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IRF4 and IRF8 act in CD11c+ cells to regulate terminal differentiation of lung tissue dendritic cells[1]

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Abstract

Dendritic cells (DCs) initiate immune responses in barrier tissues including lung and skin. Conventional DC subsets, $CD11b^-$ (cDC1s) or $CD11b^+$ (cDC2s), arise via distinct networks of transcription factors involving IRF4 and IRF8 and are specialized for unique functional responses. Using mice in which a conditional *Irf4* or *Irf8* allele is deleted in $CD11c^+$ cells, we determined if IRF4 or IRF8 deficiency beginning in $CD11c^+$ cDC precursors (pre-cDCs) changed the homeostasis of mature DCs or pre-DCs in the lung, dermis and spleen. *CD11c-cre-Irf4*−/− mice selectively lacked a lung-resident $CD11c^{hi}CD11b^+SIRP\alpha^+CD24^+$ DC subset, but not other lung $CD11b^+$ DCs or alveolar macrophages. Numbers of $CD11b^+CD4^+$ splenic DCs, but not $CD11b^+$ dermal DCs, were reduced, indicating cDC2s in the lung and dermis develop via different pathways. *Irf4* deficiency did not alter numbers of cDC1s. *CD11c-cre-Irf8*−/− mice lacked lung-resident $CD103^+$ DCs and splenic $CD8\alpha^+$ DCs, yet harbored increased IRF4-dependent DCs. This correlated with a reduced number of *Irf8*−/− pre-cDCs, which contained elevated IRF4, suggesting that *Irf8* deficiency diverts pre-cDC fate. Analyses of *Irf4* and *Irf8* haploinsufficient mice showed that while one *Irf4* allele was sufficient for lung cDC2 development, two functional *Irf8* alleles were required for differentiation of lung cDC1s. Thus, IRF8 and IRF4 act in pre-cDCs to direct the terminal differentiation of cDC1 and cDC2 subsets in the lung and spleen. These data suggest that variation in IRF4 or IRF8 levels resulting from genetic polymorphisms or environmental cues will govern tissue DC numbers and therefore regulate the magnitude of DC functional responses.

Introduction

Lung resident DCs are essential regulators of innate and adaptive immune responses to respiratory pathogens and also promote chronic inflammatory diseases such as asthma (1, 2). Tissue DC subsets, including those in the lung and dermis, are specialized for particular types of functional responses, and their development is governed by specific networks of transcription factors, such as IRF4 and IRF8, expressed in DC progenitors (3–5). Mature DCs continue to express these transcription factors, which specify gene expression programs that direct their functional responses. IRF4-expressing DCs are important for the DC-driven polarization of T_H17 responses in the intestine and lung (6, 7), for the induction of T_H2

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responses in lung allergy and skin parasite models (8–10) and for attenuation of T_H1 responses (11). In turn, IRF8-expressing DCs are often most important for T_H1 and $CD8^+$ T cell responses, although the role of specific DC subsets is context dependent (12–16). In human blood, $CD1c^+$ DCs preferentially express IRF4, while $CD141^+$ DCs preferentially express IRF8 (6, 17). Thus, investigation of the role of IRF4 and IRF8 in the differentiation and homeostasis of DC subsets will help us to understand human inflammatory responses in peripheral tissues.

In mice, conventional DCs (cDCs) in lymphoid organs and nonlymphoid tissues are broadly classified as $CD11b^-$ (cDC1s) or $CD11b^+$ (cDC2s) (18). The $CD11b^-$ DCs express $CD8\alpha^+$ in the spleen and $CD103^+$ in nonlymphoid tissues and require IRF8 and BATF3 for their terminal differentiation (19). $CD11c^+CD11b^+MHCII^+$ cells are heterogeneous in nonlymphoid tissues, and the definition of specific DC subsets has only recently been clarified by approaches to separate true $CD11b^+CD24^+$ cDC2s from macrophages and monocyte-derived DCs (20–22). Because of this diversity, it has been more difficult to discern the transcription factor networks required for terminal differentiation of the cDC2s and other $CD11c^+CD11b^+$ subsets.

IRF4 is required for differentiation of splenic $CD11b^+CD4^+$ cDCs (23, 24). However, the role of IRF4 in the development of tissue cDC2s remained unclear. We used mice globally deficient for *Irf4* to show that IRF4 is not required for development or skin residence of $CD11b^+$ dermal DCs, but does promote migration of dermal $CD11b^+$ DCs to cutaneous LNs (25). More recent work, with mice bearing a conditional *Irf4* allele and a *CD11c-cre* construct that directs Cre activity in $CD11c^{int}$ pre-cDCs and $CD11c^{hi}$ mature DCs (*CD11c-cre-Irf4* −/− mice), showed that numbers of $CD11b^+CD24^+$ lung DCs were reduced but not completely ablated by *Irf4* deficiency; this was correlated with increased apoptosis in the remaining DCs suggesting an effect on DC survival (6). A similar conclusion was reached for $CD103^+CD11b^+$ DCs in the small intestinal lamina propria (SI-LP) in these mice (6, 7). In contrast, mice bearing a conditional *Irf4* allele and a CD11c-cre construct that apparently directs Cre activity only in $CD11c^{hi}$ cells showed no reduction in $CD11b^+CD24^+$ lung DCs (8). Taken together, these studies suggested that *Irf4* deletion beginning at the $CD11c^{int}$ pre-cDC stage diminished *in vivo* survival (and therefore numbers) of $CD11b^+$ lung DCs, while *Irf4* deletion only in mature $CD11c^{hi}$ DCs did not impact their numbers.

Despite these advances, it remained unclear whether IRF4 and IRF8 must act in immediate cDC precursors (pre-cDCs) to promote DC terminal differentiation and/or survival (19). *Irf8* mRNA is present in common DC progenitors (CDPs) and pre-cDCs, while *Irf4* mRNA is present in pre-cDCs but not CDPs [reviewed in (3)]. Global *Irf8* deficiency leads to defects in the formation of the CDP and all splenic DC subsets (26), while global *Irf4* deficiency did not apparently affect CDPs but did abolish splenic $CD11b^+CD4^+$ cDCs, suggesting effects on pre-cDCs (23, 24). Pre-cDCs (defined as [CD19, CD3, NK1.1-negative] $CD11c^{int}$ $MHCII^-$ $SIRP\alpha^{int}$ $Flt3^+$) with the potential to develop into cDCs in lymphoid and nonlymphoid organs have been identified in bone marrow (BM), lymphoid organs and nonlymphoid tissues including lungs (27–29). The pre-cDC population was initially divided based on low or high CD24 expression into precursors pre-committed to either the $CD11b^+$ or the $CD11b^-$ pathways (27). Recent reports used CD24 and other markers to subdivide the

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pre-cDC population into discrete precursors for the cDC1s and cDC2s that preferentially express *Irf8* and *Batf3* mRNA or *Irf4* mRNA, respectively (30, 31). *Irf8*-deficient mice lacked pre-cDC1s (30). However, the effects of *Irf4* and *Irf8* deficiency or haploinsufficiency beginning in pre-cDCs on pre-cDC numbers and their expression of IRF4 and IRF8 proteins *in vivo* have not been well characterized.

Herein, we have reexamined the effect of *Irf4* deficiency on DC differentiation in *CD11c-cre-Irf4* −/− and +/− mice in which Cre activity is present in pre-cDCs and mature DCs. Similar analyses of CD11c-restricted *Irf8* deficiency were done using *CD11c-cre-Irf8* −/− and +/− mice. We determined the effect of *Irf4* and *Irf8* gene dosage on numbers of pre-cDCs and DC subsets and their relative expression of IRF4 and IRF8 proteins. Taken together our data show that changes in IRF4 or IRF8 levels beginning in pre-cDCs have profound effects on relative numbers of the cDC1 and cDC2 subsets in the lung and spleen in homeostasis. Thus, variation in IRF4 or IRF8 levels resulting from environmental stimuli or genetic polymorphisms may regulate tissue DC numbers, and therefore modulate the magnitude of DC functional responses in inflammation. Indeed, polymorphisms in human *IRF4* genes impart susceptibility to melanoma and lymphocytic leukemia, while polymorphisms in *IRF8* genes impart susceptibility to systemic lupus erythematosus or lead to deficient antimycobacterial immunity secondary to the absence of select DC subsets (32–36).

Materials and Methods

Mice

Mice (purchased from The Jackson Laboratory) bearing a conditional allele of *Irf4* (B6.129S1-Irf4<tm1Rdf>/J) (37) were bred to mice bearing Cre recombinase driven by the CD11c promoter (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) (38) and then interbred to yield *CD11c-cre-Irf4* −/−, +/− or +/+ mice. Mice used as "wild-type" were either Cre^{+} but bearing two wild-type alleles of *Irf4*, or Cre^{-} and bearing either wild-type or conditional *Irf4* alleles; we did not note any differences between these two groups. As noted in a prior report, the Cre activity may randomly act in $CD11c^{-}$ cells in this line of CD11c-Cre mice, leading to mice that have the conditional allele deleted in many tissues (7). Therefore, we screened for mice bearing a global deletion of *Irf4* using PCR primers (for: CAGGATGTTGCCGTCCTCCTTG and rev: CCTGCAGCCAATAAGCTTATAAC), and excluded those mice from this study. Similarly, mice bearing a conditional allele of *Irf8* (B6(Cg)-Irf8<tm1.1Hm>/J) (39) were purchased from the Jackson Laboratory, bred to B6.Cg-Tg(Itgax-cre)1-1Reiz/J mice and then interbred to produce *CD11c-cre-Irf8* −/−, +/− or +/+ mice. Since a global deletion of *Irf8* favors development of neutrophils at the expense of DCs (26), we excluded *CD11c-cre-Irf8* mice from analysis that had an enlarged spleen or BM compartment, or high numbers of Ly-$6G^{+}$ cells (identified by mAb Gr-1) in blood; we were unable to develop a PCR assay for this screening in the *CD11c-cre-Irf8* mice. Littermate mice of each genotype and of both sexes were analyzed at 6–10 weeks of age, and we did not find a sex difference in any parameter analyzed. The OMRF IACUC approved the studies.

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Isolation of cells from tissues

Lungs were perfused with PBS prior to digestion. Lung lobes were digested for 60 min with collagenase type D (2 mg/ml) and DNAse I (0.2 mg/ml) (both from Roche) in 10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$. Spleens were digested to a single cell suspension with collagenase type D (1 mg/ml) and DNAse I (0.1 mg/ml) in Ca^{2+} and Mg^{2+}-containing Hank's Balanced Salt Solution (HBSS) at 37°C for 30 min. Bone marrow (BM) cells were isolated as described (40). Red blood cells in tissues were lysed using RBC lysis buffer (BD Biosciences). In some experiments to analyze pre-cDCs, CD11c$^+$ cells in spleen and BM were enriched using a murine CD11c positive selection kit (Stem Cell Technologies).

Flow cytometry

After isolation from tissue, cells were immediately processed for flow cytometry by pre-incubating with anti-CD16/32, and labeling with optimally titered mAbs (obtained from BD Biosciences, eBioscience or Biolegend) in FACS buffer (PBS, 5% newborn calf serum, 0.1% sodium azide). To identify lung DC and macrophage populations, cells were stained with a lymphocyte marker cocktail (mAbs specific for CD19, CD3, B220 and NK1.1 linked to a common fluorochrome) to gate out lymphocytes, in conjunction with various combinations of fluorochrome-labeled mAbs specific for CD11c, MHCII, Siglec F, CD64, CD11b, CD103, SIRPα, CD24 and CD14. Splenic DCs were defined using a combination of mAbs specific for CD11c, CD8α, CD11b, CD4, and MHCII. Pre-cDCs (Lin$^-$ CD11c$^+$ MHCII$^-$ SIRPα^{lo} Flt3$^+$) in BM or spleen were identified using a Lineage cocktail (mAbs specific for CD19, CD3, B220, NK1.1, Ter119 linked to a common fluorochrome) to gate out mature cells, in conjunction with mAbs specific for CD11c, MHCII, SIRPα and Flt3. After surface marker staining, intracellular staining with fluorochrome conjugated mAbs specific for IRF4(PE) and IRF8(APC) was done using a Foxp3 buffer kit (all from eBioscience). Intracellular staining for activated capase 3 (mAb from BD Biosciences) was done in conjunction with live/dead fixable Aqua stain (Invitrogen). The data were collected on an LSRII instrument (BD Biosciences) and analyzed with FlowJo (TreeStar) software.

Statistical analyses

Significant differences between values measured in wild-type and mutant mice were determined using an unpaired t test (if two genotypes compared), or a one-way ANOVA with Tukey post tests (if three genotypes evaluated) in Prism 6 software as indicated in figure legends. Differences were considered significant when $p<0.05$.

Results

To evaluate the role of IRF4 in the terminal differentiation of tissue and lymphoid organ DCs, we bred mice with a conditional *Irf4* allele to mice bearing Cre recombinase driven by the CD11c promoter. The *CD11c-cre-Irf4* mice bear 0, 1 or 2 copies of *Irf4* in CD11c$^+$ cells, and are hereafter designated as *CD11c-cre-Irf4* –/–, +/– or +/+, respectively. CD11c$^+$ cells include pre-cDCs, mature DCs, NK cells and some macrophage populations, including alveolar macrophages. The CD11c-specific *Irf4* deletion did not significantly alter numbers of CD11c$^-$ cell types as the +/– and –/– mice had normal numbers of lung (CD45$^+$), spleen

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and bone marrow cells (Fig. S1). In these mice, the deletion of *Irf4* places an *Egfp* minigene in frame, resulting in GFP expression in CD11c$^+$ cells that deleted the *Irf4* gene in +/– and –/– mice; wild-type mice do not express any GFP. Based on GFP expression, ~100% of CD11c^{hi} DCs have deleted the *Irf4* gene (Fig. 1G, S1). However, a subset (~30%) of CD11c^{int} cells is not clearly GFP$^+$, and thus may not have deleted the *Irf4* gene (Fig. S1, S2D). Backgating of GFP$^+$ and GFP$^-$ CD11c^{hi} cells in the lung shows that all CD11c^{hi} MHCII$^+$ cells are GFP$^+$, while only a subset of CD11c^{int} cells are GFP$^+$ (Fig. S1C,D). GFP$^-$ CD11c^{hi} cells are primarily alveolar macrophages.

To also evaluate the role of IRF8 in the terminal differentiation of lung tissue DCs, we bred mice bearing a conditional *Irf8* allele to the *CD11c-cre* mice; these mice do not bear a *Gfp* reporter. Total numbers of bone marrow cells, lung (CD45$^+$) cells and splenocytes were not altered in *CD11c-cre-Irf8* +/– and –/– mice (Fig. S1).

Through comparisons of *CD11c-cre-Irf4* and *CD11c-cre-Irf8* –/–, +/– and +/+ mice, we determined if a reduction or absence of *Irf4* and *Irf8* gene expression in pre-cDCs and cDCs regulates the numbers and IRF4 or IRF8 expression of DCs and pre-cDCs in lymphoid organs and lung tissue during homeostasis.

IRF4 expression in CD11c$^+$ cells is required for development of a CD11c^{hi} MHCII^{hi} CD11b$^+$ CD24^{hi} SIRPα$^+$ DC subset in the lung

To determine if IRF4 were required for the development or survival of specific lung DC subsets, we analyzed the CD11c^{hi}SiglecF^{lo}CD64$^-$MHCII^{hi} population in perfused lungs (Fig. 1A–C). Combined with a lineage gate to exclude CD19$^+$, CD3$^+$ and NK1.1$^+$ cells, this marker combination also facilitates exclusion of CD11c^{hi}SiglecF^{hi}CD64$^+$ alveolar macrophages, and helps to clearly delineate CD11c^{hi} (R1 gate) vs. CD11c^{int} (R2 gate) cells. Once gated, the CD11c^{hi}MHCII^{hi} cells in gate R1 were divided into CD11b$^+$ and CD103$^+$ fractions (Fig. 1D). *CD11c-cre-Irf4* –/– mice showed a significant reduction of the percentage and number of CD11c^{hi} MHCII^{hi} CD11b$^+$ DCs (Fig. 1D–E). In an alternate marker scheme, the CD11c^{hi} MHCII^{hi} DCs were divided into 3 subsets based on expression of CD24 and SIRPα (CD172a) (Fig. 1F). Previous reports showed that the CD103$^+$ DCs are CD24^{hi}SIRPα^{lo}, while CD11b$^+$ DCs are CD24^{int}SIRPα^{hi}. We identified two populations of SIRPα$^+$ DCs, differing in the level of expression of CD24. Notably, *Irf4* deficiency results in a near absolute reduction in the SIRPα$^+$CD24^{hi} DCs (designated the P1 population), while the numbers of the SIRPα$^+$CD24^{int} cells (P2 population) and the SIRPα^{lo}CD24^{hi} cells (P3 population; also CD103$^+$) are present in normal numbers (Fig. 1F,H). The P1, P2 and P3 populations express high levels of GFP, reporting the deletion of *Irf4* (Fig. 1G). Since a fraction of CD11b$^+$ tissue DCs is derived from monocytes, and thus would not be reduced by CD11c-Cre activity (41), it is possible that the CD11c^{hi}MHCII$^+$ P2 cells represent this monocyte-derived fraction. However, the P2 cells do not express high levels of CD14 or CD64, relative to the CD11c^{lo}CD64$^+$ macrophage population (Fig. 1B,I).

CD11c-cre-Irf4 +/– heterozygotes showed a trend to reduced percentages and numbers of the CD11b$^+$SIRPα$^+$CD24^{hi} population relative to +/+ mice, but this reduction did not reach statistical significance (Fig. 1H). This suggests that one copy of the *Irf4* gene leads to decreased levels of IRF4, which is nearly sufficient for complete lung cDC2 development.

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Thus, separation of the $CD11c^{hi}MHCII^{hi}SIRP\alpha^{+}$ DCs into $CD24^{hi}$ and $CD24^{int}$ cells revealed that only one of two distinct DC subsets within the $CD11b^{+}$ $SIRP\alpha^{+}$ population is dependent upon *Irf4* for development. Prior reports of the *CD11c-cre-Irf4* –/– mice showed a reduction, but not absence, of the numbers of $CD11b^{+}$ lung resident DCs and $CD11b^{+}CD103^{+}$ DCs in the SI-LP (6, 7). Based on this reduction and other data showing increased apoptosis of the $CD11b^{+}$ subset, it was suggested that *Irf4* was not required for development of the $CD11b^{+}$ subset, but rather important for regulation of its survival. To address DC survival in view of our finding that the *CD11c-cre-Irf4* –/– mice do lack the $SIRP\alpha^{+}CD24^{hi}$ (P1) DC subset, we determined activated caspase 3 levels in the $SIRP\alpha^{+}CD24^{int}$ (P2) subset directly *ex vivo*. In *CD11c-cre-Irf4* –/– mice, the P2 subset did not show a significant difference in the low fraction of cells bearing activated caspase 3 (Fig. 1J), suggesting that these cells are not undergoing higher levels of apoptosis than in +/+ mice. Taken together, these data show that *Irf4* is required for the development of the P1 DC subset, without an effect on the numbers or survival of the P2 and P3 DC subsets.

cDC subsets differ in expression of IRF4 and IRF8 and those cDCs that develop in *CD11c-cre-Irf4* –/– mice do not alter their expression of IRF8

We next determined IRF4 and IRF8 protein levels in these distinct lung DC subsets using specific Abs for intracellular flow cytometry. The P1 DCs harbored the highest level of IRF4 and no IRF8, while P3 DCs expressed very low levels of IRF4 and high levels of IRF8 (Fig. 2A–B). The P2 population also expressed IRF4 and very little IRF8. Analyses of *CD11c-cre-Irf4* +/– mice showed that a single copy of the *Irf4* gene led to a reduced level of IRF4 protein, ~75% of the level in +/+ mice, in the P1 subset (Fig. 2B). In *CD11c-cre-Irf4* –/– and +/– mice, the IRF8 protein levels in the P2 and P3 subsets were not different from +/+ mice (Fig. 2A–B). Thus, a single copy of the *Irf4* gene leads to a reduced amount of IRF4 protein in DCs, yet *Irf4* deficiency or haploinsufficiency did not alter IRF8 expression in the P2 and P3 DC subsets. Furthermore, although the P2 DCs express IRF4, *Irf4* deficiency did not alter their numbers.

IRF4 was reported to regulate CIITA, a transcription factor that promotes expression of MHCII (23). Despite the reduction in IRF4 in the P1 subset of +/– mice, the MHCII level was not reduced (Fig. 2C). In the P2 subset, IRF4 deficiency, but not hemizygosity, led to decreased MHCII expression. This suggests that the amount of IRF4 in +/– DCs is sufficient for induction of CIITA. MHCII levels in the P3 subset were not affected by loss of IRF4.

Deletion of *Irf4* in $CD11c^{+}$ cells does not alter numbers of $CD11c^{int}$ DCs or macrophage subsets in the lung

We also determined if IRF4 were required for the development of the $CD11c^{int}SiglecF^{lo}$ $CD64^{-}MHCII^{hi}$ subset, which appears to represent a distinct population of DCs (R2 gate in Fig. 1A; S2A). This population is $CD11b^{+}$, with a $SIRP\alpha^{+}CD24^{int}$ phenotype similar to the $CD11c^{hi}$ P2 population described above (Fig. S2B), but only ~50% of the cells in +/– mice express GFP (Fig. S2D), suggesting that not all cells bear a deletion of *Irf4*. Numbers of this $CD11c^{int}CD11b^{+}MHCII^{hi}$ population do not differ among *CD11c-cre-Irf4* –/–, +/– and +/+ mice, even when gating only upon the GFP^{+} population in +/– and –/– mice (Fig. S2C–D). The $CD11c^{int}CD11b^{+}MHCII^{hi}$ DCs express low levels of IRF4 and IRF8 (Fig. S2E).

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$CD11c^{hi}SiglecF^{hi}CD64^{+}$ alveolar macrophages also will have deleted *Irf4*; however, numbers of these cells did not differ among *CD11c-cre-Irf4* –/–, +/– and +/+ mice (Fig. S2F–G). This is consistent with low expression of *Irf4* in alveolar macrophages (immgen.org).

IRF4 expression in CD11c^{+} cells is required for development of splenic CD11b^{+}CD4^{+}DCs

Consistent with previous reports in globally *Irf4* deficient mice, in *CD11c-cre-Irf4* –/– and +/– mice, we observed an *Irf4* dose-dependent reduction in the percentages and numbers of splenic $CD4^{+}CD8^{-}$ DCs; these DCs are also $CD11b^{+}$ (Fig. 3A–C). Numbers of the $CD4^{-}CD8^{-}$ DCs and the IRF8-dependent $CD4^{-}CD8^{+}$ DCs were not altered by *Irf4* deficiency. These data show that a reduction of IRF4 activity beginning at the pre-cDCs stage preferentially reduces the development of splenic $CD11b^{+}CD4^{+}CD8^{-}$ DCs. Consistent with this finding, in +/+ mice, the $CD4^{+}CD8^{-}$ DCs express IRF4 but little IRF8, while the $CD4^{-}CD8^{+}$ DCs express IRF8 but not IRF4 (Fig. 3D). $CD4^{-}CD8^{-}$ DCs express IRF4 but little IRF8, but their numbers and expression of IRF8 were not altered by *Irf4* deficiency. Levels of IRF4 protein in $CD4^{+}CD8^{-}$ DCs in *CD11c-cre-Irf4* +/– mice were reduced. In –/– mice, the levels of IRF8 were reproducibly increased in $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ DCs, suggesting that *Irf4* deficiency increases expression of IRF8 in the DCs that develop (Fig. 3D).

Irf4 deficiency in CD11c^{+} cells does not affect skin DC development but reduces migration of CD11b^{+} dermal DCs to local draining lymph nodes

We previously published that $CD11b^{+}$ dermal (dDCs) and epidermal (eLCs) DCs developed normally and were present in the skin of globally *Irf4*-deficient mice (25). However, these $Irf4^{-/-}$ $CD11b^{+}$ DC subsets failed to migrate to LN in homeostasis and inflammation, consistent with the failure to upregulate CCR7. To determine if CD11c-specific *Irf4* deficiency led to the same phenotype, we analyzed skin and cutaneous LN DC populations, gating as in Bajaña et al (25). The percentages of total DCs and DC subsets in the dermis were similar in *CD11c-cre-Irf4* –/–, +/– and +/+ mice, and tended toward being increased in the –/– mice as if they had accumulated (Fig. 4A–B). Although the total number of cells in the cutaneous lymph node cells was not different, we detected an *Irf4* gene dosage-dependent reduction in numbers of $CD11b^{+}$ dermal DCs in the cutaneous LN of *CD11c-cre-Irf4* –/– and +/– mice (Fig. 4C–D). However, unlike the global $Irf4^{-/-}$ mice, the numbers of migrating eLC were not significantly reduced in the *CD11c-cre-Irf4* –/– and +/– mice (Fig. 4D). Expression of GFP, indicating *Irf4* deletion, was equivalent in the three skin DC subsets (Fig. S1). As in the lung, $CD11b^{+}$ dDCs expressed high levels of IRF4 and little IRF8, while $CD103^{+}$ dDCs expressed high levels of IRF8 and little IRF4 (Fig. 4E). eLCs expressed low levels of both IRF4 and IRF8. Taken together, these data show that the IRF4-expressing tissue resident $CD11b^{+}$ DC populations in the lung and dermis show a differential dependence on IRF4 for their development and tissue residence.

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IRF4 deficiency in $CD11c^+$ cells does not alter numbers or IRF8 expression of pre-cDCs in bone marrow or spleen

The immediate precursors of conventional DCs are $CD11c^{int}$ pre-cDCs, defined as lineage-negative $CD11c^+MHCII^-SIRP\alpha^{lo}Flt3^+$ (Fig. 5A). In *CD11c-cre-Irf4* –/– and +/– mice, in which GFP expression can be used to monitor *Irf4* deletion, 60–85% of the pre-cDCs were GFP^+ (Fig. 5B). Numbers of total and GFP^+ pre-cDCs in the bone marrow (BM) and spleen did not differ in *CD11c-cre-Irf4* –/–, +/– and +/+ mice (Fig. 5B–C), suggesting that *Irf4* deficiency does not affect pre-cDC numbers. Intracellular staining with IRF4- and IRF8-specific mAbs showed that BM pre-cDCs in +/+ mice express fairly uniform levels of IRF4 and IRF8; a small percentage of pre-cDCs are $IRF8^-$, but their IRF4 expression level is unchanged (Fig. 5D,I). Thus, the pre-cDCs cannot be divided into cells expressing variable levels of IRF4. In pre-cDCs of +/– mice, IRF4 was present at a reduced level relative to +/+ mice (Fig. 5D–E). These IRF4 levels were similar to levels on mature cDCs in BM of the same +/+ and +/– mice (Fig. 5F). Despite the incomplete *Irf4* deletion predicted by the GFP expression, we did not identify two levels of anti-IRF4 binding in –/– pre-cDCs that would be indicative of IRF4 expression stemming from intact *Irf4* genes vs. nonspecific binding of the mAb. Indeed, a similar level of nonspecific binding of the anti-IRF4 mAb to –/– mature BM cDCs, which were ~88% GFP^+, was observed (Fig. 5F). We could not directly correlate IRF4 expression with GFP (indicating *Irf4* deficiency) in pre-cDCs, since GFP leaks out of cells permeabilized for intracellular staining. IRF8 levels on pre-cDCs did not change in *CD11c-cre-Irf4* +/– or –/– mice (Fig. 5G–H). Binding of the anti-IRF8 mAb to pre-cDCs of *Irf8*-deficient mice was used to show background levels of mAb binding, and this revealed that not all pre-cDCs in the *Irf8*-deficient mice have deleted *Irf8* (Fig. 5G,7D).

Taken together, these data show that *Irf4* deficiency in $CD11c^+$ cells (as judged by GFP expression) did not alter numbers of GFP^+ pre-cDCs or their expression of IRF8. This is consistent with the absence of an effect of *Irf4* deficiency on numbers of lung and splenic IRF8-dependent DCs, and suggests that IRF4 is not needed for maintenance of normal numbers of pre-cDCs. Furthermore our data using this intracellular staining approach did not reveal a relationship between levels of IRF4 and IRF8 in individual pre-cDCs in the BM.

IRF8 deficiency in $CD11c^+$ cells alters numbers of both IRF8- and IRF4-dependent lung DCs

In *CD11c-cre-Irf8* –/–, +/– and +/+ mice, we analyzed the $CD11c^{hi}SiglecF^{lo}CD64^-MHCII^{hi}$ lung DC population (gated as in Fig. 1). Unexpectedly, the $CD103^+$ DC subset was absent in both *CD11c-cre-Irf8* –/– and +/– mice (Fig. 6A,B). The numbers of $CD11b^+$ DCs increased in both *CD11c-cre-Irf8* –/– and +/– mice (Fig. 6A,B). The same results were obtained when $CD11c^{hi}MHCII^{hi}$ DCs were defined by CD24 and SIRPα: numbers of $SIRP\alpha^{lo}CD24^{hi}$ (P3) DCs were reduced while $SIRP\alpha^+CD24^{hi}$ (P1) DCs were increased in both *CD11c-cre-Irf8* –/– and +/– mice (Fig. 6C,D). Numbers of $SIRP\alpha^+CD24^{int}$ (P2) DCs did not change in the *CD11c-cre-Irf8* –/– and +/– mice (Fig. 6D). Thus, one copy of the *Irf8* gene is insufficient to promote lung $CD11c^{hi}MHCII^{hi}CD103^+$ DC development, and 0 or 1 copies of *Irf8* leads to increased differentiation of $CD11b^+SIRP\alpha^+CD24^{hi}$ DCs. Interestingly, in *CD11c-cre-Irf8* –/– and +/– mice, IRF4 protein levels were increased in the P2 but not the P1 DC subset

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(Fig. 6E), indicating that the increased numbers of the IRF4-dependent P1 DCs is not due to their significantly increased IRF4 levels in mature DCs.

CD11c-cre-Irf8 –/– and +/– mice did not show differences in the numbers of lung CD11c^{int}SiglecF^{lo}CD64^{-}MHCII^{hi} DCs (Fig. S2I), yet the *Irf8* –/– DCs expressed elevated levels of IRF4 (Fig. S2J), as observed for the CD11c^{hi} P2 population (Fig. 6E). Numbers of CD11c^{hi}SiglecF^{hi}CD64^{+} alveolar macrophages also were unaffected by *Irf8* deficiency (Fig. S2K). However, in *CD11c-cre-Irf8* –/– mice, the alveolar macrophages displayed reduced levels of CD64 (FcγRI); this was not observed on alveolar macrophages in *CD11c-cre-Irf4* –/– mice (Fig. S2H,L). This suggests that the *Irf8* –/– macrophages may be functionally impaired.

IRF8 deficiency in CD11c^{+} cells alters both IRF8 and IRF4-dependent splenic DC differentiation

Consistent with previous reports in globally *Irf8*-deficient or mutant mice (42), in *CD11c-cre-Irf8* –/– and +/– mice, we observed a reduction in the percentages and numbers of splenic CD4^{-}CD8^{+} (CD11b^{-}) DCs (Fig. 6F–G). These data show that a reduction of IRF8 activity beginning at the pre-cDC stage decreases the development of splenic CD4^{-}CD8^{+} DCs. The *CD11c-cre-Irf8* –/– mice also showed a significant increase in the numbers of CD4^{+}CD8^{-} (CD11b^{+}) DCs (Fig. 6G). This increment in CD4^{+}CD8^{-} DCs was not in proportion to the reduction in CD4^{-}CD8^{+} DCs, but suggests that the absence of IRF8 expression potentiates the development of IRF4-dependent DCs, as we observed in the lung. Numbers of CD4^{-}CD8^{-} (CD11b^{+}) DCs were not affected by IRF8 deficiency (Fig. 6G).

IRF8 deficiency in CD11c^{+} cells leads to decreased numbers of splenic pre-cDCs expressing elevated levels of IRF4

While numbers of pre-cDCs in the BM of *CD11c-cre-Irf8* –/–, +/– and +/+ mice were similar (Fig. 7A), the +/– and –/– mice harbored an IRF8-dose dependent decrease in the numbers of pre-cDCs in the spleen (Fig. 7B–C). Intracellular staining with IRF4- and IRF8-specific mAbs showed that BM pre-cDCs in +/+ mice expressed uniform levels of IRF4 and IRF8, which did not permit identification of pre-cDC subsets (Fig. 7D–G). In the –/– mice, not all of the pre-cDCs appeared to have deleted the *Irf8* gene as approximately half of the cells expressed IRF8 (Fig. 7D). Notably, gating on the IRF8^{-} pre-cDCs in the BM of –/– mice revealed an increase in IRF4 expression relative to the IRF8^{+} pre-cDCs (Fig. 7D–G). Taken together, these data show that IRF8 deficiency reduces numbers of splenic pre-cDCs, and the remaining pre-cDCs express elevated IRF4 compared to +/+ pre-cDCs. This elevated IRF4 in pre-cDCs likely explains the increased development of IRF4-dependent CD11b^{+} DCs in the lung and spleens of *CD11c-cre-Irf8* –/– mice (Fig. 6).

Discussion

Through studies of *CD11c-cre-Irf4* –/– and +/– mice, we determined if IRF4 deficiency or haploinsufficiency in CD11c^{+} cells altered the numbers of pre-cDCs and specific DC subsets in the lung, dermis and spleen. Herein, we have shown that *CD11c-Cre-Irf4* –/– mice selectively lacked a lung-resident CD11b^{+}CD11c^{hi}SIRPα^{+}CD24^{hi}MHCII^{hi} DC subset,

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while other lung CD11b$^+$ CD64$^-$ (CD11c^{hi} or CD11c^{int}) DC subsets and CD11c^{hi} alveolar macrophages were present in normal numbers. Consistent with prior studies of globally *Irf4* −/− mice, the *CD11c-Cre-Irf4* −/− and +/− mice also showed a gene dose-dependent reduction in splenic CD4$^+$CD11b$^+$ DCs, but no defect in numbers of CD11b$^+$ dermal DCs. Measurement of IRF4 and IRF8 protein levels revealed that IRF4 is expressed at the highest levels in CD11b$^+$ DCs and less so in *Irf8*-dependent DCs in the spleen and lung, while IRF8 is expressed almost exclusively in *Irf8*-dependent DCs. A reduced amount of IRF4 protein was present in +/− CD11b$^+$CD11c^{hi} DCs, but this amount was ~75% of the amount in +/+ DCs, consistent with near normal numbers of the IRF4-dependent DCs in +/− mice. IRF4 reduction or absence led to minimal changes in IRF8 expression. *Irf4* deficiency (as judged by GFP expression) did not alter numbers of pre-cDCs in the bone marrow and spleen, or pre-cDC expression of IRF8; this was consistent with normal numbers of IRF8-dependent DCs in the *CD11c-cre-Irf4* −/− and +/− mice.

Similar experiments were done with *CD11c-cre-Irf8* −/− and +/− mice, in which *Irf8* is deleted in CD11c$^+$ cells. *CD11c-cre-Irf8* −/− and +/− mice both lacked lung-resident CD103$^+$ DCs, indicating that a single copy of *Irf8* leads to insufficient amounts of IRF8 required to promote their development. In contrast, the spleens of *CD11c-cre-Irf8* −/− and +/− mice showed an *Irf8* dose-dependent reduction in CD4$^-$CD8$^+$ DCs. *Irf8* deficiency led to a reduced number of splenic pre-cDCs with increased IRF4 expression relative to +/+ mice, which correlated with increased numbers of IRF4-dependent DCs in the spleen and lung. Taken together, these data from *CD11c-cre-Irf8* −/− and *CD11c-cre-Irf4* −/− mice show that IRF4 and IRF8 are critically required in pre-cDCs to direct the terminal differentiation of select cDC subsets in the lung and spleen.

Adoptive transfer and lineage tracing studies have shown that lung resident CD11b$^+$ DCs arise from both pre-cDCs and monocytes in homeostasis (28, 29, 41, 43, 44). The CD11b$^+$ population is heterogeneous, and these initial studies did not clarify if distinct subsets of lung resident CD11b$^+$MHCII^{hi} DCs were derived from different precursors. Here we have subdivided the CD11b$^+$MHCII^{hi} DCs to show that the CD11c^{hi}SIRPα$^+$CD24^{hi} subset is uniquely dependent on IRF4 expression in CD11c$^+$ cells, while two other CD64$^-$ subsets, CD11c^{hi}SIRPα$^+$CD24^{int} and CD11c^{int}SIRPα$^+$CD24^{int}, are not. This suggests that the *Irf4*-dependent lung CD11b$^+$ DCs arise from CD11c$^+$ pre-cDCs, while the *Irf4*-independent CD11b$^+$ DCs arise from CD11c$^-$ monocytes. However, it is formally possible that *Irf4*-dependent CD11b$^+$ DCs also arise from monocytes, and are deficient in these mice because IRF4 is required at a later CD11c$^+$ stage of the monocyte to DC differentiation pathway. This latter possibility is supported by studies showing that a significant proportion of lung CD11b$^+$ DCs originate from monocytes (41, 43).

A prior report using the same *CD11c-cre-Irf4* −/− mice showed that numbers of lung resident CD11b$^+$ tissue DCs were reduced but not absent (6). Based on evidence for increased apoptosis of these CD11b$^+$ DCs, the conclusion was made that IRF4 was required in mature CD11b$^+$ DCs for their survival, but not their development from pre-cDCs. In contrast, our more detailed separation of the CD11c^{hi} CD11b$^+$ tissue DC subset in the lung revealed that in fact one CD11b$^+$ DC subset (SIRPα$^+$CD24^{hi}) is absent, while a second CD11b$^+$ DC subset (SIRPα$^+$CD24^{int}) is present in normal numbers. Furthermore, levels of

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activated caspase 3 in the remaining CD11b^{+} DCs were low in *CD11c-cre-Irf4* −/− mice, suggesting that their survival was not impaired. Thus, we favor the interpretation that IRF4 is selectively required for the terminal differentiation of the CD11c^{hi}SIRPα^{+}CD24^{hi} subset of lung CD11b^{+} DCs from pre-cDCs.

Our finding is consistent with a recent report that a developmental requirement for KLF4 subdivides IRF4-expressing CD11b^{+} DCs. KLF4 is required for the differentiation of lung CD11c^{hi}SIRPα^{+}CD24^{hi} DCs that promote Th2 responses but not CD11c^{hi}SIRPα^{+}CD24^{int} DCs that promote Th17 responses (10). This may help to explain observations in *CD11c-cre-Irf4* −/− mice that IRF4-dependent DCs are required for both Th2 and Th17 responses. The deficit of Th17 responses upon *Aspergillus fumigatus* challenge of *CD11c-cre-Irf4* −/− mice (6) is likely due to IRF4 deficiency in the CD11c^{hi}SIRPα^{+}CD24^{int} DCs, which are present in the lungs but may lack capacity to produce IL-23 or migrate to LNs.

It is surprising that *Irf4* deficiency restricted to CD11c^{+} cells led to differential effects on CD11c^{+}CD11b^{+}MHCII^{hi} DCs in the lung and the dermis. While a subset of CD11b^{+} lung DCs expresses and depends on IRF4 expression as outlined above, our data show that numbers of CD11b^{+} DCs in the dermis were not reduced by this same *Irf4* deficiency. In fact, numbers of dermal CD11b^{+} DCs were increased in *CD11c-cre-Irf4* −/− mice, consistent with their inability to migrate to LN, as we and others observed previously (11, 25). While both pre-cDCs and monocytes were reported to give rise to lung and dermal CD11b^{+} DCs, it is notable that lung CD11b^{+} DCs were found to be much more dependent on Flt3L (29). Thus, our data suggest that the Flt3L-driven IRF4-dependent pathway for pre-cDC development into CD11b^{+} DCs proceeds to a greater extent in the lung than in the dermis. Development of splenic CD4^{+}CD11b^{+} DCs also was significantly impaired with *Irf4* deficiency restricted to CD11c^{+} cells, consistent with prior work showing that most splenic DCs develop from pre-cDCs (45). The distinct developmental pathways of CD11b^{+} DCs in different peripheral tissues may arise due to differences in homeostatic tissue microenvironments, such as levels of Flt3L, M-CSF or GM-CSF directing pre-cDC-derived vs. monocyte-derived DC differentiation, or the production of chemokines that preferentially attract monocytes or pre-cDCs.

Recent reports demonstrated the subdivision of the pre-cDC population into discrete precursors for cDC1s and cDC2s (30, 31). Pre-cDC1s preferentially express *Irf8* and *Batf3* mRNA while pre-cDC2s preferentially express *Irf4* mRNA. Our own attempts to subdivide the pre-cDCs into discrete populations based on relative IRF4 and IRF8 protein expression were unsuccessful. This could be due to incomplete deletion of the *Irf4* or *Irf8* genes since use of the GFP reporter showed that the CD11c-cre did not act in all CD11c^{int} pre-cDCs in the *CD11c-cre-Irf4* −/− mice (Fig. 5B). Alternately, Grajales-Reyes *et al* reported that pre-cDC2s do express IRF8 directly *ex vivo*, and the IRF8 is lost as the pre-cDC2s differentiate *in vitro* (30).

We investigated the effect of *Irf4* and *Irf8* deficiency beginning in the pre-cDCs, thus bypassing an effect on CDPs. *Irf4* deficiency (as judged by GFP expression) did not apparently change numbers of total pre-cDCs or their expression of IRF8, consistent with unchanged numbers of IRF8-dependent DCs in the lung and spleen. Thus, IRF4 is

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apparently not required for maintenance of pre-cDC2s, although we cannot rule this out definitively since we were unable to assess IRF4 expression and GFP indicating *Irf4* deletion at the same time using intracellular flow cytometry. Furthermore, the absence of IRF4 does not significantly alter IRF8 expression nor divert pre-cDC2s into the cDC1 lineage. In contrast, *Irf8* deficiency led to normal numbers of BM pre-cDCs expressing higher levels of IRF4 and a reduction in splenic pre-cDC numbers, which correlated with increased numbers of IRF4-dependent lung and splenic DCs. This suggests that IRF8 is required for maintenance of a pre-cDC1 population in the spleen, and that the absence of IRF8 allows greater expression of IRF4 in the remaining pre-cDC2s. Indeed, in *Batf3*$^{-/-}$ mice lacking appropriate IRF8 autoactivation, the pre-cDC1s did not commit to the CD8^{+} DC lineage, and were diverted into the CD4^{+}CD11b^{+} lineage (30). More studies are needed to determine how *Irf8* deficiency might modulate the expression or activity of IRF4 in pre-cDC subsets. Our data show that IRF4 protein levels in pre-cDCs are low, which is consistent with low *Irf4* mRNA levels reported for pre-cDC2s (30). Thus, the substantial increase in IRF4 expression in the absence of IRF8 is notable and suggests that IRF8 may act to limit IRF4. IRF4 activity also may increase in the absence of IRF8, since IRF4 and IRF8 partner with common transcription factors such as PU.1 or BATF (46).

We also investigated the effect of *Irf4* or *Irf8* haploinsufficiency on cDC development. A single copy of *Irf4* in CD11c^{+} cells led to reduced IRF4 protein levels in lung and spleen cDCs; this correlated with reduced numbers of CD4^{+} splenic DCs but not lung CD11b^{+} DCs, suggesting that a single copy of *Irf4* produces sufficient amount of IRF4 protein to drive DC differentiation. In contrast, a single copy of *Irf8* in CD11c^{+} cells did not support development of lung CD103^{+} DCs, while numbers of CD8^{+} cDCs in the spleen were reduced but not absent. This suggests that development of the lung resident CD103^{+} cDCs is critically dependent on threshold levels of IRF8. Indeed, the amount of IRF8 produced from a single copy of *Irf8* was reported to be significantly less than 50%, consistent with the requirement for IRF8 to autoregulate its own transcription to maintain cDC1 fate (30). BATF3 is needed for this autoactivation of IRF8 (30), and levels of *Batf3* mRNA were reported to be lower in lung CD103^{+} DCs than spleen CD8^{+} DCs (13). This lesser amount of BATF3 likely decreases the amount of IRF8 produced from a single copy of *Irf8*, leading to deficient development of lung CD103^{+} DCs in *Irf8* +/– mice.

In future studies, we can expect to learn more about how variable levels of transcription factors regulate immune responses, as an increasing number of reports describe gene dosage effects of regulatory factors involved in DC biology (47). Indeed, polymorphisms in human *IRF8* have been linked to autoimmune diseases such as systemic lupus erythematosus (32), while *IRF4* variants predispose to nevi and melanoma and have been linked to lymphocytic leukemia (33, 48). Although we lack information about how most of these polymorphisms alter protein levels of IRF4 or IRF8 in DCs or other immune cells, one mutation in *IRF8* leads to CD1c^{+} DC deficiency and defects in antimycobacterial immunity (36). Identified polymorphisms in the human *IRF4* promoter change transcription factor binding and gene expression levels (34, 35). Interestingly, *Irf4* RNA levels also are increased or decreased by physiological cues such as prostaglandins and estrogens (40, 49). Thus, consistent with our data obtained from these murine models during homeostasis, variable IRF4 or IRF8

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expression resulting from genetic polymorphisms or environmental stimuli may govern tissue DC numbers, and therefore regulate the magnitude of DC functional responses during inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AM	alveolar macrophage
BM	bone marrow
CDP	common DC progenitor
cDC1 and cDC2	conventional DC type 1 or 2
DC	dendritic cell
IRF4 and IRF8	interferon regulatory factor 4 and 8
pre-cDC	precursor of cDCs

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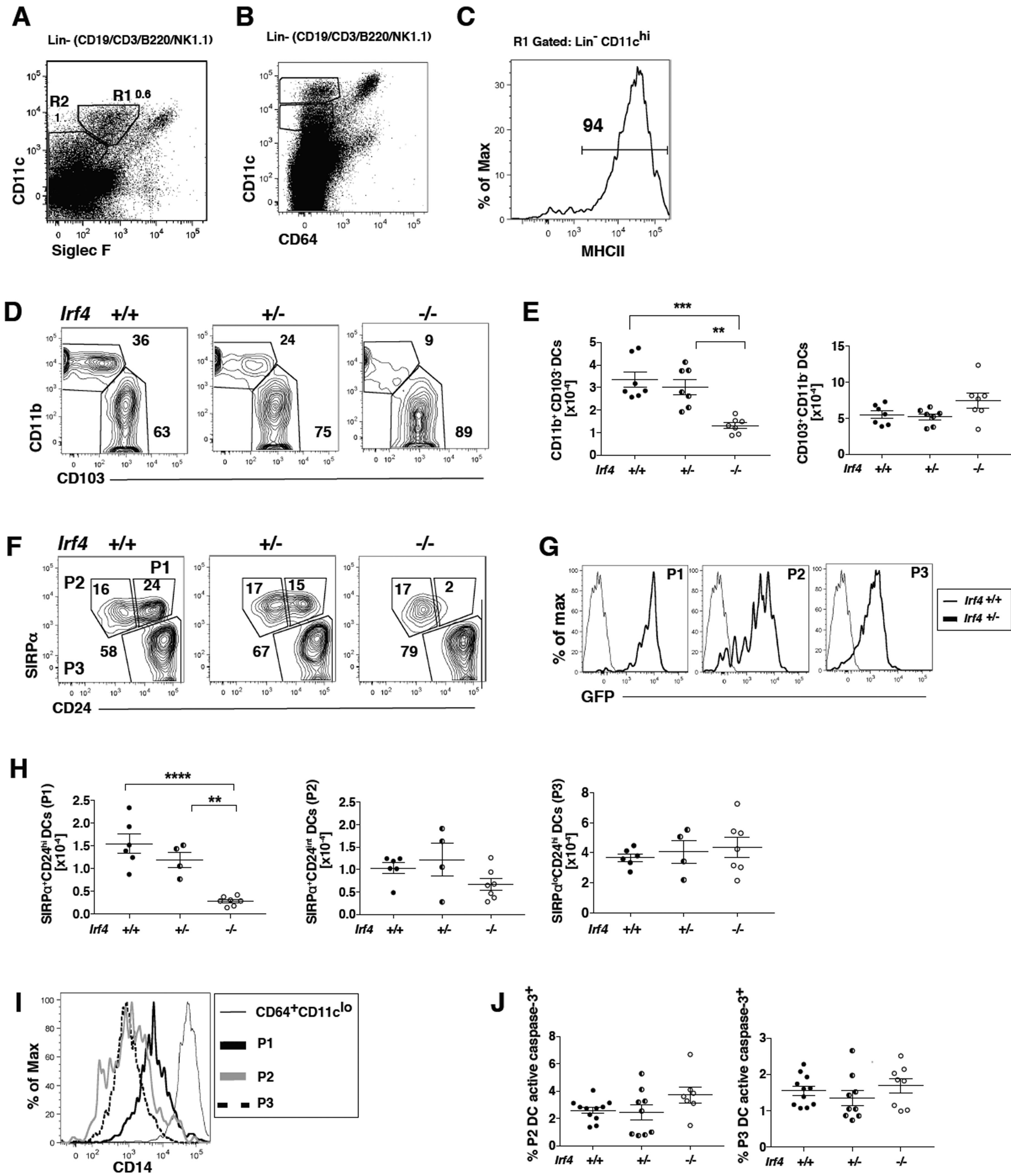

Fig. 1. IRF4 expression in CD11c^{+} cells is required for development of a CD11c^{hi}MHCII^{hi}CD11b^{+}CD24^{hi}SIRPα^{+} DC subset in the lung

(**A–C**) Definition of DC populations in *CD11c-cre-Irf4* +/+ mice. (**A**) CD11c^{+} myeloid cells displaying distinct levels of SiglecF are identified in the Lineage-negative (CD19^{-}CD3^{-}B220^{-} NK1.1^{-}) fraction of lung cells. The R1 gate defines CD11c^{hi}SiglecF^{lo} DCs that are (**B**) CD64^{-} and (**C**) MHCII^{hi}. The R2 gate defines CD11c^{int}SiglecF^{-} cells, some of which are (**B**) CD64^{-} and MHCII^{+} (see Fig. S2). The numbers within these plots from +/+ mice indicate the percentage of cells within each gate. (**D**) CD11c^{hi}MHCII^{hi} cells

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in gate R1 are divided into CD11b^{+} and CD103^{+} subsets; a comparison of +/+, +/− and −/− mice is shown. (**E**) The total numbers of CD11b^{+} and CD103^{+} DC subsets in multiple +/+, +/− and −/− mice are compiled; shown are values for individual mice, n=7 per genotype. (**F**) CD11c^{hi}MHCII^{hi} cells in gate R1 are divided into P1, P2 and P3 subsets based on SIRPα and CD24; a comparison of +/+, +/− and −/− mice is shown. (**G**) The CD11c^{hi}MHCII^{hi} DC subsets SIRPα^{+}CD24^{hi} (P1), SIRPα^{+}CD24^{int} (P2) and SIRPα^{lo}CD24^{hi} (P3) are GFP^{+} in +/− mice. (**H**) The total numbers of P1, P2 and P3 DC subsets in multiple +/+, +/− and −/− mice are compiled, n=4–7 per genotype. (**I**) DCs within the CD11c^{hi} P1, P2 and P3 subsets display lower levels of CD14 than the CD11c^{lo} CD64^{hi} macrophages. (**J**) Shown is the percentage of DCs in P2 and P3 subsets that contain activated caspase-3 in +/+, +/− and −/− mice, n=8–11 per genotype. The significance of the data in panels E, H and J was evaluated using a one-way ANOVA with a Tukey multiple comparisons test; **p<0.01, ***p<0.001, ****p<0.0001.

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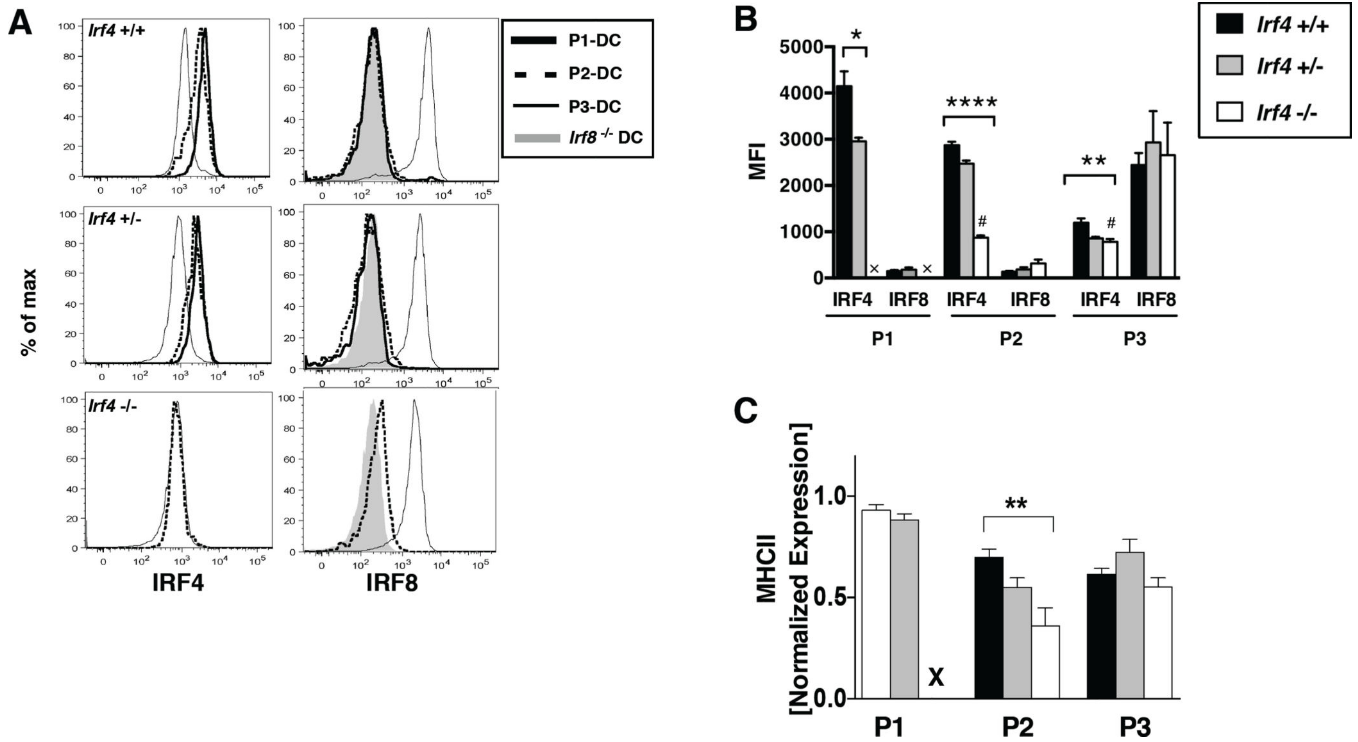

Fig. 2. cDC subsets in *CD11c-cre-Irf4* mice differ in expression of IRF4 and IRF8 while heterozygotes show reduced levels of protein

(**A**) In *CD11c-cre-Irf4* mice, lung cDCs were subdivided into P1, P2 and P3 subsets as in Fig. 1F, and intracellular IRF4 and IRF8 levels were determined using flow cytometry. For each subset in +/+, +/– and –/– mice, the binding of anti-IRF4 (left panels) and anti-IRF8 (right panels) is shown. The *CD11c-cre-Irf4* –/– and *CD11c-cre-Irf8* –/– mice were used to determine the nonspecific level of anti-IRF4 and anti-IRF8 Ab binding, respectively, to DCs. (**B**) The mean fluorescence intensity (MFI) of anti-IRF4 and anti-IRF8 binding is shown for each cDC subset (P1, P2, P3) in *CD11c-cre-Irf4* +/+, +/– and –/– mice. The # indicates the nonspecific binding of the anti-IRF4 Ab on *Irf4* –/– cells. X indicates that the P1 subset is absent in the –/– mouse. (**C**) The relative MHCII expression (normalized MFI) on the P1, P2 and P3 subsets present in *CD11c-cre-Irf4* +/+, +/– and –/– mice is shown. X indicates that the P1 subset is absent in the –/– mouse. For panels B and C, the significance of the data was evaluated using an unpaired t test (P1) or a one-way ANOVA (P2, P3); *p<0.05, **p<0.01, ****p<0.0001, n=4 per genotype.

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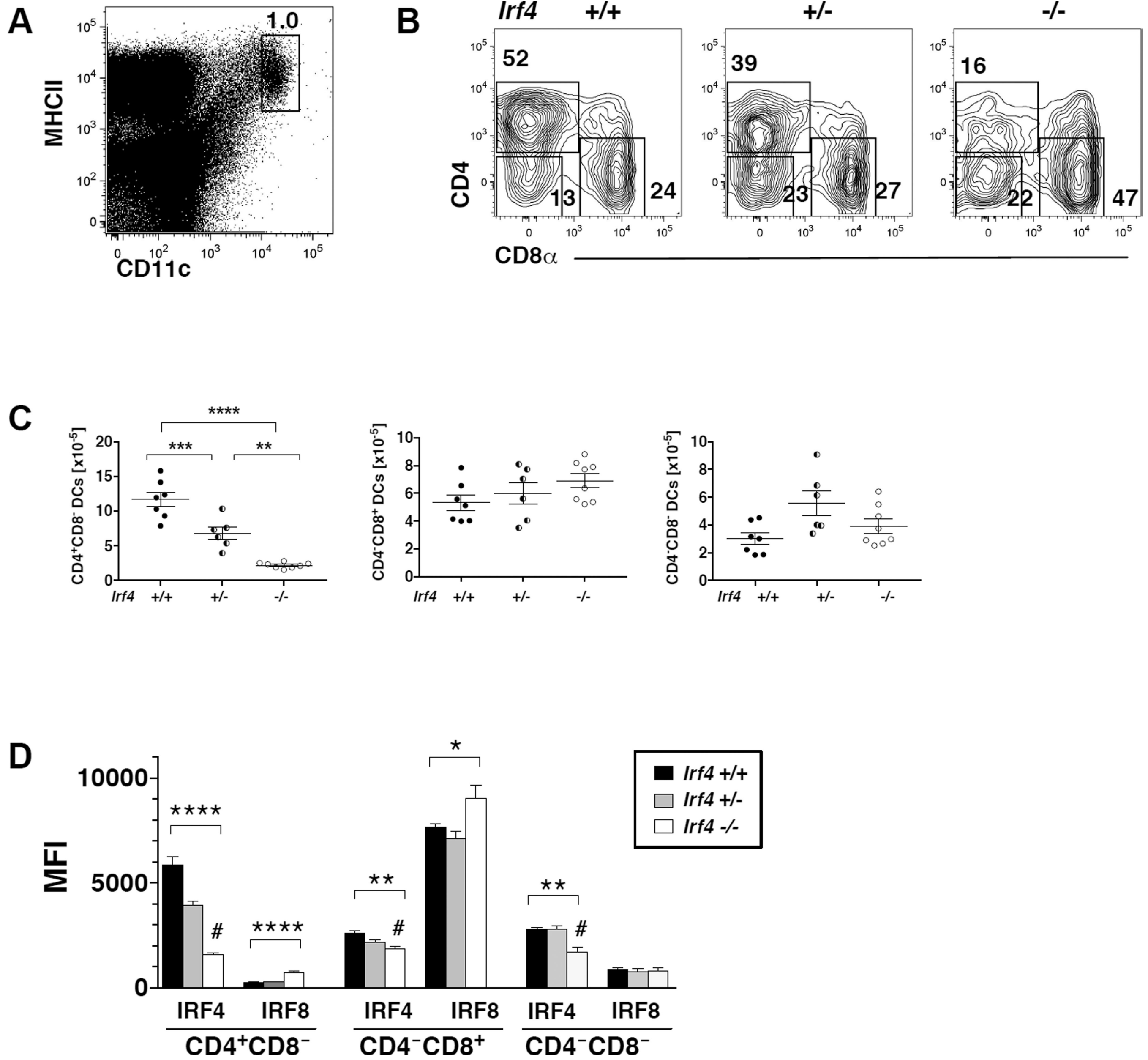

Fig. 3. IRF4 expression in CD11c^{+} cells is required for development of splenic CD11b^{+}CD4^{+}DCs

(**A**) Definition of CD11c^{hi}MHCII^{hi} cDCs in the spleen of *CD11c-cre-Irf4* +/+ mice. (**B**) CD11c^{hi}MHCII^{hi} cells are divided into CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} DC subsets; a comparison of +/+, +/– and –/– mice is shown. (**C**) The total numbers of CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} DC subsets in multiple +/+, +/– and –/– mice are compiled; shown are values for individual mice, n=6–8 per genotype. The significance of the data was evaluated using a one-way ANOVA with a Tukey multiple comparisons test; **p<0.01, ***p<0.001, ****p<0.0001. (**D**) The mean fluorescence intensity (MFI) of anti-IRF4 and anti-IRF8 binding is shown for the CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} DC subsets in +/+, +/– and –/– mice. The # indicates the background binding of the anti-IRF4 Ab on *Irf4*–/– cells. The binding of anti-IRF8 to *Irf8*–/– splenic DCs was a MFI of 195. The

significance of the data was evaluated using a one-way ANOVA; *p<0.05, **p<0.01, ****p<0.0001, n=4 per genotype.

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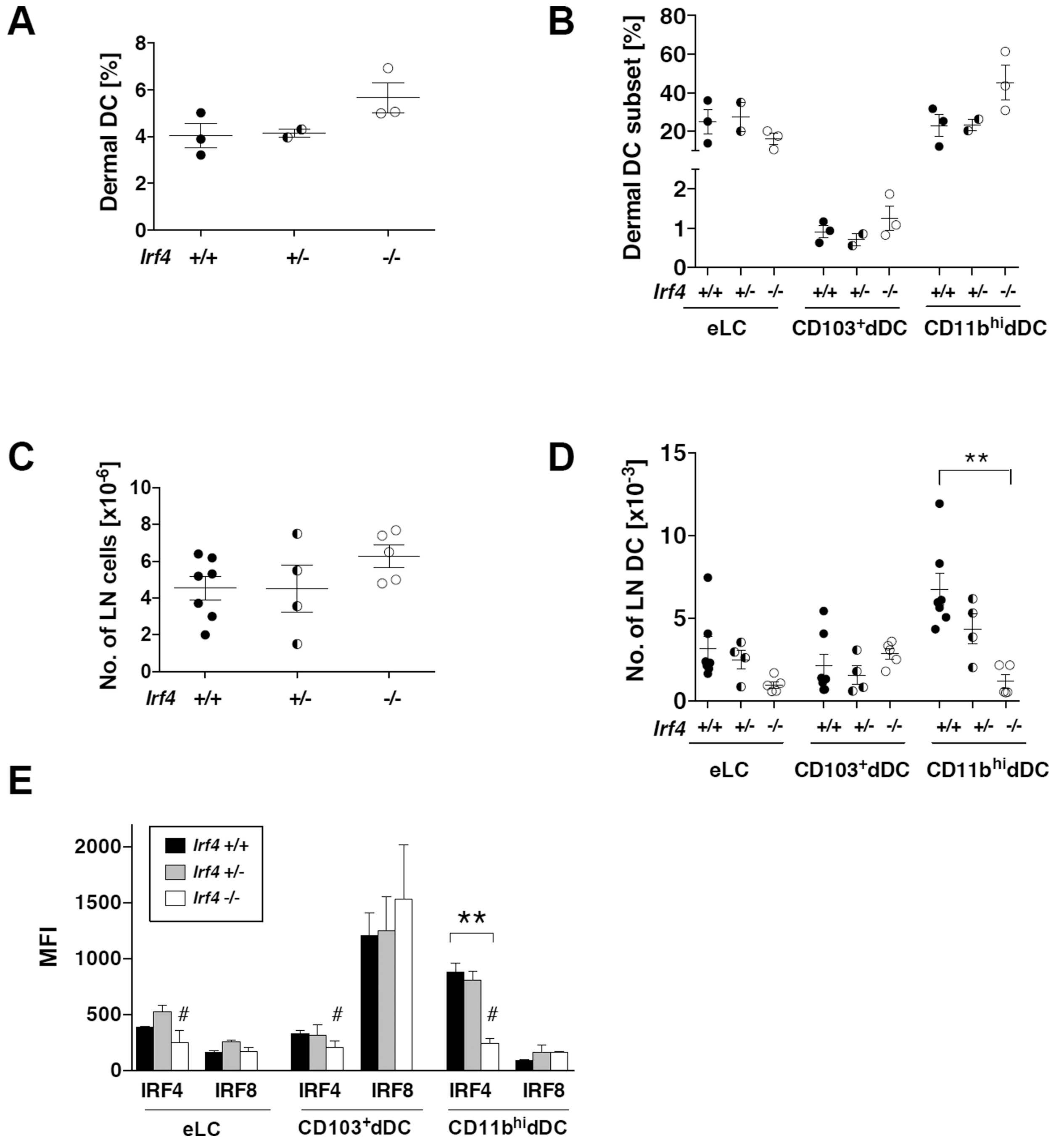

Fig. 4. *Irf4* deficiency in CD11c^{+} cells does not affect skin DC development but reduces migration of CD11b^{+} dermal DCs to local draining lymph nodes

(**A–B**) The percentages of total dermal DCs and distinct DC subsets (eLC, CD103^{+}, CD11b^{hi}) in the skin of multiple *CD11c-cre-Irf4* +/+, +/– and –/– mice, n=2–3. (**C–D**) Numbers of LN cells and migratory DC subsets in *CD11c-cre-Irf4* +/+, +/– and –/– mice, n=4–7. (**E**) The MFI of anti-IRF4 and anti-IRF8 binding is shown for the eLC, CD103^{+} and CD11b^{hi} DC subsets in +/+, +/– and –/– mice. The # indicates the background binding of the anti-IRF4 Ab on *Irf4*–/– cells. The binding of anti-IRF8 to *Irf8*–/– CD11b^{hi} DCs was a

MFI of 102. The significance of the data was evaluated with a one-way ANOVA with a Tukey's multiple comparisons test, $^{**}p<0.01$.

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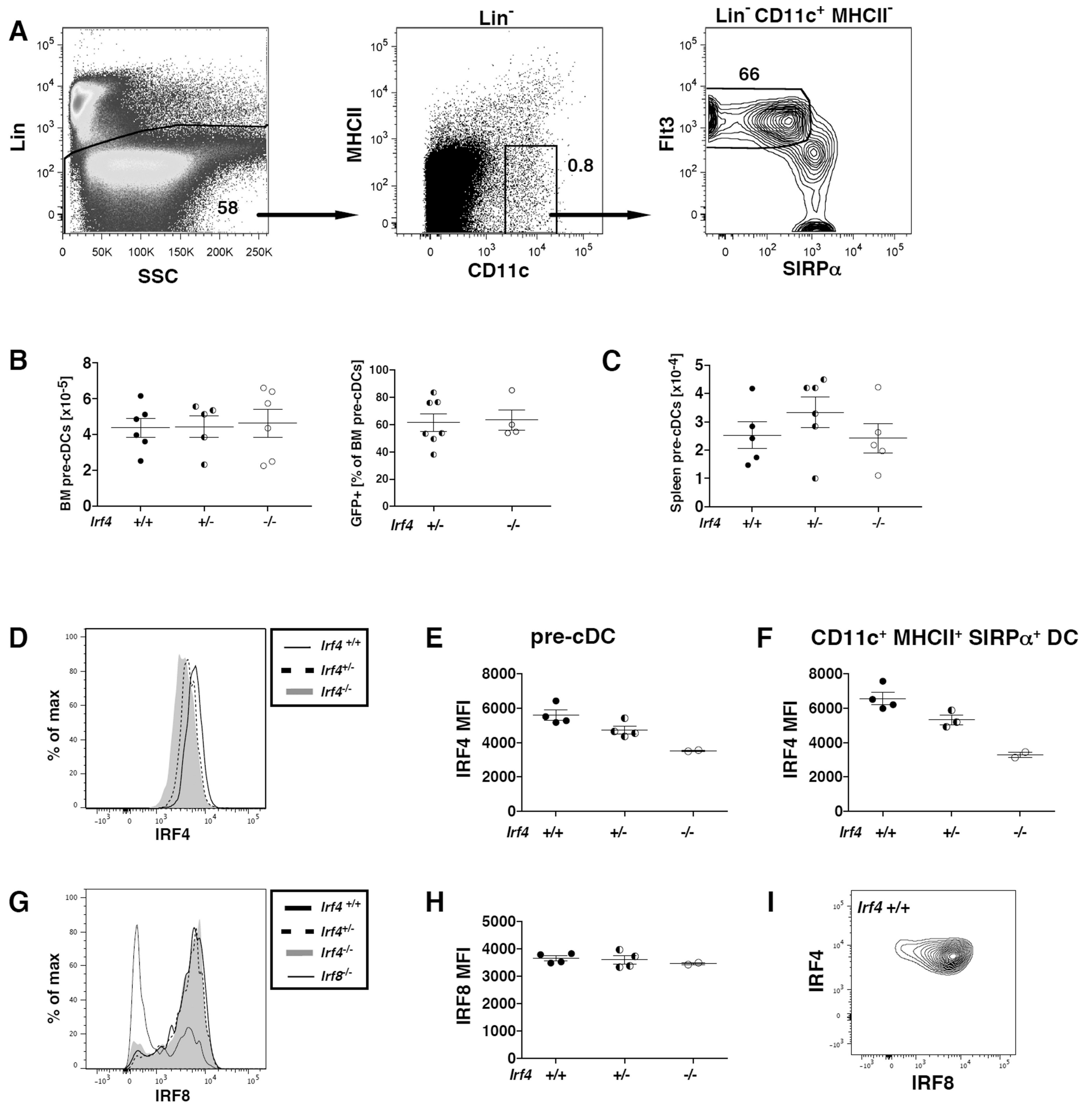

Fig. 5. IRF4 deficiency in CD11c$^+$ cells does not alter numbers or IRF8 expression of pre-cDCs in bone marrow or spleen

(**A**) Definition of pre-cDCs in the bone marrow of *CD11c-cre-Irf4* +/+ mice as lineage-negative (Lin$^-$, CD19$^-$CD3$^-$NK1.1$^-$Ter119$^-$B220$^-$) CD11c$^+$MHCII$^-$SIRPα^{lo}Flt3$^+$. (**B–C**) The numbers of total pre-cDCs and GFP$^+$ pre-cDCs in the bone marrow and spleen in multiple +/+, +/– and –/– mice are compiled, n=4–6 per genotype. (**D**) IRF4 protein levels were determined in +/+, +/– and –/– BM pre-cDCs by intracellular staining, and (**E**) the anti-IRF4 MFI values were compiled from multiple mice. (**F**) IRF4 protein levels were

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determined in $CD11c^{+}MHCII^{+}SIRP\alpha^{+}$ cDCs in BM of +/+, +/− and −/− mice by intracellular staining, and the anti-IRF4 MFI values were compiled from multiple mice. (**G**) IRF8 protein levels were determined in +/+, +/− and −/− pre-cDCs by intracellular staining, and (**H**) the anti-IRF8 MFI values were compiled from multiple mice. Binding of the anti-IRF8 mAb to pre-cDCs of *Irf8*-deficient mice was used to show background levels of mAb binding, and this revealed that not all pre-cDCs in the *Irf8*-deficient mice have deleted *Irf8* (see Fig. 7). (**I**) Binding of anti-IRF4 and anti-IRF8 mAbs to pre-cDCs in +/+ mice.

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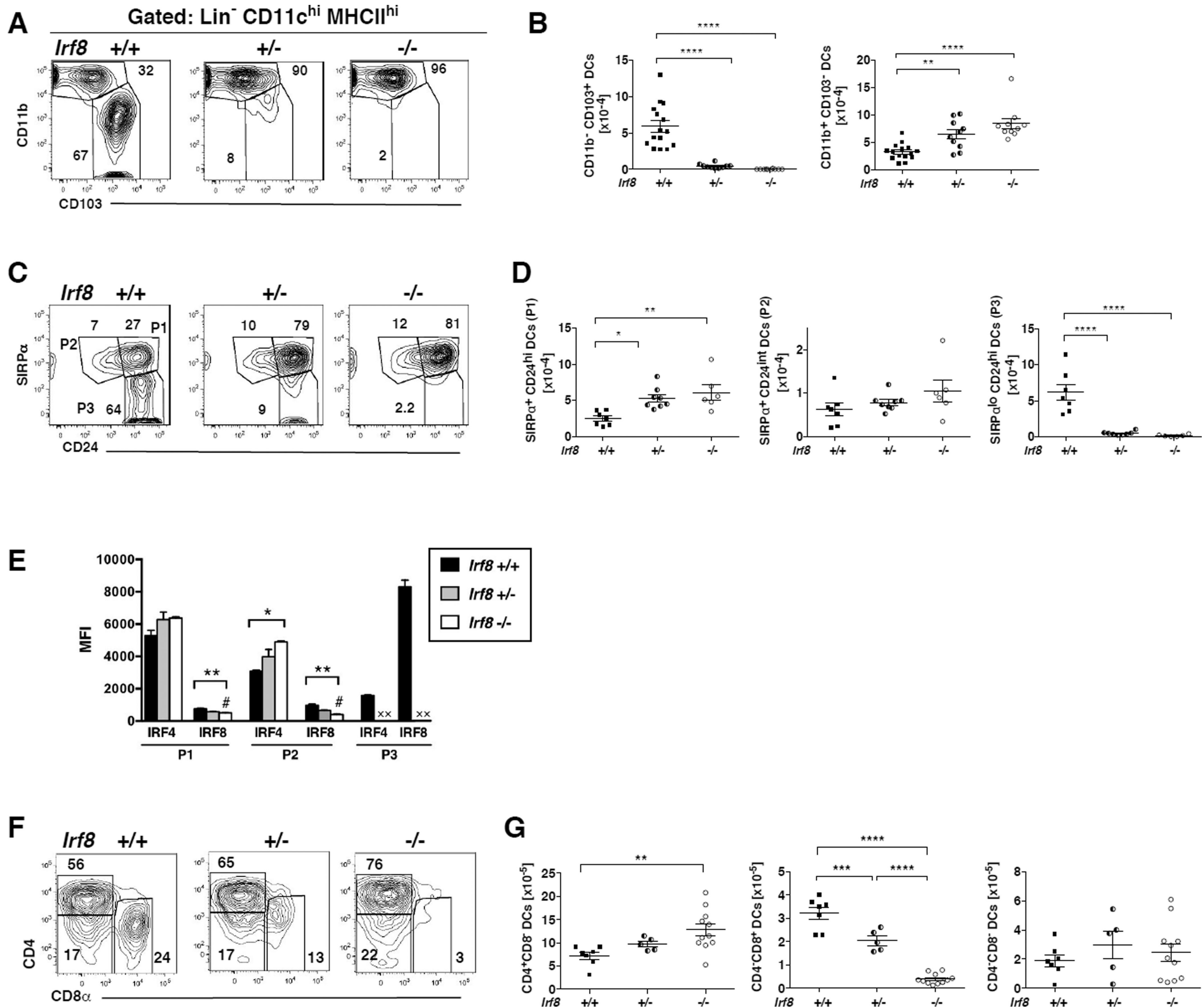

Fig. 6. IRF8 deficiency in CD11c^{+} cells alters numbers of both IRF8- and IRF4-dependent lung and spleen DCs

(**A**) CD11c^{hi}MHCII^{hi} DCs (gate R1) in the lungs of *CD11c-cre-Irf8* mice were gated as in Fig. 1A and divided into CD11b^{+} and CD103^{+} subsets; a comparison of +/+, +/– and –/– mice is shown. (**B**) The total numbers of CD11b^{+} and CD103^{+} DC subsets in multiple +/+, +/– and –/– mice are compiled; shown are values for individual mice, n=10–15 per genotype. (**C**) Lung CD11c^{hi}MHCII^{hi} cells in gate R1 (see Fig. 1A) are divided into subsets based on SIRPα and CD24; a comparison of +/+, +/– and –/– mice is shown. (**D**) The total numbers of SIRPα^{+}CD24^{hi} (P1), SIRPα^{+}CD24^{int} (P2) and SIRPα^{lo}CD24^{hi} (P3) DC subsets in multiple +/+, +/– and –/– mice are compiled, n=7–8 per genotype. For panels B and D, the significance of the data was evaluated using a one-way ANOVA with a Tukey's multiple comparisons test; *$p<0.05$, **$p<0.01$, ****$p<0.0001$. (**E**) The mean fluorescence intensity (MFI) of anti-IRF4 and anti-IRF8 binding is shown for each lung cDC subset (P1, P2, P3) in *CD11c-cre-Irf8* +/+, +/– and –/– mice. The # indicates the nonspecific binding of the anti-

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IRF8 Ab to *Irf8* −/− cells. X indicates that the P3 subset is absent in the +/− and −/− mice. The significance of the data was evaluated using a one-way ANOVA (P1, P2); *p<0.05, **p<0.01, n=3 per genotype. (**F**) Splenic CD11c^{hi}MHCII^{hi} cells (gated as in Fig. 3A) are divided into CD4^{+} and CD8α^{+} subsets; a comparison of +/+, +/− and −/− mice is shown. (**G**) The total numbers of CD4^{+} and CD8α^{+} splenic DC subsets in multiple +/+, +/− and −/− mice are compiled; shown are values for individual mice, n=5–11 per genotype.

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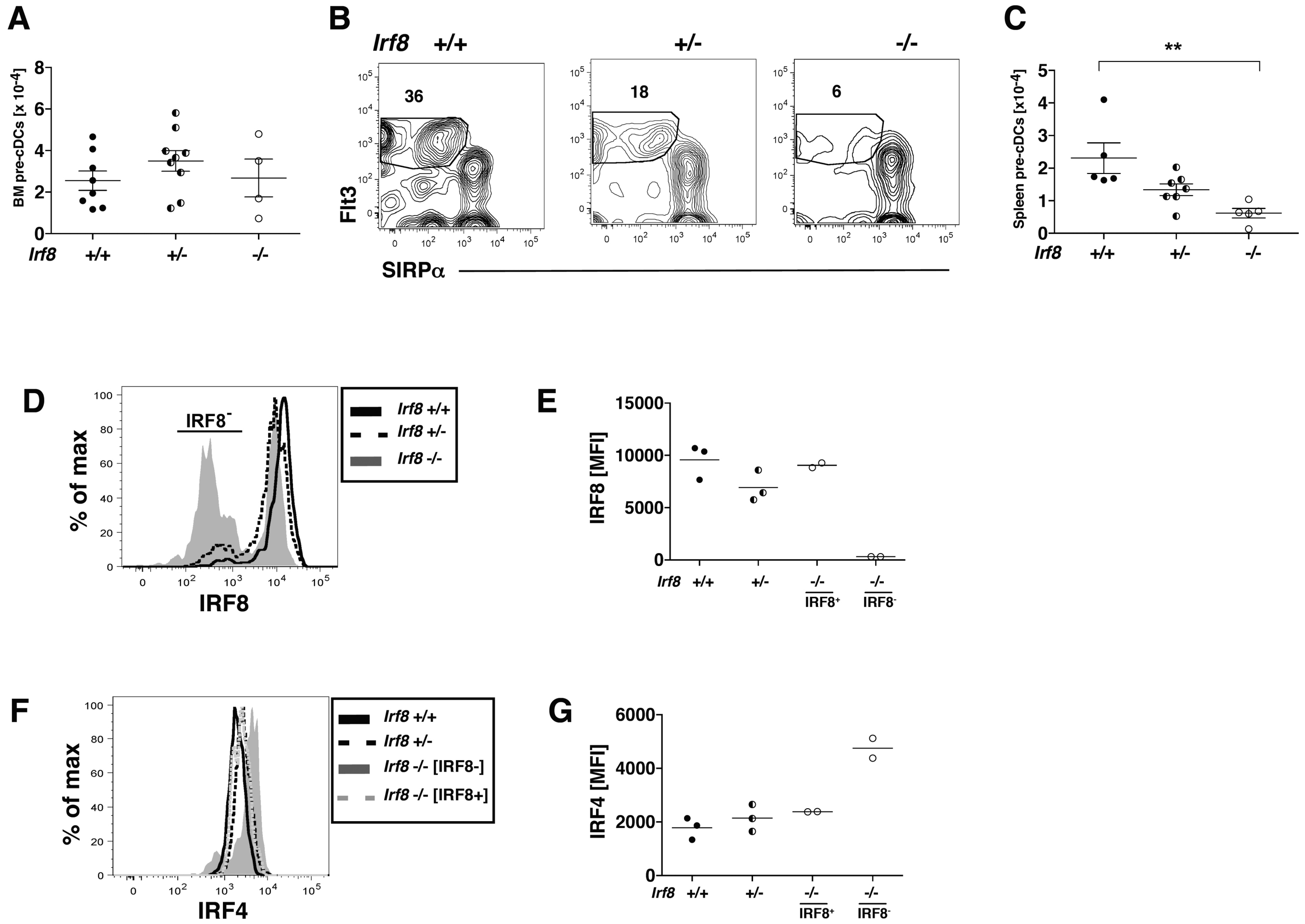

Fig. 7. IRF8 deficiency in CD11c^{+} cells leads to decreased numbers of splenic pre-cDCs expressing elevated levels of IRF4

(**A**) The numbers of pre-cDCs in the bone marrow of multiple *CD11c-cre-Irf8* +/+, +/– and –/– mice are compiled, n=4–9 per genotype. (**B**) Shown is the gating of SIRPα^{-}Flt3^{+} pre-cDCs within the lineage-negative (CD19^{-}CD3^{-}NK1.1^{-}Ter119^{-}B220^{-}) CD11c^{+}MHCII^{-} fraction of splenocytes in *CD11c-cre-Irf8* +/+, +/– and –/– mice. (**C**) The numbers of pre-cDCs in the spleens of multiple *CD11c-cre-Irf8* +/+, +/– and –/– mice are compiled, n=5–7 per genotype. (**D**) IRF8 protein levels were determined in +/+, +/– and –/– pre-cDCs by intracellular staining. Binding of the anti-IRF8 mAb to pre-cDCs of *Irf8*-deficient mice revealed that not all pre-cDCs in the *Irf8*-deficient mice have deleted *Irf8*. (**E**) The anti-IRF8 MFI values were compiled from multiple mice (n=2–3). In –/– mice, the IRF8 MFI is reported separately for the IRF8^{+} and IRF8^{-} populations. (**F**) IRF4 protein levels were determined in +/+, +/– and –/– pre-cDCs by intracellular staining, and (**G**) the anti-IRF4 MFI values were compiled from multiple mice (n=2–3). In –/– mice, the IRF4 MFI is reported separately for the IRF8^{+} and IRF8^{-} populations.