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IRF4 Promotes Cutaneous Dendritic Cell Migration to Lymph Nodes during Homeostasis and Inflammation

Sandra Bajaña, Kimberly Roach, Sean Turner, Jinny Paul, and Susan Kovats

Migration of resident dendritic cells (DC) from the skin to local lymph nodes (LN) triggers T cell-mediated immune responses during cutaneous infection, autoimmune disease, and vaccination. In this study, we investigated whether the development and migration of skin-resident DC were regulated by IFN regulatory factor 4 (IRF4), a transcription factor that is required for the development of CD11b+ splenic DC. We found that the skin of naive IRF4−/− mice contained normal numbers of epidermal Langerhans cells (eLC) and increased numbers of CD11b+ and CD103+ dermal DC (dDC) populations, indicating that tissue DC development and skin residency is not disrupted by IRF4 deficiency. In contrast, numbers of migratory eLC and CD11b+ dDC were significantly reduced in the cutaneous LN of IRF4−/− mice, suggesting a defect in constitutive migration from the dermis during homeostasis. Upon induction of skin inflammation, CD11b+ dDC in IRF4−/− mice did not express the chemokine receptor CCR7 and failed to migrate to cutaneous LN, whereas the migration of eLC was only mildly impaired. Thus, although dispensable for their development, IRF4 is crucial for the CCR7-mediated migration of CD11b+ dDC, a predominant population in murine and human skin that plays a vital role in normal and pathogenic cutaneous immunity. The Journal of Immunology, 2012, 189: 3368–3377.

Dendritic cells (DC) in barrier tissues such as skin are crucial for the initiation of immune responses to infection, vaccination, or environmental chemicals that induce allergic contact dermatitis. Cutaneous DC also contribute to the pathology of skin diseases such as psoriasis and atopic dermatitis (1) and cutaneous graft-versus-host disease secondary to alloge-

nuclear bone marrow transplantation (2). Inflammation of the skin triggers activation of epidermal DC and dermal DC (dDC), followed by their migration to cutaneous lymph nodes (cLN) where they present peripheral Ags to T cells (3). Tissue DC also constitutively migrate to cLN during homeostasis (4) and therein play an important role in the induction of tolerance to self- or environmental Ags (5). Thus, identification of the mechanisms that regulate skin DC migration will provide insight into immunity and tolerance as well as disease pathogenesis.

Multiple subsets of migratory tissue DC have been identified in the skin and within the CD11c+MHCIIhi compartment in cLN (reviewed in Ref. 6). These include the epidermal langerin+ CD11b+CD103− Langerhans cells (termed eLC), which also may be found in transit in the dermis, and at least three dDC subsets, the langerin+ CD11b+CD103+ DC (termed Cd11bhi dDC), langerin− CD11b+CD103+ CD (termed CD11blow dDC), and the langerin+ CD11b+CD103− CD (termed CD103+ dDC). The CD11b+ DC make up the greatest fraction of dDC, whereas CD103+ dDC are present in minor numbers. These phenotypically defined tissue DC subtypes also are distinguished by their developmental dependence on specific precursors, particular combinations of transcription factors, and one or more of the cytokines Flt3 ligand, GM-CSF, or M-CSF (7, 8). eLC require TGF-β, IFN regulatory factor (IRF)8, ID2, RUNX3, and M-CSF for their development and are derived from self-renewing precursors that seed the skin in fetal life, although they also develop from monocytes during inflammation (8). CD103+ dDC develop from conventional DC (cDC)-restricted precursors (pre-cDC) present in blood, peripheral tissue, and lymphoid organs in a process that requires Flt3 ligand and GM-CSF and the transcription factors IRF8, ID2, and BATF3 (9–13). In this regard, CD103+ dDC show similar developmental requirements to conventional CD8α−CD11b+low DC in the spleen (11).

In contrast, the developmental requirements for the CD11b+ dDC are less well defined. CD11b+ tissue DC depend on Flt3 li-
gand, M-CSF, and GM-CSF, and adoptive transfer and lineage-tracing experiments showed that CD11b+ tissue DC can be derived from both blood monocytes and pre-cDC (9, 14, 15). However, the transcription factors required for their development remain unknown. One candidate was IRF4 because IRF4−/− mice lack conventional CD11b+CD8α− DC in the spleen, and in this early work, a role of IRF4 in nonlymphoid tissue DC subsets was not investigated (16, 17). Furthermore, in GM-CSF–driven cultures, IRF4−/− BM precursors develop into CD11c+CD11b+ cells that have reduced CIITA mRNA and fail to significantly express MHC class II (MHCII), suggesting a block in a late developmental stage (16, 17). This prior work led us to test the hypothesis that CD11b+ dDC development requires IRF4.

Although most DC subsets have the ability to present Ag and trigger T cell responses, their different developmental requirements and mature phenotypes suggest DC are organized into distinct lineages, which are specialized for particular functional responses. These functional responses may be regulated by the same transcription factors required for DC development, because expression of these factors is usually maintained in mature DC (16, 17). Indeed, both IRF4 and IRF8 regulate inflammatory cytokine pro-

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duction, albeit by distinct mechanisms (18, 19). IRF8 promoted chemokine-induced migration of eLC in vitro and contact hypersensitivity (CHS) in vivo, although effects of IRF8 deficiency to reduce eLC development or impair migration could not be easily distinguished in vivo (12).

Tissue DC migration to cLN in homeostasis and inflammation requires the chemokine receptors CCR7 and CXCR4 (4, 20). Although eLC use CXCR4 for migration from the epidermis to the dermis (21), eLC and dDC present in the dermis require CCR7 for entry into the dermal lymphatic vessels and for localization in the T cell zone of LN (4). DC derived from bone marrow of IRF8-/- mice showed reduced levels of CCR7 mRNA, suggesting IRF8 regulates CCR7, consistent with the defect in eLC migration in these mice (12). IRF4 regulates chemokine receptor expression in pre-B cells (22), but a role for IRF4 in regulation of chemokine receptors during skin DC migration has not been reported. Studies of DC migration in CHS models of allergic contact dermatitis have revealed roles for migratory langerin+ tissue DC subsets: CD103+ dDC promote but are not absolutely required for CHS, whereas eLC may suppress CHS (3, 11, 23). However, although they constitute the majority of migrating DC, transcription factors governing the function of CD11b+ dDC during the CHS response have not been identified.

In this study, we used IRF4-/- mice (24) to determine whether IRF4 regulates skin DC development and/or migration during homeostasis and inflammation. We found that naive IRF4-/- mice

![Image of Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** IRF4-/- cLN have reduced numbers of epidermal Langerhans cells and dermal CD11b+ DC. (A) Total numbers of inguinal LN cells in IRF4+/+ and IRF4-/- mice. In all graphs, symbols indicate individual female (○) and male (●) mice. The mean and SEM are indicated. The fraction of MHCIhi CD11c+ DC (B) and the numbers of MHCIhiCD11c+ DC (C) in individual mice were determined by flow cytometry. (D) Shown is the gating of MHCIhi CD11c+ DC (percentage indicated) within total LN cells. The gating of langerin+ and langerin- DC within the MHCIhiCD11c+ fraction (E), CD103+ and CD11b+ DC within the langerin+ fraction (F), and CD11b+ DC within the langerin- fraction (G). (H) The numbers of eLC (CD11b+ langerin+ CD103+), CD11b+ dDC (CD11b+ langerin+CD103+), CD11b+ dDC (CD11b+ langerin+CD103-), and CD11b+ dDC (CD11b+ langerin-CD103-) in LN of IRF4+/+ (+/-) and IRF4-/- (-/-) mice. The data were analyzed using a two-way ANOVA with Bonferroni post tests to identify significant differences between sex and genotype. The variance was not due to an interaction between genotype and sex. Therefore, we used a nonparametric Mann–Whitney U test on combined male and female data (n = 16–20) to determine significant differences in IRF4+/+ and IRF4-/- genotypes; p values are indicated. A significant sex difference was present only within the CD103+ dDC population in both IRF4+/+ and IRF4-/- mice (two-way ANOVA; p = 0.0089).
harbor normal numbers of eLC in the epidermis and increased numbers of CD11b+ and CD103+ DC in the dermis, indicating that the development and skin residency of these DC subsets is not disrupted by IRF4 deficiency. However, numbers of migratory eLC and CD11b+ dDC were significantly reduced in the cLN of IRF4+/− mice in homeostasis, suggesting a defect in migration from dermis to LN, consistent with the increased numbers of CD11b+ dDC in the dermis. In contrast, the CD103+ dDC subset showed a modest increase in development and migration from the dermis to the cLN during homeostasis and skin inflammation.

**Materials and Methods**

**Mice**

Heterozygous IRF4+/− on the C57BL/6 background (24) were obtained from Dr. T. Mak (University of Toronto, Toronto, ON, Canada) and bred at the Oklahoma Medical Research Foundation to yield the female and male IRF4+−/− littermates analyzed in experiments. Mice were analyzed at 5–6 wk of age. Female CD45.1+ C57BL/6 mice were obtained from the National Cancer Institute’s Animal Production Program. The Oklahoma Medical Research Foundation’s Institutional Animal Care and Use Committee approved the studies.

**Cell isolation**

cLN (axillary and brachial) were digested to a single-cell suspension with collagenase type D (1 mg/ml) and DNAse I (0.1 mg/ml) (both from Roche) in Ca2+- and Mg2+-free HBSS at 37˚C for 30 min. Dermal and epidermal sheets were obtained after treatment of the skin with trypsin (0.25%) in HBSS for 1 h at 37˚C. A dermal cell suspension was obtained upon digestion of dermal sheets in RPMI 1640 medium with 10% FCS, collagenase type D (5 mg/ml), DNAse I (0.2 mg/ml), and hyaluronidase (1.5 mg/ml) (from Worthington) at 37˚C for 1 h as described previously (25). The epidermal sheets were aspirated up and down in a syringe and then filtered to obtain a single-cell suspension. When purified for RNA isolation, epidermal DC were sorted as CD45+MHCII+ cells.

**Flow cytometry**

After isolation from tissue, cells were immediately processed for flow cytometry by preincubating with anti-CD16/32 and labeling with optimally titered mAbs in FACS buffer (PBS, 5% newborn calf serum, and 0.1% sodium azide). LN, spleen, and dermal cells and bone marrow-derived DC were stained with six to seven parameter combinations of fluorochrome or biotin-labeled mAbs specific for CD45.2, CD11c, CD8a, CD11b, CD103, langerin, MHCII, B220, CCR7, and CD86 (obtained from BD Biosciences, eBioscience, or BioLegend). After surface marker staining, intracellular staining with the anti-langerin Ab was done using a buffer kit from BD Biosciences. Epidermal cells were stained with mAbs specific for CD45.2 and MHCII. Samples were run on an LSRII instrument (BD Biosciences) and data analyzed with FlowJo (Tree Star) software.

In the cLN, eLC were gated as CD11c+MHCII+CD8a−langerin−CD11b+CD103+ dDC were gated as CD11c+MHCII+CD8a−langerin−CD11bhiCD103+ and CD11b− dDC were gated as CD11c+MHCII+CD8a−langerin−CD11bhiCD103+ and CD103− dDC were gated as CD11c+MHCII+CD8a−langerin−CD11blowCD103−.

**Immunohistochemistry**

After removal of fat and cartilage, ear skin was incubated in 0.5 M EDTA for 1 h at 37˚C to separate the epidermal sheets from the dermis as described previously (26). The epidermal sheets were fixed in cold acetone for 20 min and incubated with a PE-conjugated anti-langerin Ab (eBioscience). At least eight images per sample were captured by Axiosvision software after visualization with an Axiovert 200M microscope at ×20 magnification. The numbers of eLC per square millimeter were calculated.

![FIGURE 2](http://www.jimmunol.org/) Numbers of epidermal Langerhans cells are similar in IRF4+/+ and IRF4−/− mice in homeostasis. (A) Epidermal sheets from ear skin of IRF4+/+ and IRF4−/− mice stained with an anti-langerin Ab. Lower panels show DC morphology at a higher magnification. Scale bars, 50 μm (upper panels); 20 μm (lower panels). (B) Numbers of eLC per square millimeter in individual female (○) and male (●) mice (n = 4–5/genotype). The data were analyzed using a Mann–Whitney U test. (C) Epidermal eLC within a preparation of ear skin epidermal cells were identified by expression of CD45.2 and MHCII. (D) The percentage of MHCII+ DC in the epidermis of multiple mice was determined (n = 5–9).
Induction of CHS

Dibutyl phthalate-acetone (1:1) mixed with a fluorescent cell tracker, chloromethyl fluorescein diacetate (CMFDA; 2 mM) (Invitrogen), was applied to the skin of one ear (26). After 24 or 72 h, DC subpopulations in the dermis, epidermis, and the draining auricular LN were analyzed by multiparameter flow cytometry.

Bone marrow DC cultures

DC were differentiated from bone marrow cells using a GM-CSF-driven model as described previously (27). On day 7, cells were stimulated for 12–18 h with 100 ng/ml LPS.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qPCR) of the Ccr7 gene was performed on cDNA generated from the RNA of bone marrow-derived DC and sorted epidermal eLC. Relative expression of genes was determined using the ∆∆Ct method with normalization to Gapdh expression. Specific primer sequences were as follows: sense, Gapdh, 5'-AGGTCGGTGAGGAACCCAAGTTTG-3'; and antisense, Gapdh, 5'-TGATGATGTGAGGAGTCA-3'; and sense, Ccr7, 5'-CCGTGGCAGACATCCTTTTC-3', and antisense, Ccr7, 5'-AGGTAGCGCAAAAGATCCAG-3'.

Chemotaxis assay

Bone marrow-derived DC were activated for 12 h with LPS, and 300,000 cells were plated in the upper well of 5 μM Transwell plates (Costar) in RPMI 1640 medium plus 1% BSA. CCL21 (100 ng/ml) (PeproTech) was present in the lower chamber. After 3 h, the number of migrated MHCII+ cells was determined by flow cytometry.

Adoptive DC transfer

Bone marrow-derived DC from wild-type (WT) and IRF4−/− mice (CD45.2+) were stimulated for 12 h with LPS and labeled with Cell Trace CFSE or Cell Trace Violet (Invitrogen), according to reagent instructions. Bone marrow-derived DC were activated for 12 h with LPS, and 300,000 cells were plated in the upper well of 5 μM Transwell plates (Costar) in RPMI 1640 medium plus 1% BSA. CCL21 (100 ng/ml) (PeproTech) was present in the lower chamber. After 3 h, the number of migrated MHCII+ cells was determined by flow cytometry.

Statistical analyses

Significant differences between values measured in female and male IRF4+/+ and IRF4−/− mice were determined using the nonparametric Mann–Whitney U test, the Wilcoxon matched-pairs signed rank test, and the unpaired t test. p < 0.05. To identify possible sex differences in the effect of genotype on a measured parameter, the data in Fig. 1 also were analyzed using a two-way ANOVA with Bonferroni post tests. The variance in genotype measurements was not due to an interaction between the variables of genotype and sex, so it was possible to combine female and male data in the Mann–Whitney U test.

Results

Although the total number of cLN cells is similar in young IRF4+/+ and IRF4−/− mice, the number of migratory skin-resident CD11b+ DC in cLN is significantly reduced in IRF4−/− mice during homeostasis

We used flow cytometry to analyze migratory DC populations in cLN of young ~5-wk-old mice in which the total number of cLN cells is similar in IRF4+/+ or IRF4−/− WT mice (Fig. 1A). Analysis of young mice was important because IRF4−/− mice develop lymphadenopathy at ~12 wk of age because of the accumulation of T and B cells (24).

IRF4−/− mice showed a significant reduction in the percentage and number of skin-derived migratory CD11c MHCIIP population of migratory DC, IRF4−/− mice showed a significant reduction in the percentage and number of three DC subpopulations, including eLC (langerin+CD11b−CD103−), CD11b+ dDC (langerin−).

FIGURE 3. The dermis of IRF4−/− mice has an increased proportion of both CD11b+ and CD103+ dDC subsets in homeostasis. (A) The percentage of MHCIIP cells in the dermis in individual mice. (B) The percentage of each DC subset within the MHCIIP fraction in the dermis of individual mice. (C) Gating of MHCIIP cells (percentage indicated) within a dermal cell suspension. (D) Gating of langerin+ and langerin− populations within the MHCIIP cells of the dermis. The lower panel shows a “fluorescence minus one” staining in which the anti-langerin Ab was omitted. (E) Gating of the CD11b+ eLC and CD103− dDC within the langerin+ population. (F) Gating of CD11b− dDC within the langerin− population. In all graphs, females (○) and males (●) are indicated. The p values indicate significant differences in IRF4+/+ and IRF4−/− genotypes (males and females combined), determined using a nonparametric Mann–Whitney U test; n = 5.
CD11b<sup>hi</sup>CD103<sup>−</sup>) and CD11b<sup>low</sup> dDC (langerin<sup>−</sup>CD11b<sup>low</sup>CD103<sup>−</sup>) (Fig. 1E–H). In contrast, the number of CD103<sup>+</sup> dDC (langerin<sup>+</sup>CD11b<sup>low</sup>CD103<sup>+</sup>) tended to increase in IRF4<sup>−/−</sup> mice but was not significantly different (Fig. 1H). These data led us to suspect that, like conventional splenic CD11b<sup>+</sup>CD4<sup>+</sup> DC (Supplemental Fig. 1), IRF4 was required for the development of CD11b<sup>+</sup> subsets of epidermal DC and dDC.

Previously, we showed that IRF4 expression in myeloid progenitors is increased by estrogen receptor α signaling during GM-CSF–mediated DC differentiation (27). Thus, in this study, we noted the sex of the mice to determine a possible sex bias in IRF4-regulated phenotypes. Statistical analyses showed that the effect of IRF4 deficiency on migratory DC numbers did not differ in males and females. However, we did find that females have greater numbers of CD103<sup>+</sup> dDC in cLN in both WT and IRF4<sup>−/−</sup> mice (p = 0.0089) (Fig. 1H).

In IRF4<sup>−/−</sup> mice, CD11b<sup>+</sup> eLC are present in normal numbers in the epidermis but accumulate in the dermis in homeostasis.

We next determined whether IRF4 deficiency led to reduced numbers of resident dermal and epidermal DC. We used fluorescence microscopy with an anti-langerin Ab to identify eLC in epidermal sheets. The number, distribution, and morphology of langerin<sup>+</sup> DC in the epidermis were not different between WT and IRF4<sup>−/−</sup> mice (Fig. 2A, 2B). Similar results were obtained using epidermal cell suspensions and flow cytometry to detect CD45<sup>+</sup> MHCII<sup>+</sup> eLC (Fig. 2C, 2D).

We determined the presence of DC subsets in the dermis by flow cytometry (Fig. 3C–F, Supplemental Fig. 2). Because accurate dermal cell counts are difficult to obtain, we monitored the fraction of CD45<sup>+</sup> cells in the dermis of WT and IRF4<sup>−/−</sup> mice and determined that it was similar (Supplemental Fig. 2A); thus, it was reasonable to compare the percentages of DC subsets between...
The fraction of MHCII<sup>+</sup>CD11c<sup>+</sup> cells in the dermis was not significantly different in IRF4<sup>−/−</sup> mice (Fig. 3A). Importantly, the CD11b<sup>+</sup> dDC subset was significantly increased in IRF4<sup>−/−</sup> mice (Fig. 3B). The CD103<sup>+</sup> dDC subset also was increased, whereas the eLC subset in the dermis was not significantly different (Fig. 3B, Supplemental Fig. 2). Again, we noted no significant differences between males and females.

These data show that the development of skin-resident eLC and CD11b<sup>+</sup> dDC does not require IRF4 and suggests that the reduction in these two DC subsets in IRF4<sup>−/−</sup> cLN is due to a failure of these cells to migrate from the skin to LN, leading to accumulation of the CD11b<sup>+</sup> dDC, but not eLC, in the dermis. Furthermore, the data show that IRF4 deficiency leads to the increased numbers of CD103<sup>+</sup> DC in the dermis, although there is no defect in their constitutive migration to LN.

In CHS, CD11b<sup>+</sup> dDC in IRF4<sup>−/−</sup> mice accumulate in the dermis and fail to migrate to cLN.

In a commonly used assay for CHS, increased migration of skin-resident DC into the cLN occurs in response to epicutaneous application of hapten dissolved in chemical sensitizing agents. Prior studies showed that the CD11b<sup>hi</sup> dDC and CD103<sup>+</sup> dDC migrate to the cLN within 24 h of application of the chemical sensitizing agent, whereas CD11b<sup>low</sup> dDC and eLC migrate within 2–4 d (28, 29). To evaluate the role of DC during the sensitization phase of CHS, we painted the skin of one ear in WT and IRF4<sup>−/−</sup> mice. The migration of skin DC populations 72 h after application of dibutyl phthalate-acetone/CMFDA was determined the migration of skin DC populations 72 h after application of dibutyl phthalate-acetone/CMFDA. In WT mice at 72 h post-CHS, the majority of migratory cells bearing the cell tracker (CMF<sup>+</sup>) in the draining LN were within the CD11b<sup>+</sup> dDC subset, although some CMF<sup>+</sup> eLC and CD103<sup>+</sup> dDC were present in the LN (Fig. 4C, 4D). In contrast, in IRF4<sup>−/−</sup> mice, CMF<sup>+</sup>CD11b<sup>+</sup> dDC (including both CD11b<sup>hi</sup> and CD11b<sup>low</sup> subsets) were absent in the draining LN, indicating a profound defect in their migration to the LN (Fig. 4C, 4D). This defect was also evident when the total number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC in the cLN at 24 h post-CHS was counted (Fig. 4G).

The migration of CMF<sup>+</sup>CD103<sup>+</sup> dDC was significantly increased in IRF4<sup>−/−</sup> mice at 24 h post-CHS (Fig. 4C), consistent with the increased numbers of this population in the dermis during homeostasis and upon CHS (Figs. 3B, 5B).

At 24 h post-CHS, numbers of migrating CMF<sup>+</sup> eLC tended to be reduced in IRF4<sup>−/−</sup> mice but were not significantly different from eLC numbers in WT mice (Fig. 4C); this may be due to the fact that most eLC migrate at later time points. Therefore, we also determined the migration of skin DC populations 72 h after application of dibutyl phthalate-acetone/CMFDA. In WT mice at 72 h post-CHS, the majority of migratory cells bearing the cell tracker (CMF<sup>+</sup>) in the draining LN were within the eLC subset (Fig. 4E, 4F). The numbers of migratory CMF<sup>+</sup> eLC in cLN tended to be decreased in IRF4<sup>−/−</sup> mice but were not significantly different from numbers in WT mice (Fig. 4E). Consistent with this, the total number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC was not significantly different in WT and IRF4<sup>−/−</sup> mice at 72 h post-CHS (Fig. 4G). The numbers of CMF<sup>+</sup>CD11b<sup>+</sup> dDC in LN of IRF4<sup>−/−</sup> mice remained very low at 72 h post-CHS, indicating that the defect in migration of CD11b<sup>+</sup> dDC in IRF4<sup>−/−</sup> mice was not transient (Fig. 4E).

In the dermis, the proportion of MHCII<sup>+</sup> DC in LN was reduced in IRF4<sup>−/−</sup> mice (Fig. 4A). This reduction was primarily due to a 10-fold reduction in the number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC (Fig. 4B). At 24 h post-CHS in WT mice, the majority of migratory cells bearing the cell tracker (CMF<sup>+</sup>) in the draining LN were within the CD11b<sup>+</sup> dDC subset, although some CMF<sup>+</sup> eLC and CD103<sup>+</sup> dDC also were present in the LN (Fig. 4C, 4D). In contrast, in IRF4<sup>−/−</sup> mice, CMF<sup>+</sup>CD11b<sup>+</sup> dDC (including both CD11b<sup>hi</sup> and CD11b<sup>low</sup> subsets) were absent in the draining LN, indicating a profound defect in their migration to the LN (Fig. 4C, 4D). This defect was also evident when the total number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC in the cLN at 24 h post-CHS was counted (Fig. 4G).

In CHS, IRF4<sup>−/−</sup> mice have an increased percentage of CD11b<sup>+</sup> and CD103<sup>+</sup> dDC subsets in the dermis and MHCII<sup>+</sup> DC in the epidermis. (A and B) Dibutyl phthalate-acetone (1:1) and a fluorescent cell tracker, CMFDA. After 24 h, we analyzed DC subpopulations in the draining auricular LN, using the cell tracker to identify newly migrated DC. As during homeostasis, the total number of MHCII<sup>+</sup> DC in LN was reduced in IRF4<sup>−/−</sup> mice (Fig. 4A). This reduction was primarily due to a 10-fold reduction in the number of CD11b<sup>+</sup> dDC (Fig. 4B). At 24 h post-CHS in WT mice, the majority of migratory cells bearing the cell tracker (CMF<sup>+</sup>) in the draining LN were within the CD11b<sup>+</sup> dDC subset, although some CMF<sup>+</sup> eLC and CD103<sup>+</sup> dDC also were present in the LN (Fig. 4C, 4D). In contrast, in IRF4<sup>−/−</sup> mice, CMF<sup>+</sup>CD11b<sup>+</sup> dDC (including both CD11b<sup>hi</sup> and CD11b<sup>low</sup> subsets) were absent in the draining LN, indicating a profound defect in their migration to the LN (Fig. 4C, 4D). This defect was also evident when the total number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC in the cLN at 24 h post-CHS was counted (Fig. 4G).

The migration of CMF<sup>+</sup>CD103<sup>+</sup> dDC was significantly increased in IRF4<sup>−/−</sup> mice at 24 h post-CHS (Fig. 4C), consistent with the increased numbers of this population in the dermis during homeostasis and upon CHS (Figs. 3B, 5B).

At 24 h post-CHS, numbers of migrating CMF<sup>+</sup> eLC tended to be reduced in IRF4<sup>−/−</sup> mice but were not significantly different from eLC numbers in WT mice (Fig. 4C); this may be due to the fact that most eLC migrate at later time points. Therefore, we also determined the migration of skin DC populations 72 h after application of dibutyl phthalate-acetone/CMFDA. In WT mice at 72 h post-CHS, the majority of migratory cells bearing the cell tracker (CMF<sup>+</sup>) in the draining LN were within the eLC subset (Fig. 4E, 4F). The numbers of migratory CMF<sup>+</sup> eLC in cLN tended to be decreased in IRF4<sup>−/−</sup> mice but were not significantly different from numbers in WT mice (Fig. 4E). Consistent with this, the total number of CMF<sup>+</sup> migratory MHCII<sup>+</sup>CD11c<sup>+</sup> DC was not significantly different in WT and IRF4<sup>−/−</sup> mice at 72 h post-CHS (Fig. 4G). The numbers of CMF<sup>+</sup>CD11b<sup>+</sup> dDC in LN of IRF4<sup>−/−</sup> mice remained very low at 72 h post-CHS, indicating that the defect in migration of CD11b<sup>+</sup> dDC in IRF4<sup>−/−</sup> mice was not transient (Fig. 4E).

In the dermis, the proportion of MHCII<sup>+</sup> DC was similar in WT and IRF4<sup>−/−</sup> mice at 24 h postapplication of the contact sensitizer.
Nevertheless, assessment of DC subsets (gated as in Supplementary Fig. 3) showed that relative to WT mice, the dermis of IRF4−/− mice bore an increased fraction of CD11b+ dDC and a moderately decreased fraction of eLC (Fig. 5B). In the epidermis at 24 h post-CHS, in addition to the MHCIint population observed during homeostasis (Fig. 2C), a second population of CD45−

**FIGURE 6.** IRF4−/− DC in the dermis show reduced expression of CCR7. (A) Dibutyl phthalate-acetone (1:1) and the fluorescent cell tracker CMFDA were applied to ear skin, and dermal and epidermal cells were harvested after 24 h. The gating of dermal and epidermal DC subsets is shown in Fig. 5C and (B) CD45+MHCII+ eLC were sorted from a pooled epidermal cell suspension derived from three to five mice during homeostasis (as in Fig. 2C), and separate populations of MHCIint and MHCIhi eLC were sorted from the epidermis of individual mice (n = 2–5) 24 h after application of dibutyl phthalate-acetone (1:1) and the fluorescent cell tracker CMFDA (as in Fig. 5C). The relative expression of Ccr7 RNA was determined using qPCR. For the eLC isolated during homeostasis, the data point is the mean of triplicates of the pooled sample (three to five mice) for the PCR. For the eLC isolated post-CHS, each data point is the mean of triplicates of a sample from a single mouse for the PCR reaction. The significance of the difference between Ccr7 RNA levels in populations of MHCIint and MHCIhi eLC in IRF4+/+ mice was evaluated using an unpaired t test. (C–G) DC were differentiated via GM-CSF from bone marrow cells isolated from IRF4+/+ (top panels) and IRF4−/− (bottom panels) mice, and stimulated with LPS for 12–18 h. (C) In LPS-stimulated cells, the outer box indicates the gating of total CD11c+ cells (75% IRF4+/+ versus 76% IRF4−/−) and the inset box indicates the gating of CD11c+ MHCIint DC (35% IRF4+/+ versus 95% IRF4−/−). The graphs are representative of three to five mice of each genotype. (D) The expression of CD86 on resting or LPS-stimulated DC (gated on total CD11c+ cells) is shown. (E) The expression of CCR7 on resting or LPS-stimulated DC (gated on total CD11c+ cells) is shown. (F) The expression of CCR7 RNA on LPS-stimulated CD11c+ cells (relative to CCR7 RNA on resting cells, as in E) in cultures generated from individual mice of each genotype, n = 3–5. The significance of these data were evaluated using a Mann–Whitney U test. (G) IRF4+/+ and IRF4−/− DC generated from the bone marrow of individual mice were left unstimulated or stimulated for 12 h with LPS. The relative expression of Ccr7 RNA was determined using qPCR; each data point is the mean of triplicates of an individual sample for the PCR. The significance of these data (n = 3) was evaluated using a one-way ANOVA, followed by a Bonferroni’s multiple comparison test.
from these dDC subsets to assess (Fig. 6A). Unfortunately, we were unable to isolate intact RNA present in the dermis did show reduced levels of surface CCR7

minor reduction in migration from dermis to LN, IRF4

dermis during CHS (Fig. 6B). However, despite their relatively

migration to CCL21 relative to medium alone, IRF4

migrate across a membrane barrier toward the chemokine CCL21, DC were placed into a Transwell chemotaxis assay in which DC DC were compared in assays of chemotaxis and in vivo migration.

Trace Violet, mixed 1:1, and transferred via intradermal injection of donor CD45.2+ WT and IRF4

CCR7 RNA levels were also similar in the activated MHCII

eLC isolated from WT and IRF4

expression versus RNA expression in Fig. 6A, 6B, 6E–G), it is possible that the apparently reduced CCR7 level on dermal eLC was sufficient to mediate migration of the majority of eLC from the dermis to the LN.

As previously reported, CD11b

CD11c

DC in GM-CSF–driven cultures generated from IRF4

bone marrow derived to significantly increase MHCII and CD86 expression upon LPS stimulation (Fig. 6C, 6D). IRF4

bone marrow-derived DC also expressed significantly reduced surface CCR7 and Ccr7 mRNA after LPS activation (Fig. 6E–G), suggesting that IRF4 promotes Ccr7 transcription. Taken together, these data show that IRF4 activity promotes the expression of CCR7 in skin-resident CD11b

dDC and in bone marrow-derived DC.

FIGURE 7. IRF4

DC show an intrinsic defect in migration in vitro and in vivo. (A) Migration of LPS-activated IRF4

and IRF4

bone marrow-derived DC toward the chemokine CCL21 in a transwell chemotaxis assay. Data points represent the number of migrated DC in individual wells; DC are from two IRF4

mice (three wells each). Data were evaluated using an unpaired t test. (B–F) LPS-activated IRF4

and IRF4

bone marrow-derived DC (CD45.2

) labeled with CFSE or Cell Trace Violet were mixed together in equal numbers and injected intradermally into recipient CD45.1

mice. Inguinal LN were analyzed for the presence of donor DC after 36 h. (B) Identification of CFSE-labeled IRF4

DC and Cell Trace Violet-labeled IRF4

DC among total LN cells. (C) Donor DC populations gated in B are CD45.2

IRF4

DC consistently expressed lower levels of CD45.2 than IRF4

DC, independent of the label used. (D) Identification of Cell Trace Violet-labeled IRF4

DC and CFSE-labeled IRF4

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mice each labeled with CFSE (○) or Cell Trace Violet (●). The percentage of transferred DC among total LN cells is plotted. The significance of these data (n = 10) was evaluated using a Wilcoxon matched-pairs signed rank test.

The regulation of CCR7 expression in eLC in IRF4

mice is more complex. Consistent with the only minor reduction in eLC migration in IRF4

mice, WT and IRF4

eLC (total MHCII

fraction) in the epidermis displayed similar levels of cell surface CCR7 (Fig. 6A). Induced Ccr7 RNA levels were also similar in the activated MHCII

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epidermis during CHS (Fig. 6B). However, despite their relatively minor reduction in migration from dermis to LN, IRF4

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dDC subsets in the dermis requires intracutaneous langerin staining, which involves paraformaldehyde fixation. Because the flow cytometric staining of CCR7 is relatively insensitive compared with RNA levels (e.g., compare the magnitude of the activation-induced changes in surface protein expression versus RNA expression in Fig. 6A, 6B, 6E–G), it is possible that the apparently reduced CCR7 level on dermal eLC is more complex. Consistent with the only minor reduction in eLC migration in IRF4

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mice each labeled with CFSE (○) or Cell Trace Violet (●). The percentage of transferred DC among total LN cells is plotted. The significance of these data (n = 10) was evaluated using a Wilcoxon matched-pairs signed rank test.

To determine whether IRF4 deficiency has a cell autonomous effect on DC migration, isolated WT and IRF4

bone marrow-derived DC were compared in assays of chemotaxis and in vivo migration. DC were placed into a Transwell chemotaxis assay in which DC migrate across a membrane barrier toward the chemokine CCL21, a ligand of CCR7. Although WT DC showed a ~3-fold increase in migration to CCL21 relative to medium alone, IRF4

DC failed to migrate toward CCL21 (Fig. 7A).

To assess migration in vivo, CD45.2

WT and IRF4

bone marrow-derived DC were separately labeled with CFSE or Cell Trace Violet, mixed 1:1, and transferred via intradermal injection into recipient WT CD45.1

mice. After 36 h, the relative numbers of donor CD45.2

WT and IRF4

DC in the popliteal LN of each mouse were determined (Fig. 7B–E). Within each mouse, greater numbers of WT than IRF4

DC migrated to the LN (Fig. 7F). The reduced ability of IRF4

DC to migrate to the LN was independent of the label used (Fig. 7B–E). Taken together, these data show that DC-intrinsic IRF4 deficiency results in a reduced ability to migrate toward CCL21 in vitro and to LN in vivo.
Discussion
In this work, we evaluated the role of IRF4 in the development, skin residence, and LN-directed migration of migratory tissue DC subsets that are normally present in the epidermis and dermis and also constitute a discrete population of MHCIIhi cells in cLN. Our data show that IRF4 is not required for the development and skin residence of epidermal DC and dDC but that it promotes the migration of CD11b+ dDC and eLC from the skin to the LN during homeostasis.

Upon induction of skin inflammation in IRF4−/− mice, the CD11b+ dDC also failed to migrate to the LN. This correlated with their significantly reduced surface CCR7, a chemokine receptor that is crucial for migration of skin DC to cLN during homeostasis and inflammation. Indeed, IRF4 likely promotes expression of the Ccr7 gene, because LPS-stimulated bone marrow-derived IRF4−/− DC showed a significant reduction in Ccr7 RNA. Activated IRF4−/− CD11b+ bone marrow-derived DC failed to migrate toward the CCR7 ligand CCL21 in a chemotaxis assay, suggesting a DC-intrinsic effect of IRF4 deficiency on migration.

In contrast to CD11b+ dDC, the migration of IRF4−/− eLC from skin to cLN was only minimally impaired during inflammation. This minor defect in migration was reflected in an increased fraction of MHCIIhi eLC in the epidermis and a decreased fraction of eLC in the dermis of IRF4−/− mice. MHCIIhi eLC present in the epidermis of IRF4−/− and IRF4−/− mice 24 h post-CHS contained comparable amounts of Ccr7 RNA, indicating that IRF4 is not strictly required for the increased Ccr7 transcription that occurs in activated eLC. However, CCR7 surface expression on eLC did not always correlate with the Ccr7 RNA levels and eLC migration pattern during inflammation. Although CCR7 levels on eLC in the epidermis were similar in IRF4−/− and IRF4−/− mice, eLC in the dermis in IRF4−/− mice did show a reduction in CCR7. Taken together, these data suggest that IRF4-independent chemokine receptors, perhaps regulated by IRF8, may have a stronger effect on eLC migration in an inflammatory environment than during homeostasis.

Our data suggest that the Ccr7 gene may be a direct transcriptional target of IRF4. IRF4 acts alone by binding IFN-stimulated response elements or is recruited to composite IRF/PU.1 binding sites on target genes by phosphorylated PU.1. Predicted IFN-stimulated response elements and PU.1 binding sites are present upstream of the Ccr7 gene, consistent with direct binding of IRF4. Alternately, IRF4 may indirectly regulate Ccr7 transcription through collaboration with other transcription factors such as NF-kB and AP-1, which do bind to conserved regions in the promoter of Ccr7 gene, a pathway likely to occur secondary to TLR signaling in DC (31).

CCR7 may be regulated by both IRF4 and IRF8 because Ccr7 RNA levels were decreased in IRF8−/− bone marrow-derived DC (12). Because IRF4−/− CD103+ dDC migrate efficiently, our data suggest that in the absence of IRF4, the putative IRF8 expression in CD103+ dDC was sufficient to promote CCR7 expression, which was previously shown to be required for entry of CD103+ dDC into the cLN but not the dermis (32).

Our data raise questions about the role of IRF4 in the development of the diverse CD11b+ DC subsets found in non-lymphoid tissue and lymphoid organs. In contrast to the finding that splenic CD11b+CD4+ dDC do not develop in IRF4−/− mice (Supplemental Fig. 1) (16, 17), our data show that IRF4 is not required for the initial development of CD11b+ dDC. This is puzzling because splenic CD11b+ dDC and dermal CD11b+ DC can develop from a population of transferred pre-cDC, a process that one might expect to require IRF4 expression in pre-cDC (9, 13). However, lineage-tracing and monocyte transfer experiments showed that CD11b+ tissue DC can arise from monocytes in homeostasis (9, 15, 33, 34). Thus, it is possible that the dermal CD11b+ DC present in the skin of IRF4−/− mice are derived primarily from monocytes in an IRF4-independent process, whereas the pre-cDC to CD11b+ DC pathway in the dermis and spleen is disrupted by IRF4 deficiency. We also found that MHCIIhiCD11b+ resident DC in cLN are not reduced in IRF4−/− mice in homeostasis (Supplemental Fig. 1). These data suggest that this resident DC subset also may derive primarily from monocytes in the absence of IRF4.

We also noted a modest increase in CD103+ DC in the dermis in IRF4−/− mice, suggesting that their IRF8-dependent development is enhanced by IRF4 deficiency. This could result from the disruption of the competition between IRF4 and IRF8 for common promoter binding sites or partners such as PU.1 (30). Thus, the absence of IRF4 may increase the efficiency of recruitment of IRF8/PU.1 complexes to composite IRF/PU.1 sites in target genes, thereby enhancing CD103+ dDC development.

DC migration from peripheral tissue to LN is critical for initiation of T cell-mediated immune responses that occur in infection, autoimmune disease, and vaccination. Our study now places IRF4 in a vital regulatory role during the cLN-directed migration of CD11b+ dDC, the most abundant DC subset present in murine and human skin (1). Indeed, in humans, this IRF4-regulated DC migration pathway is likely to be critical for initiation of adaptive immune responses in skin diseases such as psoriasis and atopic dermatitis, as well as cutaneous infections, graft-versus-host disease, allergic contact dermatitis, and dermal vaccination. Furthermore, our study reveals the diverse roles of IRF4 within the complex network of transcription factors that regulates differentiation of DC from precursors as well as mature DC function.

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Disclosures
The authors have no financial conflicts of interest.

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