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J Immunol 2014; 193:2504-2511; Prepublished online 23 July 2014; doi: 10.4049/jimmunol.1400536
http://www.jimmunol.org/content/193/5/2504

Supplementary Material http://www.jimmunol.org/content/suppl/2014/07/23/jimmunol.1400536.DCSupplemental

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CD326loCD103loCD11blo 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 CD326loCD103loCD11blo 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 ligation (1). TSLP binds to a receptor complex composed of an IL-7Rα 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). 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+CD11c+ 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 CD326loCD11b+CD103lo, 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...
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-Ptpa−/− 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 (Gamacell 40 exactor, Nordion International) given 4 h apart. Twenty-four hours later the irradiated hosts received 8–10 × 10^6 BM cells from B6.SJL-Ptpa−/− (CD45.1) donor mice. For competitive repopulation experiments, 18 B6. SJL-Ptpa−/− hosts were sublethally irradiated with 700 cGy and received a BM graft (8–10^6 BM cells) consisting of wild-type (WT) B6.SJL-Ptpa−/− 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 μl of a solution of 0.5% FITC (Sigma-Aldrich, St Louis, MO) in 1:1 DBP (BDH Laboratory Supplies, Poole, U.K.)/acetone (Pacific SphereLimited, 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 μg HDM/or (24). For bacterial treatments, Mycobacterium haemophilus haemophilus Calmette-Guérin Pasteur (BCG, provided by Dr. J Triccas, Centenary Institute) or Escherichia coli DH5α (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 10^6 (BCG) or 10^7 (E. coli) CFU/μl/mouse as described (25). TSLP (R&D Systems, Minneapolis, MN) was injected i.d. at 200 ng TSLP/30 μl 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-μm nylon cell strainer (BD Falcon).

Flow cytometry

Multicolor flow cytometry was performed on a BD LSRII SORP or cuseq-10-laser LSRII cytometer (Becton Dickinson, San Jose, CA) with FACSDiva software (BD Biosciences) for data acquisition and FlowJo software (Tree Star, Ashland, OR) for analysis. All MBF incubations were in PBS containing 2% FCS, 10 mM EDTA, and 0.02% sodium azide. Nonspecific MBF binding was blocked using anti-CD16/CD32 (clone 2.4G2, made in house). Brilliant Violet 785-anti-B220 (RA3-6B2), Pacific Blue-anti-IA/IE (MS/14.15.2), allophycocyanin-Cy7-anti-CD326 (G8.8), PE-anti-CD301b (URA-1), PE-anti-PD-1.2 (TY25), and biotin-anti-CD11b (M1/70) were from BioLegend (San Diego, CA); PE-anti-CD11c (A2F10.1), PE-anti-CD45R (30F-11) were from BD Biosciences. PE-anti-CD45.2 (104), PE-Cy7-CD11c (HL3), and allophycocyanin–biotin–anti-CD45.2 (A20), PE-anti-CD54, PE-anti-CD64 (X54-5/7.1.1), PerCP-Cy5.5-anti-CD45.1 (A20), and biotin-anti-CD103 (2E7), PE–anti-CD14 (Sa2-8), and PerCP–anti-CD103 (2E7) were from BioLegend (San Diego, CA); PE-anti-CD68 (ED1), PE-anti-CD11b (M1/70) were from BioLegend (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 fixed samples and fixed samples, respectively. Compensation was set in each experiment using compensation beads (Life Technologies).

Morphology

Cell suspensions were sorted using BD FACSVantage 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+MHC class II (MHCII)3 (Fig. 1A, subset I–IV), and the CD11cintMHCIIhi subset was further subdivided into a CD326hi and CD103hi DC population that included epidermal Langerhans cells (LC) (26) (Fig. 1A, subset V), and the CD11cintMHCIIhi subset was further subdivided into a CD326hi and CD103hi population that included epidermal Langerhans cells (LC) (26) (Fig. 1A, subset V), and the CD11cintMHCIIhi subset was further subdivided into a CD326hi and CD103hi population that included epidermal Langerhans cells (LC) (26) (Fig. 1A, subset V). FITC uptake was detectable in each of the CD11c+MHCIIint 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 CD11bhi DC were FITC+ at early time points, whereas the proportions of FITC+ CD326hi and CD103hi DC were lower. The CD11c+MHCIIint 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 CD11bhi DC. Some CD86 upregulation was also observed on CD103hi DC, whereas CD86 upregulation on CD326hi 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).
expressed as means ± SD; each dot refers to one mouse. The 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 ∼3- to 4-fold compared with controls, whereas CD11bhi DC were increased ∼2.5-fold and CD103hi 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 CD11bhi and CD103hi 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 ∼2 for the CD326hi and CD103hi subsets in WT mice, but increased to ∼6 for TN DC and ∼3 for CD11bhi DC (Fig. 2E). In TSLPR-deficient mice, all ratios were ∼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 ∼2-fold in rTSLP-treated compared with untreated mice, and this increase required TSLP expression (Fig. 3A). The number of CD11bhi DC was also slightly increased, but this was not statistically significant. No changes were detectable in the numbers of CD326hi or CD103hi 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 instead of anti-CD103 to exclude LC (28) and the small CD103loCD24hi 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 ∼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 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.
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 CD11bhi DC, whereas the numbers of TN and CD103hi DC were unchanged, and CD326hi DC were transiently decreased (Fig. 4A). Injection of BCG or *E. coli* bacterial suspensions also induced a substantial increase in the number of CD11bhi DC, with no change in the number of TN DC (Fig. 4B). The numbers of CD326hi and CD103hi 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+MHCIIhi DC population expressing low levels of CD24 and CD11b (Fig. 5A, Supplemental Fig. 3). Compared to CD103hi (in dLN) and CD24hi (in skin) dermal DC, the TN DC expressed low to intermediate CD11c and were CD200R+, CD14+, CD135hi, 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 CD11bhi 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 CD103hi DC from the same LN. Both populations exhibited a DC-like morphology but, compared with CD103hi 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%.
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 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 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 CD11bhi DC, and less pronounced in other DC subsets. This observation was somewhat surprising with regard to the CD326hi DC subset, as CD326hi 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 CD326hi 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 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...
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^hi 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 sensitization, 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 DBP-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

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**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 μm.
CD326<sup>lo</sup>CD103<sup>lo</sup>CD11b<sup>lo</sup> SKIN DC RESPOND TO TSLP

**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<sup>-</sup>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<sup>-</sup>)−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<sup>-</sup>)−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.

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, which had typical DC morphology, and expressed a CD64<sup>+</sup>CD135<sup>+</sup> 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 monocyte precursors. Lastly, TN DC could efficiently phosphorylate STAT3 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 over-expressing 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<sup>+</sup> 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.

**Acknowledgments**

We are sincerely grateful to Dr. Warren Leonard (National Institutes of Health) for the gift of TSLPR-deficient mice, Prof. Bernard Malissen (Centre d’Immunologie de Marseille-Luminy) for Langerin-EGFP mice, Prof. Thor Lenzschoka (Mount Sinai Medical Center, New York, NY) for provision of FLT3-deficient mice, and Dr. Niroshana Andanabapathy (Harvard Medical School) for discussion. We thank colleagues at the Malaghan Institute of Medical Research for advice and discussion, and we acknowledge the staff of the Cell Technology Suite for cell sorting and support during flow cytometry experiments, as well as Biomedical Research Unit for expert animal husbandry.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


