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

Sandra Bajana, 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.

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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...
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 in IRF4−/− mice. Upon induction of skin inflammation in a CHS assay, CD11b+ dDC in IRF4+/+ and IRF4−/− mice did not express CCR7 and failed to migrate to the draining LN. However, eLC migration after induction of CHS was only mildly impaired in IRF4−/− mice, suggesting that IRF4 governs eLC migration primarily in the absence of inflammation. Taken together, these data show that although not required for their development, IRF4 is crucial for CD11b+ dDC expression of CCR7 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 littermates analyzed in experiments. Mice were analyzed at 5–6 wk of age. Female CD45.1+ C57BL/6 mice 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 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

eLC (auxillary 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 dDC were stained with biotin-labeled mAbs specific for CD45.2, CD11c, CD8α, 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+CD8α−langerin+CD11b−CD103−, CD11b+CD103−, CD11bCD103− dDC were gated as CD11c+MHCII+CD8α−langerin+CD11b−CD103−, CD11b+CD103−, and CD103− dDC were gated as CD11c+MHCII+CD8α−langerin+CD11b−CD103−.

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. 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, 20 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**

Dibutyryl 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 culture 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′-AGGTGGTGTGAAGGAGA-3′; antisense, Gapdh, 5′-TGTAGACCATGTTGAAGTCA-3′; sense, Ccr7, 5′-CCGTGGCAGACATCCTTTTC-3′; and antisense, Ccr7, 5′-AGGTAGACGCCAAAGATCCAG-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. A total of 10⁶ cells of each genotype were mixed 1:1 and injected intradermally into recipient CD45.1+ C57BL/6 mice as described previously (4). In independent transfers, WT and IRF4+/− DC were labeled with each cell tracer to ensure that migration was not affected by the cell tracer or labeling procedures. After 36 h, popliteal LN cells were analyzed for the presence of CD45.2+ cell tracer-labeled donor DC 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, the unpaired t test, or a one-way ANOVA with Bonferroni post tests in Prism software as indicated in the figure legends. Differences were considered significant when 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+MHCII+ DC in cLN (Fig. 1B–D). Within this CD11c+MHCII+ 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−CD11b−CD103−), and CD11b−dDC (langerin+CD11b−CD103+).
CD11bhiCD103+), and CD11blow dDC (langerin+CD11blow CD103+) (Fig. 1E–H). In contrast, the number of CD103+ dDC (langerin+CD11b+CD103+) tended to increase in IRF4−/− mice but was not significantly different (Fig. 1H). These data led us to suspect that, like conventional splenic CD11b+CD4+ DC (Supplemental Fig. 1), IRF4 was required for the development of CD11bhi 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+ dDC in cLN in both WT and IRF4−/− mice (p = 0.0089) (Fig. 1H).

In IRF4−/− mice, CD11b+ 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+ DC in the epidermis were not different between WT and IRF4−/− mice (Fig. 2A, 2B). Similar results were obtained using epidermal cell suspensions and flow cytometry to detect CD45+ MHCIIC+ 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+ cells in the dermis of WT and IRF4−/− mice and determined that it was similar (Supplemental Fig. 2A); thus, it was reasonable to compare the percentages of DC subsets between

**FIGURE 4.** In CHS, CD11b+ dDC fail to migrate to cLN, whereas cell tracker-bearing CD103+ dDC migrate in increased numbers in IRF4−/− mice. (A–D) Dibutyl phthalate-acetone (1:1) and the fluorescent cell tracker CMFDA were applied to ear skin, and cLN cells were harvested after 24 h. (A) The number of MHCIIC+CD11c+ DC in the draining auricular LN. (B) Relative to eLC and CD103+ dDC, the total number of CD11b+ dDC was significantly reduced in LN of IRF4−/− mice. (C) The number of CMF cells within the CD11b+ dDC subset was significantly reduced, and the number of CMF cells within the CD103+ dDC subset was significantly increased, 24 h post-CHS in LN of IRF4−/− mice. (D) Gating of CMF cells within the indicated migratory DC subset in cLN at 24 h. (E and F) Dibutyl phthalate-acetone (1:1) and CMFDA were applied to ear skin, and cLN cells were harvested after 72 h. (E) The number of CMF cells within each migratory DC subset was determined. (F) Gating of CMF cells within the indicated migratory DC subset in cLN at 72 h. (G) The number of CMF cells with the gated MHCIIC+CD11c+ fraction (as in Supplemental Fig. 3A) in IRF4+/+ and IRF4−/− cLN at 24 and 72 h post-CHS was determined. In all graphs, females (○) and males (●) are indicated. The p values indicate significant differences in the IRF4+/+ and IRF4−/− genotypes (males and females combined), determined using a nonparametric Mann–Whitney U test; n = 3–8.
mice. The fraction of MHCII$^{+}$CD11c$^{+}$ cells in the dermis was not significantly different in IRF4$^{+/+}$ mice (Fig. 3A). Importantly, the CD11b$^{+}$ dDC subset was significantly increased in IRF4$^{−/−}$ mice (Fig. 3B). The CD103$^{+}$ 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$^{+}$ dDC does not require IRF4 and suggests that the reduction in these two DC subsets in IRF4$^{−/−}$ cLN is due to a failure of these cells to migrate from the skin to LN, leading to accumulation of the CD11b$^{+}$ dDC, but not eLC, in the dermis. Furthermore, the data show that IRF4 deficiency leads to the increased numbers of CD103$^{+}$ DC in the dermis, although there is no defect in their constitutive migration to LN.

In CHS, CD11b$^{+}$ dDC in IRF4$^{−/−}$ 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 haptens dissolved in chemical sensitizing agents. Prior studies showed that the CD11b$^{hi}$ dDC and CD103$^{+}$ dDC migrate to the cLN within 24 h of application of the chemical sensitizing agent, whereas CD11b$^{lo}$ 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$^{−/−}$ mice (Fig. 4A). This reduction was primarily due to a 10-fold reduction in the number of CD11b$^{+}$ dDC (Fig. 4B). At 24 h post-CHS in WT mice, the majority of migratory cells bearing the cell tracker (CMF$^{+}$) in the draining LN were within the CD11b$^{+}$ dDC subset, although some CMF$^{+}$ eLC and CD103$^{+}$ dDC also were present in the LN (Fig. 4C, 4D). In contrast, in IRF4$^{−/−}$ mice, CMF$^{+}$CD11b$^{+}$ dDC (including both CD11b$^{hi}$ and CD11b$^{lo}$ 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$^{+}$ migratory MHCII$^{+}$CD11c$^{+}$ DC in the cLN at 24 h post-CHS was counted (Fig. 4G).

The migration of CMF$^{+}$CD103$^{+}$ dDC was significantly increased in IRF4$^{−/−}$ 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$^{+}$ eLC tended to be reduced in IRF4$^{−/−}$ 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$^{+}$) in the draining LN were within the eLC subset (Fig. 4E, 4F). The numbers of migratory CMF$^{+}$ eLC in cLN tended to be decreased in IRF4$^{−/−}$ mice but were not significantly different from numbers in WT mice (Fig. 4E). Consistent with this, the total number of CMF$^{+}$ migratory MHCII$^{+}$CD11c$^{+}$ DC was not significantly different in WT and IRF4$^{−/−}$ mice at 72 h post-CHS (Fig. 4G). The numbers of CMF$^{+}$CD11b$^{+}$ dDC in LN of IRF4$^{−/−}$ mice remained very low at 72 h post-CHS, indicating that the defect in migration of CD11b$^{+}$ dDC in IRF4$^{−/−}$ mice was not transient (Fig. 4E).

In the dermis, the proportion of MHCII$^{+}$ DC was similar in WT and IRF4$^{−/−}$ mice at 24 h postapplication of the contact sensitizer

**FIGURE 5.** In CHS, IRF4$^{−/−}$ mice have an increased percentage of CD11b$^{+}$ and CD103$^{+}$ dDC subsets in the dermis and MHCII$^{+}$ DC in the epidermis. (A and B) Dibutyl phthalate-acetone (1:1) and a fluorescent cell tracker CMFDA were applied to ear skin, and dermal cells were harvested after 24 h. DDC subsets were identified by flow cytometry as in Supplemental Fig. 3. The percentage of total MHCII$^{+}$ cells (A) and each DC subset (B) within the MHCII$^{+}$ population in the dermis of multiple mice was determined. (C–E) Dibutyl phthalate-acetone (1:1) and the fluorescent cell tracker CMFDA were applied to ear skin, and epidermal cells were harvested after 24 h. (C) CD45$^{+}$ epidermal DC were distinguished by two distinct levels of MHCII. The percentage of total MHCII$^{+}$ DC (D) and MHCII$^{+}$ DC (E) in the epidermis of multiple mice was determined. In all graphs, females (○) and males (●) are indicated. The p values indicate significant differences in the IRF4$^{+/+}$ and IRF4$^{−/−}$ genotypes (males and females combined), determined using a nonparametric Mann–Whitney U test, n = 3–9.
However, assessment of DC subsets (gated as in Supplemental Fig. 3) showed that relative to WT mice, the dermis of IRF4<sup>−/−</sup> mice bore an increased fraction of CD11b<sup>+</sup> dDC and a moderately decreased fraction of eLC (Fig. 5B). In the epidermis at 24 h post-CHS, in addition to the MHCI<sup>int</sup> population observed during homeostasis (Fig. 2C), a second population of CD45<sup>+</sup>

**FIGURE 6.** IRF4<sup>−/−</sup> 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 Supplemental Fig. 3. (A) Expression of CCR7 on CD11b<sup>+</sup> dDC, CD103<sup>+</sup> dDC, and eLC subsets in dermis and total MHCI<sup>+</sup> eLC in the epidermis of IRF4<sup>−/−</sup> (thick solid line) and IRF4<sup>+/+</sup> (shaded histogram) mice. The dotted line (control) indicates cells stained for DC markers but not CCR7 ("fluorescence minus one" control). The graphs are representative of three to four mice of each genotype. (B) CD45<sup>+</sup>MHCI<sup>+</sup> 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 MHCI<sup>int</sup> and MHCI<sup>hi</sup> 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 MHCI<sup>int</sup> and MHCI<sup>hi</sup> eLC in IRF4<sup>+/+</sup> mice was evaluated using an unpaired t test. (C–G) DC were differentiated via GM-CSF from bone marrow cells isolated from IRF4<sup>+/+</sup> (top panels) and IRF4<sup>−/−</sup> (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<sup>+</sup> cells (75% IRF4<sup>+/+</sup> versus 76% IRF4<sup>−/−</sup>) and the inset box indicates the gating of CD11c<sup>+</sup> MHCI<sup>+</sup> DC (35% IRF4<sup>+/+</sup> versus 9% IRF4<sup>−/−</sup>). 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<sup>+</sup> cells) is shown. (E) The expression of CCR7 on resting or LPS-stimulated DC (gated on total CD11c<sup>+</sup> cells) is shown. (F) The expression of 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<sup>−/−</sup> and IRF4<sup>+/+</sup> 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.
MHCI\textsuperscript{hi} eLC was observed (Fig. 5C). Relative to WT mice, IRF4\textsuperscript{-/-} mice showed a moderate increase in this fraction of activated MHCI\textsuperscript{hi} eLC in the epidermis, suggesting that migration of the most activated eLC was mildly impaired (Fig. 5C–E). Taken together, these analyses of migratory DC in the dermis, epidermis, and draining eLN show that in IRF4\textsuperscript{-/-} mice, CD11b\textsuperscript{+} dDC cannot migrate from dermis to eLN. Furthermore, as manifested by their slight increase in the epidermis and decrease in the dermis, eLC in IRF4\textsuperscript{-/-} mice may have an attenuated ability to migrate from epidermis to dermis during inflammation.

In IRF4\textsuperscript{-/-} mice, dermal CD11b\textsuperscript{+} DC display reduced levels of CCR7 upon contact sensitivity

CCR7 promotes the migration of skin-resident DC into eLN during homeostasis and during CHS (4, 20). Thus, we evaluated the expression of CCR7 on DC subsets in the dermis (gated as in Supplemental Fig. 3) and epidermis (gated as in Fig. 5C) 24 h after application of the contact sensitizer. Expression of CCR7 on IRF4\textsuperscript{-/-} CD11b\textsuperscript{+} dDC and CD103\textsuperscript{+} dDC correlated with their migratory phenotype. IRF4\textsuperscript{-/-} CD11b\textsuperscript{+} dDC displayed reduced CCR7 levels, whereas CD103\textsuperscript{+} dDC showed normal levels of CCR7 (Fig. 6A).

The regulation of CCR7 expression in eLC in IRF4\textsuperscript{-/-} mice is more complex. Consistent with the only minor reduction in eLC migration in IRF4\textsuperscript{-/-} mice, WT and IRF4\textsuperscript{-/-} eLC (total MHCI\textsuperscript{hi}) fraction) in the epidermis displayed similar levels of cell surface CCR7 (Fig. 6A). Induced Ccr7 RNA levels were also similar in the activated MHCI\textsuperscript{hi} eLC isolated from WT and IRF4\textsuperscript{-/-} epidermis during CHS (Fig. 6B). However, despite their relatively minor reduction in migration from dermis to LN, IRF4\textsuperscript{-/-} eLC present in the dermis did show reduced levels of surface CCR7 (Fig. 6A). Unfortunately, we were unable to isolate intact RNA from these dDC subsets to assess Ccr7 mRNA levels because distinction of eLC and CD11b\textsuperscript{+} dDC subsets in the dermis requires intracellular 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 was sufficient to mediate migration of the majority of eLC from the dermis to the LN.

As previously reported, CD11b\textsuperscript{+}CD11c\textsuperscript{+} DC in GM-CSF–driven cultures generated from IRF4\textsuperscript{-/-} bone marrow failed to significantly increase MHCI and CD86 expression upon LPS stimulation (Fig. 6C, 6D). IRF4\textsuperscript{-/-} 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\textsuperscript{+} dDC and in bone marrow-derived DC.

IRF4\textsuperscript{-/-} DC show an intrinsic defect in migration in vivo and in vitro

To determine whether IRF4 deficiency has a cell autonomous effect on DC migration, isolated WT and IRF4\textsuperscript{-/-} 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\textsuperscript{-/-} DC failed to migrate toward CCL21 (Fig. 7A).

To assess migration in vivo, CD45.2\textsuperscript{+} WT and IRF4\textsuperscript{-/-} 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\textsuperscript{+} mice. After 36 h, the relative numbers of donor CD45.2\textsuperscript{+} WT and IRF4\textsuperscript{-/-} DC in the popliteal LN of each mouse were determined (Fig. 7B–E). Within each mouse, greater numbers of WT than IRF4\textsuperscript{-/-} DC migrated to the LN (Fig. 7F). The reduced ability of IRF4\textsuperscript{-/-} 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 MHCII\(^{+}\) 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 MHCII\(^{+}\) eLC in the epidermis and a decreased fraction of eLC in the dermis of IRF4\(^{-/-}\) mice. MHCII\(^{+}\) 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 (30). Thus, IRF4 may indirectly regulate Ccr7 transcription through collaboration with other transcription factors such as NF-kB and AP-1, which do bind to consensus 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 nonlymphoid tissue and lymphoid organs. In contrast to the finding that splenic CD11b\(^{+}\)CD4\(^{+}\) cDC 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\(^{+}\)CDC 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 MHCII\(^{+}\)CD11b\(^{+}\) 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.

References


Supplemental Fig. 1. Conventional CD11b+ CD4+ CD8α- DC are decreased in the spleen but not the cLN of IRF4−/− mice. [Related to Fig. 1.] The fraction (A) and number (B) of CD11b+ CD4+ CD8α- DC within the MHCII+ B220- CD11c+ splenic DC population in individual female and male mice during homeostasis were identified by flow cytometry. The fraction (C) and number (D) of CD11b+ CD4+ CD8α- DC within the MHCII+ B220- CD11c+ DC population in cLN of individual mice (the same mice as in panel A-B) were identified by flow cytometry. The significance of these data (n=4-5) was evaluated using an unpaired t test; ***, p<0.001.

Supplemental Fig. 2. The dermis of IRF4−/− mice has an increased proportion of both CD11b+ DC and CD103+ dDC subsets. [Related to Fig. 3.] Here we show an alternate staining and gating strategy to identify dermal DC during homeostasis. In this strategy, the intracellular anti-langerin staining was omitted, which allows better definition of the minor CD103+ dDC subset. (A) The percentage of CD45+ cells in the ear dermis in individual IRF4+/+ and IRF4−/− mice. (B) Gating of MHCII+ cells in the dermis. (C) The percentage of MHCII+ cells in the dermis of individual mice. (D) Gating of CD11b+ DC (combined dDC and eLC) and CD103+ dDC populations within the MHCII+ cells (gated as in panel B) of the dermis. The proportion of (E) CD11b+ cells (including dDC and eLC) and (F) CD103+ dDC within the MHCII+ cells of the dermis in individual mice was determined. Females (open circles) and males (closed circles) are indicated. The p values indicate significant differences in IRF4+/+ and IRF4−/− genotypes (males and females combined), determined using a nonparametric Mann-Whitney test, n=9-10.
Supplemental Fig. 3. Gating of DC subsets in the dermis at 24 hr post-CHS. [Related to Fig. 5A,B and 6A]. Dibutyl phthalate-acetone (1:1) and the fluorescent cell tracker CMFDA were applied to ear skin and dermal cells harvested after 24 hr. Cells were stained with Ab specific for MHCII, CD11c, langerin, CD11b, CD103 and CCR7. (A) Definition of MHCII$^+$ cells. (B) Definition of langerin$^+$ and langerin$^-$ fractions within the MHCII$^+$ cells. The control is a “fluorescence minus one” staining in which the anti-langerin Ab was omitted. (C) Definition of CD11b$^+$ eLC and CD103$^+$ dDC within the langerin$^+$ fraction. (D) Definition of CD11b$^{hi}$ dDC within the langerin$^-$ fraction.
Supplemental Fig. 2

A. CD45^+ cells [%]

B. Total Dermis

C. MHCII

D. Gated: MHCII^+

E. CD11b DC [%]

F. CD103^+ DC [%]
Supplemental Fig. 3

A

IRF4^{+/+}  IRF4^{-/-}

CD11c

B

Gated: MHCII^{+}

Control  IRF4^{+/+}  IRF4^{-/-}

MHCII  langerin

C

Gated: langerin^{+}

IRF4^{+/+}  IRF4^{-/-}

CD11b  CD103

D

Gated: langerin^{-}

IRF4^{+/+}  IRF4^{-/-}

CD11b  langerin