility (Sydney, NSW, Australia). Age- and sex-matched 6- to 16-wk-old mice were used in all experiments. All experimental procedures were approved by the Victoria University Animal Ethics Committee (Wellington experiments) and Sydney University Animal Ethics Committee (Sydney experiments) and carried out in accordance with each Committee's guidelines for the use and care of animals.

BM chimeras

BM chimeras were generated as described (23). Briefly, C57BL/6 (CD45.2) hosts were lethally irradiated with two doses of 600 cGy (Gammacell 40 exactor, Nordion International) given 4 h apart. Twenty-four hours later the irradiated hosts received $8-10 \times 10^6$ BM cells from B6.SJL-*Ptprca*^a (CD45.1) donor mice. For competitive repopulation experiments, 18 B6.SJL-*Ptprca*^a hosts were sublethally irradiated with 700 cGy and received a BM graft ($8-10 \times 10^6$ cells) consisting of wild-type (WT) B6.SJL-*Ptprca*^a BM mixed 1:1 with either WT C57BL/6 BM (control chimeras) or knockout (FLT3- or CCR7-deficient) (CD45.2) BM. All chimeric mice were allowed to reconstitute for at least 6 wk before use in experiments.

Immunizations and TSLP treatment

Mice were anesthetized and sensitized with 20 μ l of a solution of 0.5% FITC (Sigma-Aldrich, St Louis, MO) in 1:1 DBP (BDH Laboratory Supplies, Poole, U.K.)/acetone (Pacific Sphere Limited, Auckland, New Zealand) applied epicutaneously on both ears (8, 23). House dust mite (HDM) (Greer Laboratories, Lenoir, NC) was injected intradermally (i.d.) in both ears of anesthetized mice at a dose of 200 μ g HDM/ear (24). For bacterial treatments, *Mycobacterium bovis* bacillus Calmette-Guérin Pasteur (BCG, provided by Dr. J. Triccas, Centenary Institute) or *Escherichia coli* DH5 α (Life Technologies) were grown to midlog phase, washed, resuspended in PBS, and injected i.d. into the ear pinna of anesthetized mice at a dose of 10^5 (BCG) or 10^7 (*E. coli*) CFU/4 μ l/mouse as described (25). rTSLP (R&D Systems, Minneapolis, MN) was injected i.d. at 200 ng rTSLP/30 μ l PBS/ear.

Cell preparations

Both ear-dLN were harvested and pooled as one sample and digested with collagenase IV (Sigma-Aldrich) and DNase I (Roche, Mannheim, Germany) for 25 min at 37°C as described (23). For skin DC preparation, ears were split into dorsal and ventral layers and digested in collagenase IV and DNase I for 1 h at 37°C. Single-cell suspensions were obtained by passing digestion products through a 70- μ m nylon cell strainer (BD Falcon).

Flow cytometry

Multicolor flow cytometry was performed on a BD LSR II SORP or custom 10-laser LSR II cytometer (Becton Dickinson, San Jose, CA) with FACSDiva software (BD Biosciences) for data acquisition and FlowJo software (Tree Star, Ashland, OR) for analysis. All mAb incubations were in PBS containing 2% FCS, 10 nmol EDTA, and 0.02% sodium azide. Nonspecific mAb binding was blocked using anti-CD16/CD32 (clone 2.4G2, made in house). Brilliant Violet 785-anti-B220 (RA3-6-B2), Pacific Blue-anti-IA/IE (M5/14.15.2), allophycocyanin-Cy7-anti-CD326 (G8.8), PE-anti-CD301b (URA-1), PE-anti-PD-L2 (TY25), and biotin-anti-CD11b (M1/70) were from BioLegend (San Diego, CA); PE-anti-CD135 (A2F10.1), PE-anti-CD64 (X54-5/7.1.1), PerCP-Cy5.5-anti-CD45.1 (A20), biotin-anti-CD45.2 (104), PE-Cy7-CD11c (HL3), and allophycocyanin-anti-CD45 (30F-11) were from BD Biosciences. PE-anti-CD200R (OX110), PE-anti-CD103 (2E7), PE-anti-CD14 (Sa2-8), and PerCP-Cy5.5-anti-CD24 (M1/69) were from eBioscience. Streptavidin-Qdot 565 and streptavidin-Pacific Blue were from Life Technologies (Carlsbad, CA). Rabbit anti-CX3CR1 was from Sapphire Biosciences (Auckland, New Zealand) and PE-donkey anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). For pSTAT5 intracellular staining, cells were

fixed, surface stained, and permeabilized using lyse/fix buffer and permeabilization buffer III, respectively (both from BD Biosciences) before incubation with anti-STAT5 pY694 (47/Stat5, BD Biosciences). For Ki-67 intracellular staining, cells were fixed and permeabilized using Fix/Perm buffer (eBioscience) before incubation with anti-human Ki-67 Alexa Fluor 647 (BD Biosciences). DAPI and a Live/Dead fixable blue dead cell stain kit (Life Technologies) were used for dead cell exclusion for unfixed samples and fixed samples, respectively. Compensation was set in each experiment using compensation beads (Life Technologies).

Morphology

Cell suspensions were sorted using BD FACS Vantage DiVa (Becton Dickinson) and plated onto a slide using a Shandon CytoSpin 4 cytocentrifuge (Thermo Scientific) followed by methanol fixation. Bright-field images were obtained using a compound BX51 microscope with DP70 digital camera (both from Olympus, Tokyo, Japan) and Cell F software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Statistical calculations were carried out using Prism software version 5.0d (GraphPad Software, La Jolla, CA). All *p* values were calculated by a two-tailed Mann-Whitney *U* test or a Kruskal-Wallis test followed by Dunn's test. A *p* value >0.05 was considered not significant and is not shown in the figures.

Results

Sensitization with DBP-FITC induces the activation and migration of skin DC to the dLN

To investigate the DC subsets involved in the response to DBP-FITC, we treated C57BL/6 mice on ear skin using conditions that resulted in strong priming of IL-4 production by T cells (data not shown), and we analyzed DC subsets in the ear-dLN at different times after treatment. DC were gated into CD11c^{int}MHC class II (MHCII)^{hi} (Fig. 1A, subsets I–IV) and CD11c^{hi}MHCII^{int} (Fig. 1A, subset V), and the CD11c^{int}MHCII^{hi} subset was further subdivided into a CD326^{hi} population that included epidermal Langerhans cells (LC) (26) (Fig. 1A, subset I) and three populations of dermal DC that were CD11b^{hi}CD103^{lo}, CD11b^{lo}CD103^{hi}, and CD11b^{lo}CD103^{lo}, or “TN” (23) (Fig. 1A, subsets II–IV, respectively).

FITC uptake was detectable in each of the CD11c^{int}MHCII^{hi} DC subsets at all time points (Fig. 1B, Supplemental Fig. 1), which is consistent with this population containing skin-derived DC (27). About 50% of the TN and CD11b^{hi} DC were FITC⁺ at early time points, whereas the proportions of FITC⁺ CD326^{hi} and CD103^{hi} DC were lower. The CD11c^{hi}MHCII^{int} population showed no FITC uptake at any time point and was therefore excluded from further analysis.

As reported (23), all skin-derived DC subsets were increased in number in the dLN of DBP-FITC-treated mice compared with untreated controls (Fig. 1C). This increase was especially marked for the TN DC subset, which was the most abundant skin-derived DC subset in the dLN at both 24 and 48 h after DBP-FITC treatment. By 72 h, the numbers of each DC subset had stabilized or were starting to decline, and thus no further time points were examined.

The expression of the costimulatory molecule CD86 on the various DC subsets was also examined. Within each subset, CD86 was highest on FITC⁺ cells (Supplemental Fig. 1). When CD86 upregulation was compared across each DC subset, including both FITC⁺ and FITC[−] DC, it followed a similar trend to cell numbers (Fig. 1D). The highest increase in CD86 expression compared with control, and the highest expression overall, was observed in TN DC followed by CD11b^{hi} DC. Some CD86 upregulation was also observed on CD103^{hi} DC, whereas CD86 upregulation on CD326^{hi} cells was very low to undetectable. This is consistent with the inefficient upregulation of CD80/86 previously reported for LC (23). CD80 was also examined and was found to reflect the pattern of CD86 expression (not shown).

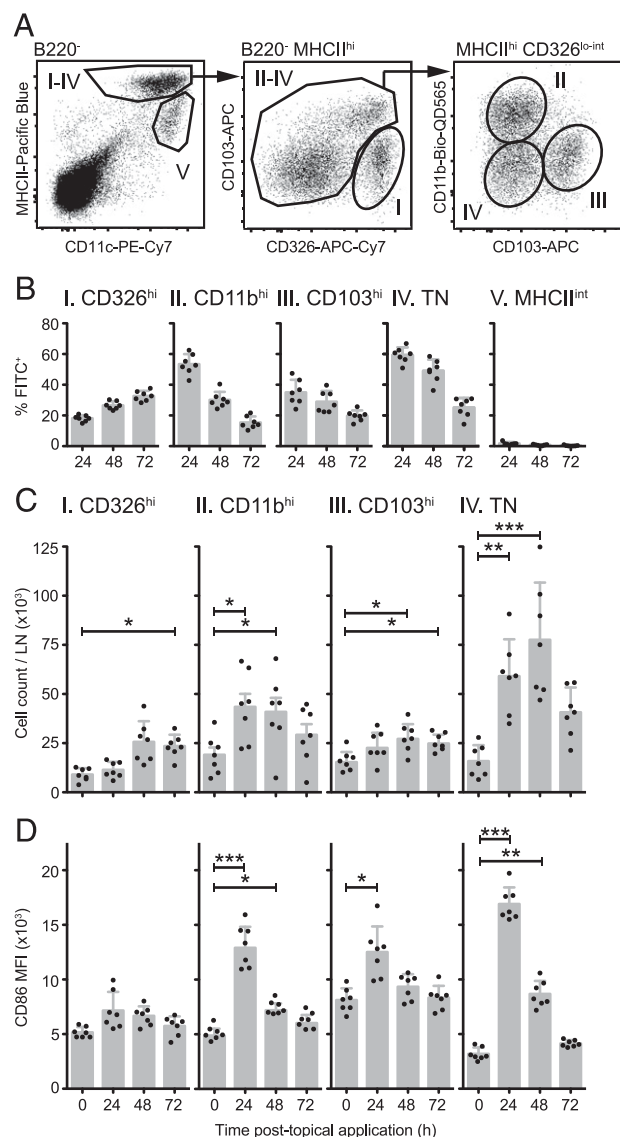


FIGURE 1. Skin application of DBP-FITC induces increased numbers and activation of skin DC in the dLN. C57BL/6 mice were treated on ear skin with DBP-FITC and DC subsets in ear-dLN were analyzed at the times specified. Time 0 refers to untreated mice. **(A)** Gating strategy used to identify DC subsets. Representative dot plots from a naive mouse are shown. **(B)** FITC uptake for each DC subset. **(C)** Numbers and **(D)** median fluorescence intensity (MFI) of CD86 expression on total DC, including both FITC⁻ and FITC⁺, in dLN at different times after treatment. Data are expressed as means $\pm$ SD; each dot refers to one mouse. The *p* values were calculated using the Kruskal–Wallis followed by Dunn’s test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data are from one of two repeat experiments, each with four–seven mice per group, which gave similar results.

Similar experiments carried out in BALB/c mice confirmed that the TN DC population was present in the skin-dLN of these mice, although in lower proportions compared with C57BL/6 mice, and was readily activated by DBP-FITC treatment (data not shown), indicating that the involvement of TN DC in the response to DBP-FITC is not restricted to the C57BL/6 strain.

The increase in the number of TN DC in dLN after DBP-FITC treatment requires TSLP

We used TSLPR-deficient mice to investigate whether TSLP was involved in the migration and activation of the DC subsets identified in Fig. 1. Preliminary experiments showed that the same DC subsets identified in the ear-dLN of WT C57BL/6 mice could also be

detected in naive TSLPR-deficient mice (Supplemental Fig. 2), indicating that TSLP was dispensable for their development, subset specialization, and steady-state migration. Additionally, as already reported (8), Th2 priming was ablated in these mice (not shown).

TSLPR-deficient and WT mice were treated with DBP-FITC, and dLN were analyzed 24 h later. In WT mice, treatment with DBP-FITC increased the numbers of TN DC $\sim$ 3- to 4-fold compared with controls, whereas CD11b^{hi} DC were increased $\sim$ 2.5-fold and CD103^{hi} DC by 30–50%. These increases were not observed in TSLPR-deficient mice (Fig. 2A). Treatment with DBP-FITC also increased expression of CD86 on DC (Fig. 2B–E); this increase was most marked (3- to 4-fold) in the TN DC subset and required expression of TSLPR. Modest but significant increases in CD86 expression were observed on the CD11b^{hi} and CD103^{hi} dermal DC subsets; however, these were independent of TSLPR expression. Although DC numbers were lower in TSLPR-deficient mice compared with WT mice, the proportions of FITC⁺ DC in each subset were similar in both strains (Fig. 2D), suggesting that some DC migration was still occurring in TSLPR-deficient mice. The ratio of CD86 expression in FITC⁺ versus FITC⁻ DC was $\sim$ 2 for the CD326^{hi} and CD103^{hi} subsets in WT mice, but increased to $\sim$ 6 for TN DC and $\sim$ 3 for CD11b^{hi} DC (Fig. 2E). In TSLPR-deficient mice, all ratios were $\sim$ 2 irrespective of the DC subset. Therefore, the activation and enhanced migration of TN DC after DBP-FITC application require TSLPR expression.

To further investigate the origin of TN DC in the dLN of DBP-FITC-treated mice, we compared the proportion of proliferating Ki-67⁺ DC in WT and TSLPR-deficient mice at 24 h after treatment with DBP-FITC (Fig. 2F). We found the proportion of Ki-67⁺ cells in each DC subset to be similar in naive and DBP-FITC-treated mice, suggesting that increased proliferation is not sufficient to account for the increased numbers of TN DC in the dLN.

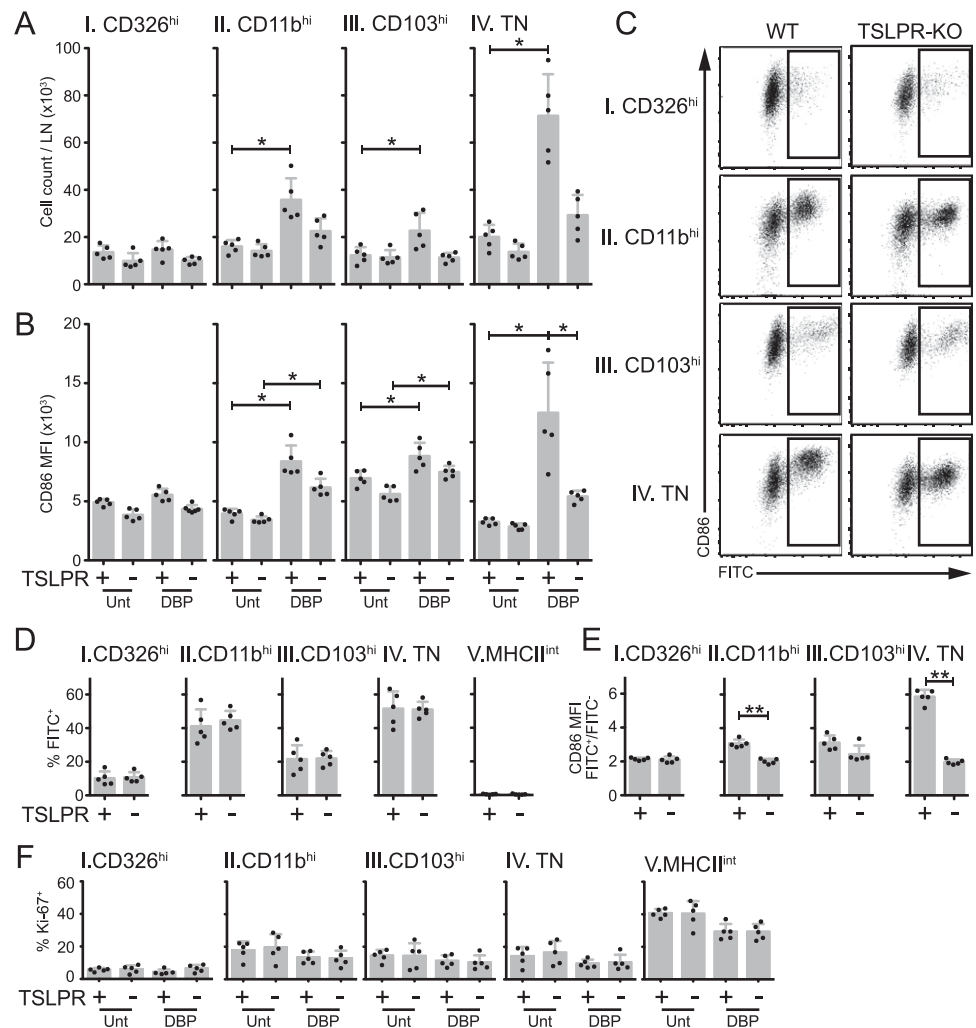
TN DC are preferentially responsive to TSLP

DC from TSLPR-deficient hosts could respond weakly to DBP-FITC treatment, suggesting that cytokines and/or factors other than TSLP were also contributing to the observed response. To assess the effects of TSLP on DC in the absence of other confounding factors, we injected rTSLP in the ear of WT and TSLPR-deficient mice and analyzed DC subsets in dLN 48 h later. The number of TN DC was increased $\sim$ 2-fold in rTSLP-treated compared with untreated mice, and this increase required TSLPR expression (Fig. 3A). The number of CD11b^{hi} DC was also slightly increased, but this was not statistically significant. No changes were detectable in the numbers of CD326^{hi} or CD103^{hi} DC.

To investigate whether TSLP was acting directly on TN DC, cell suspensions from skin-dLN of WT and TSLPR-deficient mice were treated in vitro with different doses of rTSLP or with rGM-CSF as a positive control. These experiments used anti-CD24 to exclude LC (28) and the small CD103^{lo}CD24^{hi} population that is found in skin and skin dLN (Supplemental Fig. 3 and data not shown) from the TN DC subset. Phosphorylation of STAT5 was examined by intracellular flow cytometry in all DC subsets. Preliminary experiments indicated that phospho-STAT5 was low 15 min after TSLP treatment, but clearly detectable at 30 min and still high at 2 h. Thus, further experiments used DC treated with TSLP for 45 min.

As shown in Fig. 3B, phospho-STAT5 was detectable in $\sim$ 30% of TN DC treated with 5 ng/ml TSLP; this percentage did not increase with higher TSLP doses. Phospho-STAT5 was also detected in other DC subsets; however, the percentage of phospho-STAT5⁺ cells was lower than in TN DC and did not increase by increasing TSLP dose (Fig. 3C). Little or no phospho-STAT5 was detected in any of the DC subsets in TSLPR-deficient mice.

FIGURE 2. The increase in the number of TN DC and upregulation of CD86 after DBP-FITC treatment require TSLPR expression. C57BL/6 and TSLPR-deficient (TSLPR-KO) mice were treated on ear skin with DBP-FITC and dLN were examined by flow cytometry 24 h after sensitization. **(A)** Numbers, **(B)** CD86 expression, and **(C)** representative dot plots for various DC subsets in dLN are shown. **(D)** Percentages of FITC⁺ DC within each DC subset. **(E)** CD86 expression on FITC⁺ versus FITC⁻ DC in each subset, expressed as median fluorescence intensity (MFI) ratios. **(F)** Percentages of Ki-67⁺ DC within each DC subset. Data are from one of two repeat experiments, each with four–seven mice per group, which gave similar results. Data are expressed as means $\pm$ SD; each dot refers to one mouse. The *p* values were calculated using the Mann–Whitney *U* test. **p* < 0.05, ***p* < 0.01.



Treatment with rGM-CSF induced STAT5 phosphorylation in all DC populations regardless of TSLPR expression (Fig. 3D and data not shown). These data suggest that TN DC are directly responsive to TSLP.

We then investigated whether other forms of immunization, which do not involve TSLP production, result in the preferential increase of TN DC numbers in dLN. Injection of HDM preparations by the i.d. route resulted in a moderate increase in the number of CD11b^{hi} DC, whereas the numbers of TN and CD103^{hi} DC were unchanged, and CD326^{hi} DC were transiently decreased (Fig. 4A). Injection of BCG or *E. coli* bacterial suspensions also induced a substantial increase in the number of CD11b^{hi} DC, with no change in the number of TN DC (Fig. 4B). The numbers of CD326^{hi} and CD103^{hi} were also not increased. Therefore, the increase in numbers of TN DC in LN is restricted to specific types of immune responses.

TN DC represent a population of dermal DC that migrate from skin to dLN and require FLT3 expression to develop from BM precursors

The presence of a TN DC subset in the skin dLN has been reported in the literature (23, 27), but its developmental origin and function have not been fully characterized. We confirmed the presence of a TN DC population in skin by carrying out flow cytometry analysis of ear skin cell suspensions from WT mice. This analysis revealed a CD45⁺MHCII^{hi} DC population expressing low levels of CD24 and CD11b (Fig. 5A, Supplemental Fig. 3). Compared to

CD103^{hi} (in dLN) and CD24^{hi} (in skin) dermal DC, the TN DC expressed low to intermediate CD11c and were CD200R⁺, CD14⁺, CD135^{hi}, CD64⁻, and Langerin-GFP⁻ (Fig. 5B, 5C). The expression of these markers was consistent between skin and dLN, further supporting a skin origin of TN DC in LN. Additionally, TN DC in LN expressed variable levels of CX3CR1 (Fig. 5C, Supplemental Fig. 4) and are therefore distinct from the Langerin⁻CD11b⁻CX3CR1⁻CD24⁺ DC subset recently described by Mollah et al. (29).

We also examined expression of CD301b and PD-L2. TN DC were negative or low for CD301b, and they expressed variable levels of PD-L2 (Fig. 5B, 5C), whereas CD11b^{hi} DC were partly positive for these markers (Supplemental Fig. 4). The morphology of TN DC flow sorted from skin-dLN was also examined and compared with CD103^{hi} DC from the same LN. Both populations exhibited a DC-like morphology but, compared with CD103^{hi} DC, TN DC had smaller cell bodies and more extensive and finer dendrites (Fig. 5D).

The developmental origin of TN DC was investigated in CD45.2→CD45.1 BM chimeras. In chimeric mice, 98% of TN DC in skin dLN were of donor origin, confirming that, as previously shown (23), the replenishment of this population relied on continuous BM supply (Fig. 6A). Mixed BM chimera experiments also showed that differentiation of TN DC from BM precursors required expression of the receptor for the DC growth factor FLT3L, as in mice reconstituted with equal mixtures of WT and FLT3-deficient BM, FLT3-deficient BM cells contributed to <5%

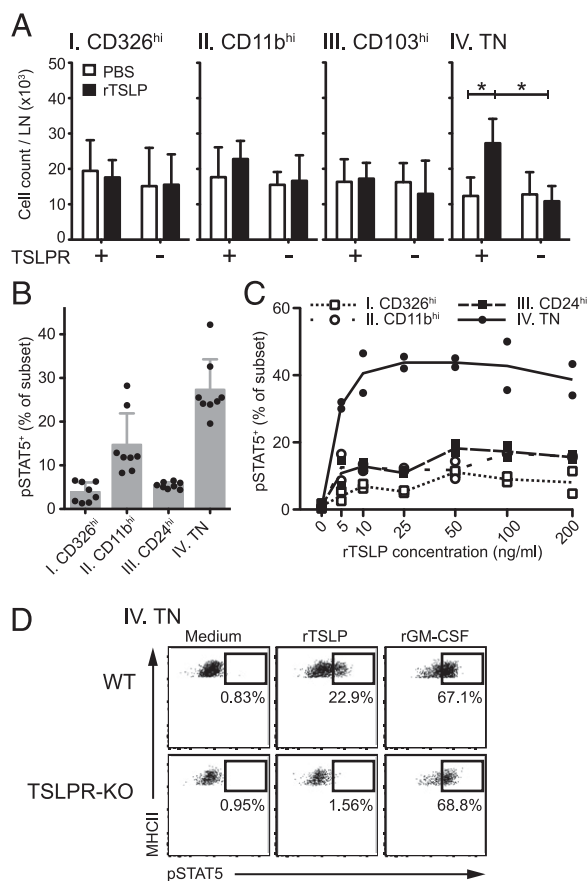


FIGURE 3. Treatment with rTSLP increases the number of TN DC in skin-dLN and induces STAT5 phosphorylation in vitro. **(A)** C57BL/6 and TSLPR-deficient (TSLPR-KO) mice were injected i.d. in the ear with rTSLP, and dLN were examined 48 h later by flow cytometry. The numbers of each subset of skin-derived DC in dLN are shown. Data are from one of two repeat experiments, each with four–six mice per group, which gave similar results. Mean values $\pm$ SD are shown. The p values were calculated using the Kruskal–Wallis test followed by Dunn’s test. $*p < 0.05$. **(B–D)** Cell suspensions from ear-dLN were treated as indicated before intracellular staining for pSTAT5. **(B)** Treatment with 50 ng/ml rTSLP for 45 min; mean values $\pm$ SD for three combined experiments each with two samples per group are shown. **(C)** Treatment with various doses of rTSLP for 45 min; each symbol refers to one sample. **(D)** Treatment with 50 ng/ml rTSLP or 10 ng/ml GM-CSF for 45 min; representative dot plots are shown. Results are from one of two experiments that gave similar results.

of the TN DC pool in the skin and $<15\%$ in the LN (Fig. 6B). We also generated mixed BM chimeras using CD45.1⁺ CCR7-sufficient and CD45.2⁺ CCR7-deficient BM cells. In chimeric mice, almost all TN DC in the dLN were CD45.1⁺ (CCR7-sufficient) (Fig. 6C), indicating that TN DC require CCR7 expression to migrate to the dLN. Thus, TN DC represent a true DC population that shares the markers and developmental origin of conventional DC.

Discussion

In this study we found that a subset of dermal DC lacking expression of CD326, CD103, and CD11b, which we refer to as TN DC, was especially prominent in the TSLP-dependent response to the contact sensitizer DBP-FITC. The TN DC were the most abundant skin-derived DC subset in the dLN of DBP-FITC-treated mice at 24 and 48 h, and they expressed the highest levels of CD86. Experiments in TSLPR-deficient mice showed that TSLPR signaling did not result in increased proliferation of TN DC, but

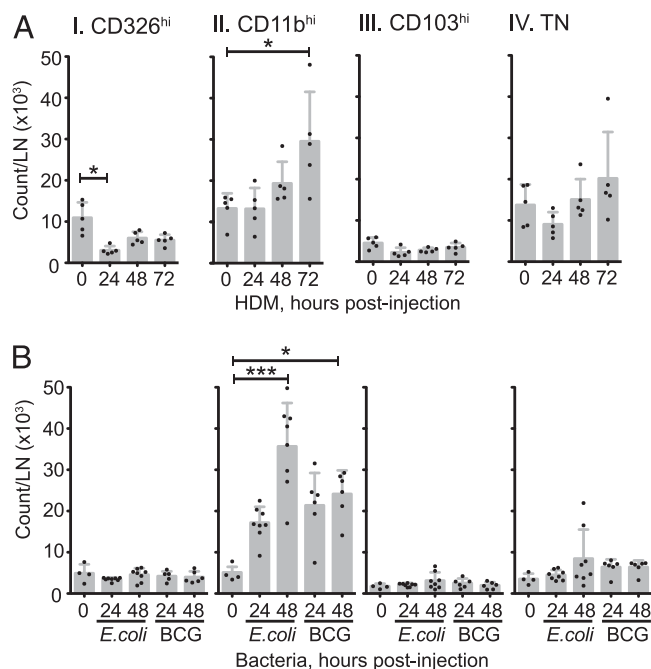


FIGURE 4. The number of TN DC in LN is not increased by treatment with HDM or bacteria. C57BL/6 mice were treated on ear skin as indicated and DC subsets in ear-dLN were analyzed at the times specified. Time 0 refers to untreated mice. DC populations were defined as in Fig. 1A. **(A)** Mice were injected i.d. with HDM; data are from one of two experiments with five mice per group that gave similar results. **(B)** Mice were injected i.d. with *E. coli* or BCG. Data are expressed as means $\pm$ SD and are representative of two independent experiments; $n = 4$ –8 mice/group; each dot refers to one mouse. The p values were calculated using the Kruskal–Wallis test followed by Dunn’s test. $*p < 0.05$, $***p < 0.001$.

was necessary for both the increase in TN DC numbers and for the upregulation of CD86 on TN DC, whereas other DC subsets were only partially affected by TSLPR deficiency. In support of this finding, in vivo treatment with rTSLP selectively increased the numbers of TN DC in dLN, and rTSLP in vitro induced STAT5 phosphorylation in TN DC. Thus, TN DC are directly and readily responsive to TSLP, and they presumably play a role in immune responses involving production of this cytokine.

In vitro experiments assessing STAT5 phosphorylation in LN DC suggested that the levels of phospho-STAT5 were highest in TN DC followed by CD11b^{hi} DC, and less pronounced in other DC subsets. This observation was somewhat surprising with regard to the CD326^{hi} DC subset, as CD326^{hi} LC are reported to respond to TSLP (4). There are two potential explanations for this finding. First, on the basis of BM chimera experiments, the CD326^{hi} subset in LN does not consist exclusively of LC but also includes other DC subsets; these subsets might be poorly responsive to TSLP and mask the response of LC. Alternatively, the slow migration of LC to the LN after maturation (20) may result in a more marked TSLPR downregulation in LC as compared with other, more mobile DC subsets, with consequent lower ability to respond to TSLP. Data in this study do not enable us to distinguish between these possibilities.

Experiments in mice that were depleted of LC (20) or lacked CD103⁺ dermal DC due to BATF3 deficiency (12) showed that these DC subsets are not required for sensitization to DBP-FITC. In contrast, dermal DC typed as CD11b⁺CD301b⁺ (16–18) or CD11b⁺CCL17⁺ (15) were found to be important in the Th2 response to DBP-FITC. The evidence provided in those studies included experiments of in vivo DC depletion (17), FITC uptake

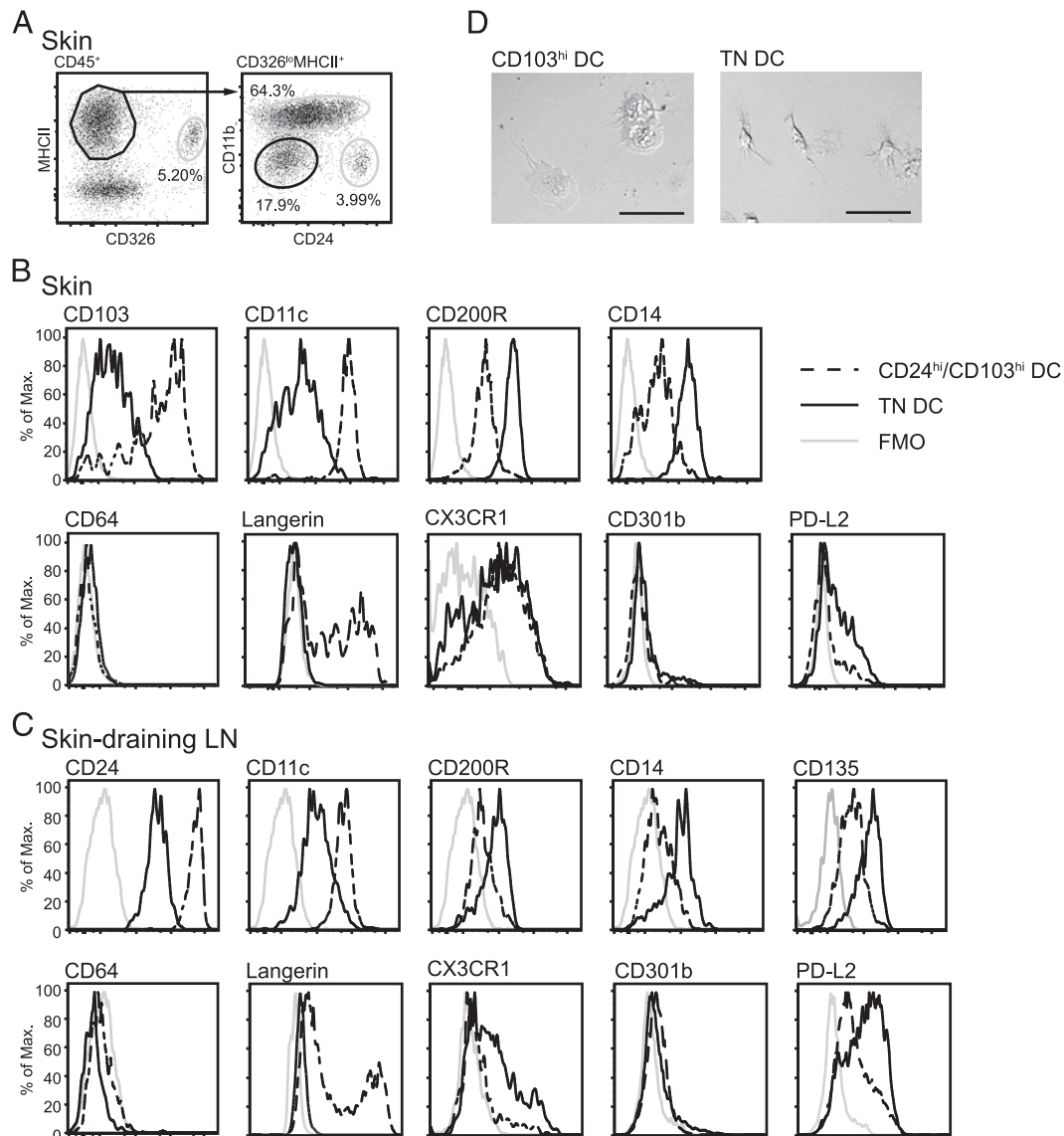


FIGURE 5. TN DC express phenotypic and morphologic features of conventional DC. Single-cell suspensions were prepared from ear skin or dLN of naive C57BL/6 mice, and DC subsets were examined by flow cytometry. **(A)** Representative gating of DC subsets in the skin; the TN DC population is highlighted by a black line. **(B)** Expression of surface markers on skin and **(C)** skin-dLN DC subsets. Solid lines indicate TN DC; dotted lines, CD24^{hi}/CD103^{hi} dermal DC; gray lines, fluorescence-minus-one controls. Data are representative of two experiments involving four–six mice. **(D)** Bright-field images of flow-sorted CD103^{hi} DC and TN DC. Scale bar, 50 μ m.

(17, 18), and stimulation of Th2 responses in vivo and in vitro (15, 16). The TSLP responsiveness of those populations was clearly established for CD11b⁺CCL17⁺ cells (15) but was not directly tested in the case of CD301b⁺ cells (17, 18). In partial agreement with those reports, we and others (23) show that TN DC were not the only population responding to DBP-FITC treatment, and that CD11b^{hi} DC were also involved. However, rTSLP injection experiments revealed marginal effects on CD11b^{hi} DC, suggesting that the response of this DC population to DBP-FITC may involve other signals in addition to TSLP. In contrast to CD11b^{hi} DC, TN DC could readily respond to in vivo treatment with rTSLP, and they were predominant in the response to DBP-FITC. Additionally, TN DC were CD301b[−] and expressed variable levels of PD-L2, and thus they are clearly distinct from the CD11b⁺CD301b⁺ PD-L2⁺ population that was recently reported to be necessary, but not sufficient, for the Th2 response to DBP-FITC (16, 17, 30).

The precise role of TN DC in immune responses remains undefined owing to the lack of experimental models to selectively

target this cell population in vivo. The rapid increase in numbers of TN DC in the dLN after DBP-FITC application, as well as their efficient uptake of FITC and upregulation of costimulatory molecules, may suggest an involvement of TN DC in direct Ag presentation to CD4⁺ T cells. The high responsiveness of TN-DC to TSLP, which is critically required for the development of Th2 responses after DPB-FITC sensitization (8), may also suggest a role in the priming of naive CD4⁺ T cells. We did not directly test the ability of resting or TSLP-activated TN DC to prime T cells or induce their differentiation into effector cells. Published data indicate that TSLP production is induced by a broad range of stimuli including allergens but also bacterial and viral infections (1). Accordingly, TSLP is also required for optimal CD8 responses to infection with influenza virus (7). These observations would suggest that TSLP is involved in other types of immune responses in addition to Th2 priming after DBP-FITC sensitization.

Increased numbers of TN DC were also observed in other models of immune response that involve TSLP production (data not

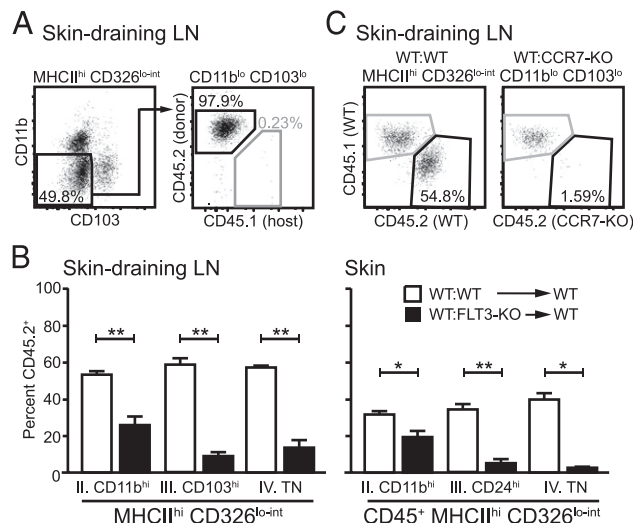


FIGURE 6. TN DC originate from BM precursors and require FLT3 and CCR7 for their development and migration from skin to dLN. Single-cell suspensions were prepared from ear skin or dLN of naive chimeric mice, and DC subsets were examined by flow cytometry. **(A)** Ear-dLN from CD45.2→CD45.1 BM chimeras were collected; the TN DC subset was identified as in Fig. 1A and examined for CD45.1 versus CD45.2 expression. Representative dot plots from one experiment with three mice are shown. **(B)** Ear-dLN from (CD45.1 WT plus CD45.2 WT)→CD45.2 and (CD45.1 WT plus CD45.2 FLT3-deficient)→CD45.2 mixed BM chimeras were collected, and TN DC were examined for CD45.1 versus CD45.2 expression. Data are representative of three independent experiments with five mice per group. The *p* values were calculated using the Mann–Whitney *U* test. **p* < 0.05, ***p* < 0.01. **(C)** Ear-dLN from (CD45.1 WT plus CD45.2 WT)→CD45.2 and (CD45.1 WT plus CD45.2 CCR7-deficient)→CD45.2 mixed BM chimeras were collected, and TN DC were examined for CD45.1 versus CD45.2 expression. Representative dot plots from one of two independent experiments with three mice per group are shown.

shown), including treatment with the vitamin D analog MC903 (31). These data further confirm the relationship between TSLP and TN DC activation. We also examined the effect of the allergen HDM and the bacterial species *E. coli* and BCG on DC subsets in skin, and we show that all of these forms of immunization led to increased numbers of CD11b^{hi} DC in dLN without increasing TN DC. In the conditions used in our experiments, HDM did not elicit TSLP production and did not require TSLPR expression to induce Th2 priming (data not shown) (24). Thus, these results confirm that type 2 immunity can be generated via both TSLP-dependent and -independent pathways (32), and they suggest that any potential role played by TN DC in the Th2 responses to DBP-FITC may not extend to other models of Th2 induction.

TN DC have been identified as one of the steady-state subsets in skin (23, 27); however, their responsiveness to innate signals, functional properties, likely heterogeneity, and role in skin immune responses are essentially unknown. This lack of information is due at least in part to the lack of unique markers that positively identify and differentiate this DC subset from other skin DC populations. Thus, our present study begins to shed light on the properties of this relatively unknown DC population. We confirm that TN DC were of BM origin and required the DC growth factor FLT3 to differentiate into mature DC. TN DC were found in the dermis and required CCR7 expression to accumulate in the dLN, had typical DC morphology, and expressed a CD64[−]CD135⁺ phenotype that is characteristic of conventional DC. This phenotype was maintained in DBP-FITC-treated mice (data not shown), indicating that the dramatic increase in TN DC numbers after sensitization was not due to differentiation from monocytes

precursors. Lastly, TN DC could efficiently phosphorylate STAT5 in response to GM-CSF, a cytokine recently reported to support the survival of conventional DC in the periphery (33). Other authors have reported that a DC population phenotypically similar to our TN DC was induced to proliferate, accumulate in dLN, and express increased levels of CD86 in mice overexpressing TGF-β1 in keratinocytes (34), and that these changes resembled those induced by DBP treatment. Exploring the potential similarities between the responses to TGF-β1 and DBP-FITC may yield interesting information on the properties of TN DC.

The DC subsets that control the induction of allergic responses including contact sensitivity are the object of intense study. The roles of LC and dermal CD103⁺ DC subsets in tolerance induction, and during immune responses to vaccination and infection, are progressively being defined. Dissecting the function of the additional DC subsets that form the complex DC network in the skin, both during contact sensitization and other models of immune responses, remains a challenge for future investigations.

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Disclosures

The authors have no financial conflicts of interest.

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CD326^{lo}CD103^{lo}CD11b^{lo} dermal dendritic cells are activated by Thymic Stromal Lymphopoietin during contact sensitization in mice.

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Supplementary Figures and Legends

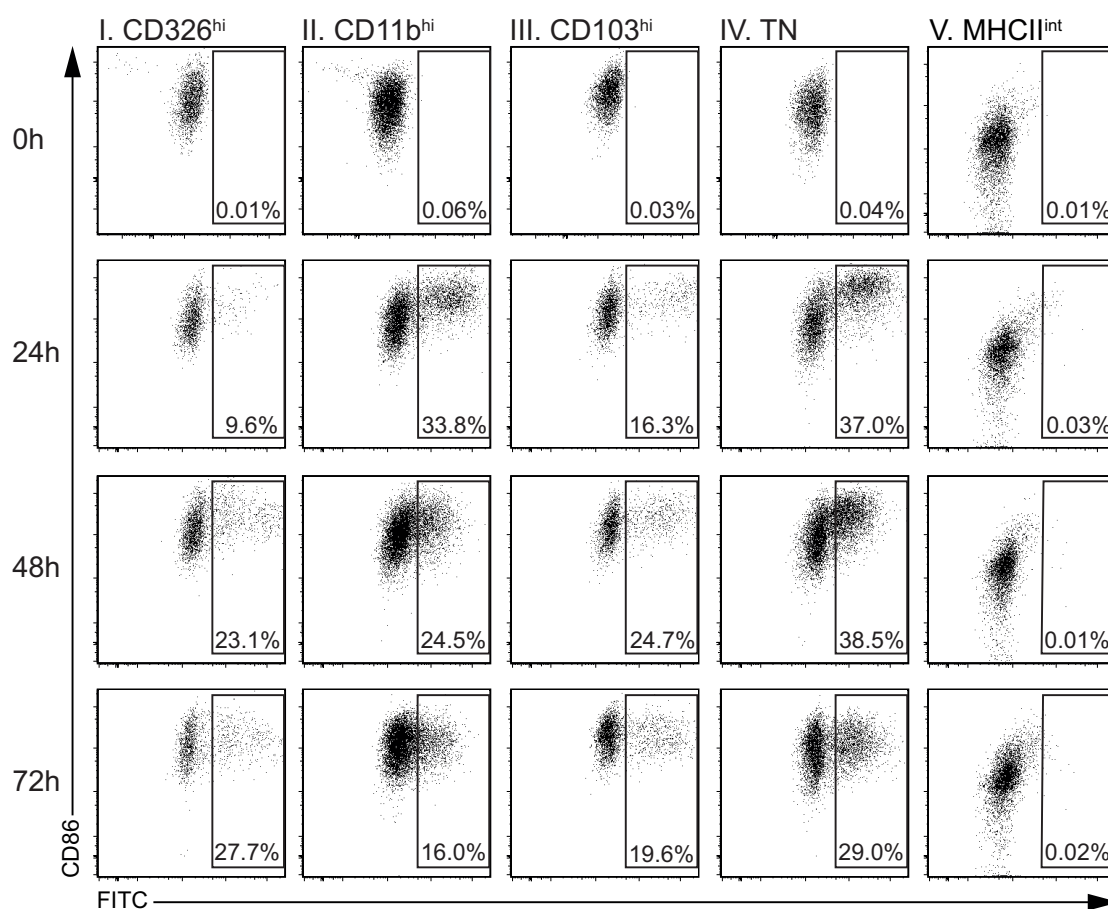


Figure S1: FITC uptake and increased CD86 expression in LN DC subsets from DBP-FITC-treated mice. C57BL/6 mice were treated with DBP-FITC on ear skin and DC subsets in the ear dLN were examined by flow cytometry at the indicated times, using the gating strategy described in Figure 1. One set of representative dot plots is shown. Summary data are shown in Figure 1.

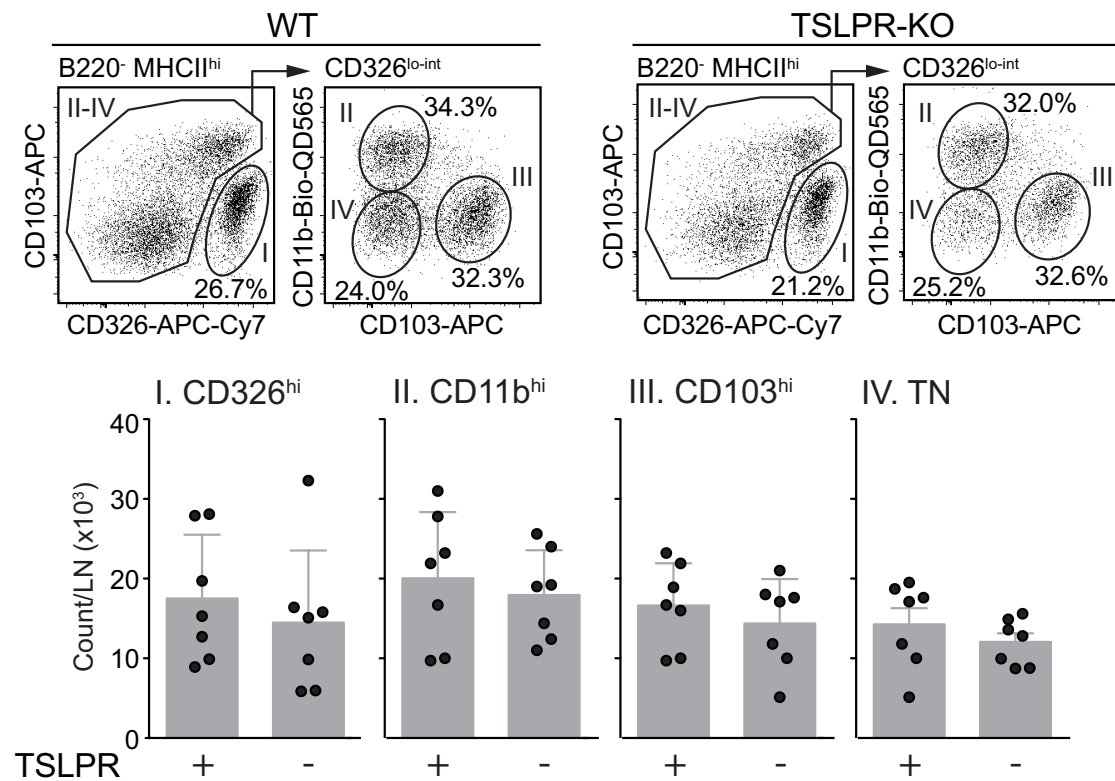


Figure S2. The ear-dLN of WT and TSLPR-deficient mice contain the same DC subsets. DC subsets in the ear-dLN of naïve C57BL/6 (WT) and TSLPR-deficient mice were identified by flow cytometry. Representative flow plots from one of 7 mice per group are shown at the top. The bar graphs show numbers of DC/LN, data are expressed as means + SD. Each dot refers to one mouse. Data are from one of two repeat experiments (n = 5-7) that gave similar results.

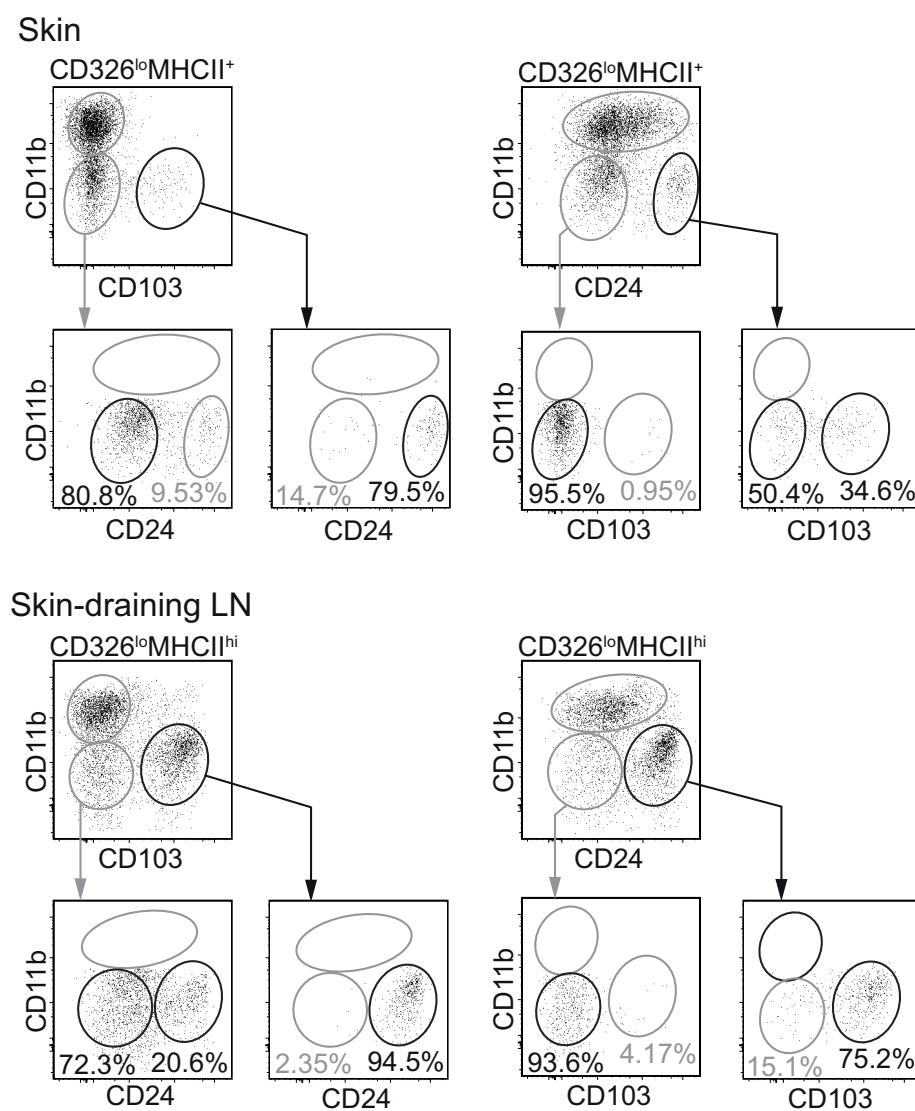


Figure S3. The ear skin and ear-dLN contain a population of
CD11b^{lo}CD24^{hi}CD103^{lo} DC. Skin preparations from the ears and ear-dLN of naïve C57BL/6 mice were made into single cell suspensions and analyzed by flow cytometry using the indicated surface markers. For each tissue, all the flow plots show data acquired from the same sample.

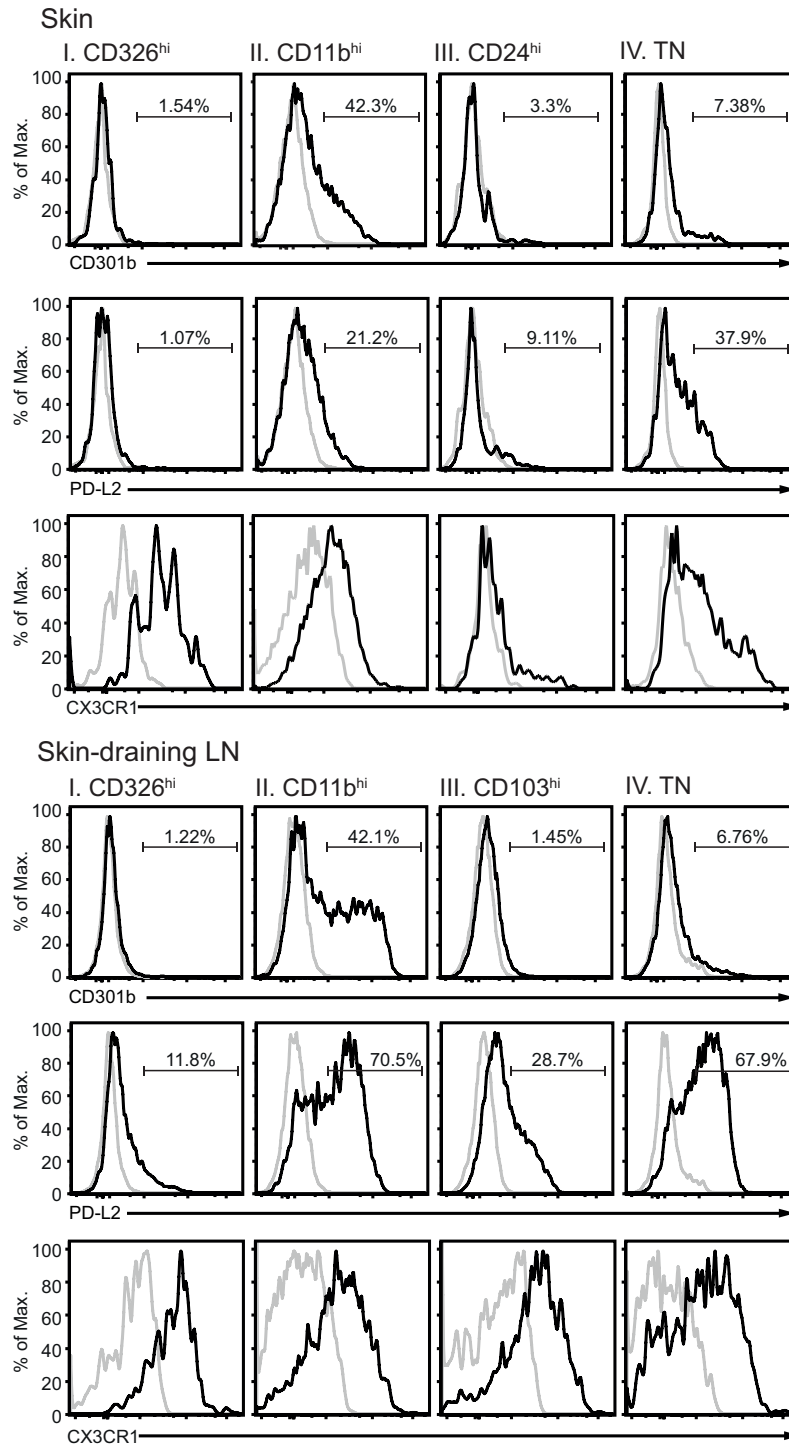


Figure S4. Expression of CD301b, PD-L2 and CX3CR1 on DC subsets in skin and skin-draining LN. Ear skin and ear-dLN were harvested from naïve C57BL/6 mice, and DC subsets, defined as in Figure 5A and Figure 1, respectively, were examined for expression of CD301b, PD-L2 and CX3CR1 by flow cytometry. Black lines, staining; gray lines, FMO controls.