Klf4 Expression in Conventional Dendritic Cells Is Required for T Helper 2 Cell Responses

Graphical Abstract

Highlights

- Klf4 is required for the development of a subset of IRF4-expressing cDCs
- Klf4 deletion results in reduced pre-cDCs
- A specific Klf4-dependent subset can be identified in several tissues
- Klf4\textsuperscript{fl/fl} Itgax-cre mice have selectively impaired Th2 cell immunity

Authors
Roxane Tussiwand, Bart Everts, ..., Edward J. Pearce, Kenneth M. Murphy

Correspondence
kmurphy@wustl.edu (K.M.M.), r.tussiwand@unibas.ch (R.T.)

In Brief
The IRF4-expressing cDCs subset is highly heterogeneous and has been implicated in priming Th17, as well as Th2 cell immunity. Murphy and colleagues dissect this heterogeneity showing a developmental requirement for Klf4 on specific subsets across several tissues. Moreover, Klf4 guides a transcriptional program necessary for Th2 cell immunity.

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**Klf4 Expression in Conventional Dendritic Cells Is Required for T Helper 2 Cell Responses**

Roxane Tussiwand,1,3,* Bart Everts,1,4 Gary E. Grajales-Reyes,1 Nicole M. Kretzer,1 Arifumi Iwata,5 Xiaodi Wu,1 Rachel Wong,1 David A. Anderson,1 Theresa L. Murphy,1 Edward J. Pearce,1 and Kenneth M. Murphy1,2,∗

1Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA
2Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110, USA
3Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058, Basel, Switzerland
4Department of Parasitology, Leiden University Medical Center, Albinusdreef 2, 2333 Leiden, the Netherlands
5Department of Pediatrics, Washington University School of Medicine, 1 Children’s Place, St. Louis, MO 63110, USA

*Correspondence: kmurphy@wustl.edu (K.M.M.), r.tussiwand@unibas.ch (R.T.)

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**SUMMARY**

The two major lineages of classical dendritic cells (cDCs) express and require either IRF8 or Klf4 transcription factors for their development and function. IRF8-dependent cDCs promote anti-viral and T-helper 1 (Th1) cell responses, whereas IRF4-expressing cDCs have been implicated in controlling both Th2 and Th17 cell responses. Here, we have provided evidence that Kruppel-like factor 4 (Klf4) is required in IRF4-expressing cDCs to promote Th2, but not Th17, cell responses in vivo. Conditional Klf4 deletion within cDCs impaired Th2 cell responses during *Schistosoma mansoni* infection, *Schistosoma* egg antigen (SEA) immunization, and house dust mite (HDM) challenge without affecting cytotoxic T lymphocyte (CTL), Th1 cell, or Th17 cell responses to *herpes simplex virus*, *Toxoplasma gondii*, and *Citrobacter rodentium* infections. Further, Klf4 deletion reduced IRF4 expression in pre-cDCs and resulted in selective loss of IRF4-expressing cDC subsets in several tissues. These results indicate that Klf4 guides a transcriptional program promoting IRF4-expressing cDCs heterogeneity.

**INTRODUCTION**

Conventional dendritic cells (cDCs) are professional antigen-presenting cells that play a key role in shaping appropriate immune responses (Banchereau and Steinman, 1998; Merad et al., 2013; Satpathy et al., 2012b; Mildner and Jung, 2014). Several transcription factors have been implicated in cDCs development, but the basis for specification and commitment of cDC subsets is still incompletely understood (Belz and Nutt, 2012; Murphy, 2013). One major subset of cDCs identified by the expression of CD8α in spleen, and CD24 or CD103 in the periphery, requires the transcription factors IRF8 (Hambleton et al., 2011; Tailor et al., 2008), BATF3 (Edelson et al., 2010; Hildner et al., 2008; Ginhoux et al., 2009), NFIL3 (Kashivada et al., 2011), and ID2 (Hacker et al., 2003; Spits et al., 2000). Selective loss of CD8α+ and CD103+ cDCs in Batf3−/− mice demonstrated the specialization of this cDC subset in promoting anti-viral immunity, tumor rejection, and protection against *Toxoplasma gondii* infection (Mashayekhi et al., 2011; Hildner et al., 2008; Tussiwand et al., 2012; Pinto et al., 2011; Torti et al., 2011).

The second major branch of cDCs is characterized by the expression of IRF4 and CD11b and is developmentally impacted by the transcription factors RelB, Traf6, Notch2, Irf4, and Klf4 (Mildner and Jung, 2014).

The function of CD11b+ cDCs in controlling different classes of immune responses has been recently examined (Lewis et al., 2011; Satpathy et al., 2013; Persson et al., 2013; Schlitzer et al., 2013; Williams et al., 2013; Gao et al., 2013; Kumamoto et al., 2013; Zhou et al., 2014). Conditional Notch2 deletion in cDCs impaired development of CD11b+ cDCs expressing CD4 and the endothelial cell-selective adhesion molecule (ESAM) (Lewis et al., 2011; Satpathy et al., 2013). These mice are susceptible to infection with *Citrobacter rodentium*, resulting from a requirement for interleukin-23 (IL-23) production by Notch2-dependent cDCs early during infection (Satpathy et al., 2013). Similarly, conditional deletion of *Irf4* in cDCs causes a reduction in the numbers of CD11b+ cDCs, and reduced IL-23 production leading to impaired Th17 cell development in both lung and intestine (Persson et al., 2013; Schlitzer et al., 2013). Consistently, mice lacking IRF4 expression in cDCs are therefore susceptible to pulmonary infection with *Aspergillus fumigatus* (Schlitzer et al., 2013).

Subsequent studies showed that Irf4-dependent CD11b+ cDCs are involved in Th2 cell responses in skin and lung (Gao et al., 2013; Kumamoto et al., 2013; Williams et al., 2013; Zhou et al., 2014). However, other reports have demonstrated that IRF4 acts in both migration of CD11b+ cDCs (Bajaña et al., 2012) and expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules in cDCs (Vander Lugt et al., 2014). As a result, it is unclear whether there is a specific cDC subset dedicated to Th2 cell priming that is selectively dependent on IRF4, or whether IRF4 acts in all CD11b+ cDCs, affecting their migration and maturation in both Th17 and Th2 cell responses.

We identified Kruppel-like factor 4 (Klf4) as a potential candidate for regulating the development of the IRF4-expressing CD11b+ cDCs. Klf4 can act as a repressor or activator of transcription and regulates development in several epithelial tissues, including skin, lung, and intestine (Segre et al., 1999; Dang et al., 2000; Katz et al., 2002; Dang et al., 2000; Ghaleb et al., 2005; Feinberg et al., 2007; Alder et al., 2008; Zheng et al., 2009; McConnell and Yang, 2010; Yamanaka, 2008; Yoshida and
RESULTS

Conditional Deletion of Klf4 Alters Development of IRF4-Expressing pre-cDCs

Klf4 expression was transiently upregulated at the bone marrow (BM) pre-cDC stage, whereas Irf8 was induced in common DC progenitors (CDPs) (Liu et al., 2009; Akashi et al., 2000; Onai et al., 2007) (Figure 1A). Klf4 expression within mature splenic cDC subsets was reduced compared to Irf8 and Irf4 (Figure 1B). We crossed the Klf4fl/fl allele (Katz et al., 2002) onto Vav1-icre, Itgax-cre, and Ly22-cre deleter strains (Cat on et al., 2007; de Boer et al., 2003; Claussen et al., 1999). Vav1-icre induced general hematopoietic Klf4 deletion as expected, whereas Itgax-cre deleted Klf4 only within cDCs (Figure S1A). Deletion of Klf4 by Vav1-icre resulted in loss of Ly6C+ monocyte development (Figures S1B and S1C), as previously reported (Feinberg et al., 2007). Neither Ly22-cre- nor Itgax-cre-mediated deletion of Klf4 impaired Ly6C+ monocyte development, confirming an early developmental requirement for Klf4 in monocyte differentiation and validating the use of Itgax-cre mice for a cDC restricted deletion of Klf4 (Figures S1B and S1C). Klf4 deletion by Vav1-icre reduced the expression of IRF4 on pre-cDC (Figure S1E) and impaired development of SiglecH- pre-cDCs (Figures 1C–1E), which also had reduced IRF4 expression (Figure S1E). Macrophage and DC precursors (MDPs) and cDCs were unaltered in Klf4fl/fl Vav1-icre mice (Figures 1C–1E, S1I). CD11c is induced at the pre-cDC stage (Naik, 2010; Liu et al., 2007) and comparison of Klf4fl/fl Itgax-cre and cre-negative progenitors showed few changes in gene expression in CDPs, but increased differences in pre-cDCs (Figures S1F–S1I). Deletion of Klf4 by Vav1-icre or Itgax-cre reduced IRF4 expression in progenitors but still

Figure 1. KLF-4 Deletion Impairs Development of Bone-Marrow pre-cDC Progenitors

(A) Relative expression of Klf4, Irf8, and Irf4 determined by microarray analysis is shown for the indicated stages of DC progenitors.
allowed the divergence of DC progenitors into two major subsets of IRF8+ and IRF4+ cDCs in BM cultures (Figure S1D).

**Klf4 Regulates Development of Specific Subsets of IRF4-Expressing cDCs in Peripheral Tissues**

We next examined various lymphoid and peripheral tissue-resident DC subsets under conditional deletion of Klf4 (Figures 2 and S2). Consistent with a previous report (Park et al., 2012), there was approximately 50% reduction in total CD11c+ MHC-II+ cells in spleen (Figures 2D and S2G), mostly compromising Sirp-α+ CD24+ cDCs (Figures 2D and S2G). Approximately a 50% reduction of CD11b+ cDCs occurred across all lymphoid and peripheral tissues examined (Figures 2 and S2). In skin draining lymph nodes (sLN), one subset within the migratory cDCs was completely absent (Figures 2A, 2B, and S2B). In skin, Langerhans cells (LCs) and at least four other cDCs subsets can be distinguished on the basis of CD207, CD103, and CD11b (Henri et al., 2010). The CD103+/CD207− cDC subset represents the Batf3-dependent cDC lineage, but three other subsets (CD103−CD207+, CD207−CD11b+, and CD103−CD11b−CD207− cDCs) are independent of Batf3 (Edelson et al., 2010; Ginhoux et al., 2009) and have distinct transcriptional identities (Miller et al., 2012; Robbins et al., 2008; Bar-On and Jung, 2010). Among these migratory cDC subsets, deletion of Klf4 completely impaired development of CD11b−CD24− cDCs, previously called “double negative” (DN) cDCs (Maissen et al., 2014; Henri et al., 2010) and characterized as CD207−CD11b−Serp-α+ CX3CR1+ (Figures 2B, S2A, and S2B and data not shown). In the dermis, deletion of Klf4 reduced CD11b+ cDCs and increased CD24+ and CD103+ cDCs (Figure S2D).

In the lung, Klf4-deficiency selectively eliminated a Sirp-α−CD24+ subset (Figures S2E and S2J, S2K), which comprised 20% of cDCs (Figures S2J and S2K), and reduced Sirp-α−CD24+ cDCs. Further, Mgl-2 expression identified the Klf4-dependent Sirp-α−CD24+ cDCs in lung (Figure 2E), but not in sLN (Figure S2C). In mediastinal lymph nodes, Klf4 deficiency severely reduced Sirp-α−cDCs, which expressed PD-L2, but not Mgl2 (Figures S2L and S2M and data not shown). In the liver, Klf4 deficiency reduced the frequency of Sirp-α−CD24− cDCs, which also expressed Mgl2 (Figure S2H). In mesenteric lymph nodes, lamina propria, and thymus, Sirp-α−cDCs were reduced and CD24+CD103+ respectively increased in the absence of Klf4 (Figures S2E and S2F; Figure 2F and S2I). These results highlight the requirement for Klf4 on the IRF4-expressing, but not IRF8-dependent cDCs, and suggest heterogeneous expression of PD-L2 and Mgl2 across the different peripheral tissues (Figures 2E and S2 and data not shown). Expression amounts of IRF4 and IRF8 were analyzed on Klf4Cre+ mice (Figure S2C and data not shown). Among the different IRF4 expressing cDCs, the Klf4-dependent DN subset showed the highest expression level for IRF4 (Figure 2C).

**A Cell-Intrinsic Requirement for Klf4 in Development of CD11b−cDCs in sLNs**

Previous studies concluded that dermal CD11b+ MHC-II+ cells were conventional DCs based on hematopoietic origin and dependence on Flt3L (Henri et al., 2010; Mollah et al., 2014), although these properties also identify progenitors of other hematopoietic lineages (McKenna et al., 2000; Mackarehtschian et al., 1995). Thus, we independently assessed the identity of the sLN Klf4-dependent cells (Figure 3). Klf4-dependent CD11b+ cells had morphology similar to other migratory and resident cDC subsets (Figures 3A and S3A), were not affected by Lys2-cre-mediated Klf4 deletion, and were present in Csf2rb−/− mice (Figures 3B and 3C). In contrast, IRF8-dependent CD103+ cDCs develop in Csf2rb−/− mice but have reduced surface CD103 expression (Figure 3C) (Edelson et al., 2011). Moreover, the Klf4-dependent CD11b+ cells in sLNs were absent in Ifi31−/− and Itgax−/− mice (Figures 3B and 3C), confirming their identity as IRF4-dependent cDCs. Among migratory cDCs, IRF4 expression was highest on the Klf4-dependent DCs (Figure S1W). Klf4 deficiency did not impair the development of CD24+ cDCs, which were absent in Ifi31−/− mice (Figures 3B and 3C), nor the development of LCs, which were severely reduced in Ifi34−/− mice (Wang et al., 2012; Greter et al., 2012) (Figure S3F). Klf4-dependent CD11b+ cells were most similar in gene expression to migratory CD11b+ cDCs and more distantly related to resident CD11b+ cDCs and LCs (Figures 3D and S3G). In Zbtb46fl/fl mice, the Klf4-dependent CD11b+ cells expressed GFP at amounts similar to migratory CD11b+ cDCs and resident cDCs in sLN (Figures 3E and S3H–S3J). These results suggest that Klf4-dependent CD11b− cells are bona fide dendritic cells.

To test for a non-cell intrinsic action of Klf4, we analyzed single- and mixed-BM chimeras reconstituted with Klf4Cre+Itgax-cre and Zbtb46fl/fl BM (Figures 3F and S3B–S3D). A hematopoietic requirement of Klf4 was confirmed by the selective loss of migratory CD11b− cDCs in sLN (Figure 3F) and the reduction of splenic CD11b+ cDCs (Figure S3B) in chimeras reconstituted with Itgax-cre Klf4+BM. We also excluded an indirect action of Klf4 deficiency on other hematopoietic lineages in regulating development of migratory CD11b+ cDCs, because this subset was positive for GFP expression in chimeras reconstituted with a 10:1 mixture of Itgax-cre Klf4+BM and Zbtb46fl/fl BM (Satpathy et al., 2012a) (Figures S3C and S3D). As a control, we observed that similar ratios of GFP-positive and -negative cells developed within the CD24+ and CD11b− migratory DC subsets (Figure S3D). Collectively, these results argue for a cell-intrinsic requirement for Klf4 acting on migratory CD11b− cDCs.

**Klf4-Dependent Migratory cDCs Transport Antigen from Skin to sLNs**

cDCs residing in the migratory gate transport the majority of fluorescein isothiocyanate (FITC) from skin to sLNs and the Klf4-dependent CD11b− migratory cDC subset accounted for a substantial fraction of total antigen transport (Figures 4A–4C). Itgax-cre Klf4fl/fl and Vav1-cre Klf4fl/fl mice showed reduced percentage and absolute numbers of FITC+ cells present in sLNs after FITC painting (Figures 4A, 3B, and S4A). In control mice, direct examination of FITC+ cells showed roughly similar contributions to antigen transport by CD11b+ and Klf4-dependent CD11b− cDCs, which together accounted for about 90% of FITC+ cells (Figures 4B, 4C, and S4B). In the absence of migratory CD11b− cDCs, the relative contribution to antigen transport by CD24+ cDCs was increased from 10% to roughly 40% (Figures 4B, 4C, and S4B). In contrast to Itgax−/− mice (Bajaña et al., 2012; Gao et al., 2013), deletion of Klf4 did not affect antigen transport, as Klf4-independent migratory CD11b+ cDCs...
Figure 2. A Discrete Subset of Migratory cDCs in Skin-Draining Lymph Nodes Requires Klf4

(A and B) Two-color histograms for CD11b and CD24 expression are gated on live cells for the resident (A) and migratory (B) gates shown in (S2A).

(C) Single color histograms for intracellular IRF4 expression of the indicated subsets gated as in Figures 2A and 2B for resident and migratory DCs isolated from Klf4^{fl/fl} cre-negative mice. Dotted line shows Irf4^{-/-} control cDCs. Numbers represent geometrical mean values of IRF4 expression on the gated subsets.

(D) Splenocytes from mice of the indicated genotypes were pre-gated as cDCs (MHC-II^{hi} CD11c^{+}) and analyzed for CD24 and Sirp-α expression. Numbers indicate the percent of cells in the gate.

(E and F) Two-color histograms for Sirp-α, CD24, CD11b, CD103, and Mgl2 as indicated in the plots, are shown for live cells from lung (E) and liver (F) of mice of the indicated genotype. Cells were pre-gated as CD45^{+} MHC-II^{hi} CD11c^{+} CD64^{-}. Numbers indicate percent cells in the gates. All stainings were repeated in at least three independent experiments using at least two mice per genotype.

See also Figure S2.
Figure 3. Klf4-Dependent CD11b- cDCs Require IRF4 and FLT3L, Express Zbtb46gfp, and Most Closely Resemble CD11b+ cDCs
(A) Wright's stain of sort-purified migratory CD11b- cDCs (mCD11b-), CD11b+ (mCD11b+), or CD24+ (mCD24+) and resident CD11b+ (resCD11b+) cDCs. Images were collected from two independent experiments, cells from two mice were pooled and at least ten cells were evaluated per subset.
(B and C) Representative two-color histograms for CD11b, CD24 (B), and CD103, Sirp-a (C) for cells pre-gated as migratory CD24+ cDCs as in Figure 2B are shown for sLN cells harvested from mice of the indicated genotypes. Analysis was performed in at least two independent experiments and at least three mice per genotype were analyzed.
(D) Microarray analysis of gene expression presented as M-plots for sort-purified mCD11b- cDCs, mCD11b+ cDCs, CD24+ migratory cDCs, and resident CD11b+ cDCs harvested from sLN. Colors indicate higher (red) or lower (blue) expression. Shown at the bottom right is the number of genes differing between paired samples by more than 2-fold.

(legend continued on next page)
subset transported substantial antigen from skin to sLNs, in both Itgax-cre Klf4^{fl/fl} and Vav1-icre Klf4^{fl/fl} mice (Figures 4B, 4C, and S4B).

**Deletion of Klf4 in cDCs Selectively Impairs Th2 Cell Responses to Pathogens**

IRF4-expressing DCs produce IL-23 that drives ILC3 and Th17 cell activation and protect against Citrobacter rodentium and Aspergillus fumigatus infection (Schlitzer et al., 2013; Satpathy et al., 2013; Persson et al., 2013). We tested the role of Klf4-dependent cDCs in IL-23 production and Th17 cell response by infecting Itgax-cre Klf4^{fl/fl} mice with C. rodentium. We confirmed that susceptible to C. rodentium infection was increased in mice where Notch2 was deleted by Itgax-cre (cNotch2^{−/−}). These mice lost 10%–15% of their body weight and succumbed to infection around day 12 (Figures 5A and 5B). In contrast, Itgax-cre Klf4^{fl/fl} mice did not lose weight and survived infection similar to wild-type mice, suggesting normal in vivo activation of ILC3 and Th17 cell responses. While cNotch2^{−/−} mice have severely reduced splenic ESAM^{+} cDCs, Klf4 deficiency did not compromise development of this subset (Figures S5A–S5C).

Protection against the helminth pathogen Schistosoma mansoni requires a Th2 cell response (Brunet et al., 1997; Fallon et al., 2000; Herbert et al., 2004). We infected Klf4^{fl/fl} Itgax-cre and Il4^{−/−} mice with S. mansoni (Figure 5C). Il4^{−/−} mice succumbed to infection after 50 days with an overall mortality of approximately 75% at 60 days after infection (Figure 5C), as expected (Brunet et al., 1997; Fallon et al., 2000; Pearce et al., 1996). Klf4^{fl/fl} Itgax-cre mice succumbed to infection with a similar rate and kinetic as Il4^{−/−} mice, while wild-type control mice survived beyond 100 days after challenge (Figure 5C).

Because Itgax-cre can induce deletion in cells other than DCs, including T cells (Figure S1A) (Abram et al., 2014), we tested Th2 and Th17 cell polarization after Klf4 deletion. Deletion of Klf4 by either Vav1-icre or Itgax-cre did not decrease IL-17, IL-4, and IL-10 production in Th17 or Th2 cells compared to cre-negative controls (Figures 5D and 5E), but Baff^{−/−} Batf3^{−/−} T cells were strongly impaired in all of these cytokines, as expected (Tussiwand et al., 2012). Further, deletion of Klf4 by either Vav1-icre or Itgax-cre also did not reduce intracellular expression of GATA3 (Figures 5D and 5E). Thus, deletion of Klf4 in T cells by Vav-cre leaves Th2 cell polarization intact, suggesting that susceptibility to S. mansoni infection occurred through a mechanism other than T cell intrinsic action.

We also immunized mice with S. mansoni egg antigen (SEA) (Sabin et al., 1996; Everts et al., 2012; Kane et al., 2008; Pearce et al., 1991). IL-4 and IL-5 were both produced by T cells harvested from sLNs 7 days after SEA immunization of wild-type cre-negative control mice, but this was substantially and selectively reduced in Klf4^{fl/fl} Itgax-cre mice (Figures S5D–S5G). Moreover, switching of germinal center B cells from surface immunoglobulin M (IgM) to IgG1 after SEA-immunization was reduced in Klf4^{fl/fl} Itgax-cre mice relative to cre-negative control mice (Figures S7H and S7I).

To test whether Klf4 deletion within cDCs impaired the function of the IRF8- and Batf3-dependent cDCs, we examined infections by T. gondii and herpes simplex virus (HSV) (Masihyekhi et al., 2011; Tussiwand et al., 2012). Control mice cre-negative Klf4^{fl/fl} and Itgax-cre Klf4^{fl/fl} mice were similarly resistant to T. gondii infection, while Batf3^{−/−} mice were as expected highly susceptible (Figures S5J and S5K). Second, Itgax-cre Klf4^{fl/fl} mice, but not Batf3^{−/−} mice, generated a robust Th1 cell response specific for the Gb2 peptide of herpes simplex virus (HSV) 7 days after infection (Figures S5L and S5M). Also, cross-presentation of cell-associated antigens was preserved in Itgax-cre Klf4^{fl/fl} mice (Figure S5N) suggesting that IRF8-expressing DC were phenotypically and functionally fully preserved in Itgax-cre Klf4^{fl/fl} mice.

We also excluded that klf4 deletion resulted in a general priming defect in the remaining IRF4-dependent cDCs by performing several DC-T cell cultures, where we did not observe any difference using either BM derived or isolated DCs from control mice cre-negative, Itgax-cre or Vav1-icre Klf4^{fl/fl} mice (Figure S5O). Further, Klf4-dependent DCs did not show superior in vitro priming ability, but only increased expression of the co-stimulatory molecules CD80 and CD86 after SEA (data not shown).

Collectively, because deletion of Klf4 in Itgax-cre Klf4^{fl/fl} mice did not compromise the development of other myeloid subsets other than cDCs and these mice had normal Th17, Th1, and CTL cell response to several infections and antigens, Klf4 appears to be selectively required on DCs for inducing Th2 immunity.

**Deletion of Klf4 in cDCs Prevents House Dust Mite-Induced Allergic Inflammation**

Development of asthma is associated with a Th2 cell type of immunity characterized by the accumulation of eosinophils (Robinson, 2000; Robinson et al., 1992). In mice, intra-nasal challenge with extracts of house dust mite (HDM) causes acute allergic peribronchial Th2 cell inflammation mediated by CD11b^{+} cDCs (Hammad et al., 2009; Plantinga et al., 2013; Williams et al., 2013). Because pulmonary macrophages express CD11c, we used Klf4^{fl/fl} Lyz2-cre mice as a control for potential requirements for Klf4 in pulmonary macrophages. Klf4^{fl/fl} Itgax-cre mice showed a dramatic reduction in pulmonary accumulation of eosinophils compared to both cre-negative Klf4^{fl/fl} control mice and Klf4^{fl/fl} Lyz2-cre mice, which had robust eosinophilia (Figures 6A and 6B). Control cre-negative and Klf4^{fl/fl} Lyz2-cre mice, but not Klf4^{fl/fl} Itgax-cre mice, had typical asthmatic features with peribronchial inflammation with substantial leukocyte infiltrations (Figure 6C).

**DISCUSSION**

Klf4 regulates DCs development beginning in the pre-cDC, where it impacts the IRF4-expressing branch of cDCs. Deletion

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(E) Shown are single-color histograms for Zbtb46^{−/−} expression for B cells, mCD11b^{+}, or mCD11b^{−} cDCs from sLN of Zbtb46^{−/−} mice (Satpathy et al., 2012a). Numbers are the percent of live cells within the indicated gate.

(F) Chimera were generated by reconstitution of lethally irradiated C57BL/6 mice (WT) using BM from Klf4^{fl/fl} cre-negative or Klf4^{fl/fl} Itgax-cre mice as indicated. Shown are two-color histograms as in (B) for cells pre-gated as migratory cDCs as in Figure S2A. Experiment was performed twice and at least four mice per group were analyzed. See also Figure S3.
Figure 4. Klf4-Dependent CD11b+ cDCs Account for Substantial Antigen Transport from Skin to Lymph Nodes

(A) Shown is the contribution to total FITC+ cells by migratory (open bars) and resident (closed bars) cDCs in skin draining lymph nodes (sLN) 16 hr after FITC painting, as a percentage of total FITC+ sLN cells. Error bars ± SD, n = 6, Student’s t test. ***p < 0.001.

(B) Shown is contribution to total FITC+ cells by each migratory DC subset as a percentage of total FITC+ sLN cells in mice of the indicated genotypes 16 hr after FITC painting. Error bars ± SD, n = 6, Student’s t test. ***p < 0.001.

(C) FITC transport by individual migratory cDC subsets is show as single color histogram from mice of the indicated genotypes and gates (left panels). N.P., not present. Numbers indicate the percent of cells in the indicated gates. The experiment was performed three times, and two mice per group were used.

See also Figure S4.
Figure 5. Conditional Deletion of *Klf4* in DCs Selectively Impairs Th2 Cell Responses to *S. mansoni*

(A and B) Survival (A) and weight loss (B) of mice after oral inoculation of *C. rodentium* (2 x 10^9 colony-forming units). Mice used were *Klf4*^{fl/fl} (n = 20) or *Notch2*^{fl/fl} (n = 5) (Satpathy et al., 2013) crossed to *Itgax*-cre (n = 10) (Caton et al., 2007) (*Notch2^{−/−}* or *Klf4*^{fl/fl} cre-negative (cre-neg) (n = 10) as indicated. Experiment was repeated two times.

(C) Survival of mice after skin inoculation with 100 *Schistosoma mansoni* cercariae. Mice used were *Klf4*^{fl/fl} crossed to *Itgax*-cre, (n = 7) or a cre-negative background, (n = 11), or were *Il4*^{−/−} (Kopf et al., 1993) (n = 7) experiment was repeated three times.

(D) Shown are histograms for intracellular GATA3 and IL-4 staining for CD4^+ T cells isolated from mice of the indicated genotypes purified by MACS beads and passed twice in vitro with anti-CD3 and anti-CD28 under Th2-inducing (solid lines) or Th1-inducing conditions (dotted and shaded) as described (Schraml et al., 2009). Mice used were *Klf4*^{fl/fl} crossed to *Itgax*-cre, *Vav1*-icre, cre-negative, or *Batf3*^{−/−} double-deficient mice (Tussiwand et al., 2012). Numbers are the percent of cells in the indicated gates.

(E) MACs purified T cells from mice of the indicated genotypes as in (D) were polarized under Th1-, Th17-, or Th2-inducing conditions as described (Schraml et al., 2009). Shown are the percent of T cells positive for intracellular expression of the indicated cytokine or factor for each genotype as a percent of the maximum expression obtained for cre-negative control T cell samples. Two mice per genotype were used per experiment and the experiment was repeated four times. Error bars ± SD, n = 8, Student's t test.

See also Figure S5.
of Klf4 by Vav1-icre altered the expression of relatively few genes, but among these, IRF4 was severely downregulated as measured by microarray expression and intracellular staining. Reduction of IRF5 and induction of IRF4 occurs at the SiglecH- pre-cDCs stage, a stage that was severely reduced in Vav1-icre Klf4+/mice. However, IRF4 expression was not altered in mature cDCs or in Itgax generated BM-derived cDCs implying a developmental rather than an absolute requirement for Klf4 in the induction of IRF4 expression.

In mature cDCs, Klf4 deletion selectively compromised the development of particular subsets of IRF4-expressing cDCs. The subset most clearly dependent on Klf4 was the previously described migratory subset of CD11b-CD24- (DN) cDCs in sLN. This subset had the highest expression of IRF4 among migratory cDCs. However, it is still unclear whether impaired Klf4-dependent cDCs development is caused by reduced IRF4-expression at a progenitor stage or due to some other action of Klf4.

The DN migratory cDCs identified here as being Klf4 dependent have been described recently to acquire fluorescently labeled antigen from non-viable Nippostrongylus larvae and suggested to be involved in Th2 cell responses based on in vitro analysis (Ochiai et al., 2014; Connor et al., 2014). Our results would support those conclusions by drawing a correlation between the loss of the DN cDC subset and reduced in vivo Th2 cell responses in Klf4-deficient mice. We also observed a higher co-stimulatory potential of the Klf4-dependent DN migratory cDCs relative to other cDC subsets after SEA stimulation. Nonetheless, despite the genetic requirement for Klf4 for DN cDC development, it is still possible that Klf4 controls Th2 responses by actions in other cDC subsets that are not seemingly affected by the deletion. Excluding such possibilities awaits the development of deleter strains with higher DC-subset specificity.

IRF4-expressing cDCs were recently shown to control Th2 cell responses induced in skin and lungs (Schlitzer et al., 2013; Gao et al., 2013; Kumamoto et al., 2013; Zhou et al., 2014; Williams et al., 2013), although no mechanism for this action was determined. Irf4-/- cDCs fail to migrate to sLN (Bajana et al., 2012) and show reduced expression of co-stimulatory molecules (Vander Lugt et al., 2014), which could explain reduced T cell priming, at least in skin. Klf4 deletion selectively impaired Th2 cell responses without preventing migration of the remaining cDCs.

IRF4-expressing cDCs also control Th17 cell responses in lungs and intestine (Schlitzer et al., 2013; Lewis et al., 2011; Satpathy et al., 2013). We find that in the lung, Klf4 deletion selectively eliminated an IRF4-dependent cDC subset characterized by the expression of Sirp-a-CD24+ Mgl-2+. Conceivably, Klf4-dependent Sirp-a-CD24+ lung cDCs might mediate Th2 cell responses to HDM, while Klf4-independent Sirp-a-CD24+ cDCs mediate Th17 cell immunity to fungal infections.

Figure 6. Conditional Deletion of Klf4 in DCs Prevents Development of HDM-Induced Allergic Inflammation
(A) HDM sensitized mice of the indicated genotypes were challenged intranasally with PBS or HDM extract. After 3 days, bronchial alveolar lavage (BAL) was analyzed for eosinophils by FACS. Shown are eosinophils as a percentage (left panel) or total numbers recruited into BAL (right panel) and pre-gated as CD45+ live cells. Mice used were Klf4+/cre (n = 8) or a cre-negative background (n = 8) 3 mice per genotype used per experiment. Experiment was repeated three times. Error bars, ± SD, n = 8. Student’s t test. ***p < 0.001.

(B) Mice of the indicated genotypes were challenged as in (A) and BAL analyzed by flow cytometry after 3 days. Shown are two-color histograms pre-gated on CD45+ BAL cells. Numbers indicate percentage of cells in the indicate gates for eosinophils (SiglecF+CD11c+) or pulmonary macrophages (SiglecF+CD11c+), respectively.

(C) Shown are hematoxylin and eosin stained sections of lungs from mice described in (A) as indicated. Scale bars represent 200 μm.
Alternately, Klf4 might act on both IRF4-expressing cDC subsets but selectively influence Th2, but not Th17, cell priming. Similarly, in the small intestine, Th17 cell immunity against *C. rodentium* infection has been attributed to the action of a Notch2-dependent subset of IRF4-expressing cDCs (Lewis et al., 2011; Satpathy et al., 2013). Klf4 deletion did not increase susceptibility to *C. rodentium* infection and did not impair development of the splenic Notch2-dependent ESAM-expressing cDC subset. Thus, in the intestine as well, distinct transcriptional programs regulated by either Notch2 or Klf4 appear to control different types of immunity mediated by the IRF4-expressing cDCs.

Our results show a genetic requirement for Klf4 in cDCs for Th2 cell immunity; however, they do not reveal the cellular mechanism mediating Th2 cell responses. The in vivo trigger for Th2 cell development has been elusive, with many mechanisms proposed over time. Conceivably, a particular cDC subset could directly induce Th2 development, for example by production of a cytokine or expressing a specific surface receptor. Alternately, the cDC subset could recruit an accessory cell that favors and creates the necessary microenvironment for Th2 cell priming. Indeed, recent evidence suggests the involvement of innate lymphoid cells type 2 (ILC2s) in the lung (Halim et al., 2014; Licona-Limón et al., 2013; Halim et al., 2012) and of eosinophils in the intestine (Chu et al., 2014). It will be important to determine whether Klf4-dependent cDCs utilize these cells, because such a mechanism would not be re-capitulated by in vitro studies using isolated co-cultures of cDCs and T cells.

**EXPERIMENTAL PROCEDURES**

**Mice**

All animals were bred and maintained in a specific pathogen-free animal facility according to institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis. Mice of the following genotypes were purchased from Jackson Laboratories: wild-type mice C57BL6 (C57BL/6J), Igta-gcre (B6.Cg-Tg(Igta-gcre)1Balwi/J) (Caton et al., 2007), Vav1-icre (B6.Cg-Tg(Vav1-cre)2Akijs/J) (Harker et al., 2002), Lyz2-cre (B6.129P2-Lyz2tm1Jlskr+), (Claussen et al., 1999), and Cx3cr1cre (B6.129P2-Cx3cr1tm1Litt/J) (Jung et al., 2000). Irf4-/- were generated in our facility by deletion of exon 1 (Mashayekhi et al., 2011). For infection mice were infected with the KOS strain s.c. with 1.5 × 10^6 plaque-forming units (pfu) per mouse in the footpad.

**In Vitro T Cell Re-stimulation after SEA and HSV Infection**

One week after HSV infection, spleens and popliteal lymph nodes (LN) were collected and re-stimulated with the HSV peptide HSV-gB2 (498-505) (Anaspec). Briefly, 2 × 10^6 splenocytes or LN cells were re-stimulated for 5 ch in the presence of brefeldin A at 1 μg/ml and analyzed by FACs for intracellular IFN-γ and TNF-α production as described later. Similarly, 1 week after SEA injection, 3 × 10^6 cells harvested from popliteal lymph nodes were collected and restimulated under different conditions: SEA (20 μg/ml), plate-bound anti-CD3, PMA and Lonomycin (50ng/ml and 1 μg/ml), or media alone. For detection of IL-4, 2.5 μg/ml of anti-IL-4R antibody (M1) was added to the culture. Supernatants were collected after 72 hr and analyzed with the BD CBA mouse Th1/Th2 Kit (BD Biosciences), and data were analyzed with FCP Array software (Soft Flow).

**HDM-Induced Asthma**

HDM (Dermatophagoidespteronyssinus extracts, Greer Laboratories) was dissolved in PBS. To induce allergic airway inflammation, we sensitized mice i.n. with 10 μg HDM subsequently challenged with 50 μg HDM i.n. on days 7 and collected lungs, BAL, and mediastinal LNs 3 days after challenge.

**Statistical Analysis**

Differences between groups in survival were analyzed by the log rank test. Analysis of all other data was done with an unpaired, two-tailed Student’s t test with a 95% confidence interval (Prism; GraphPad Software). p values less than 0.05 were considered significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**ACCESSION NUMBERS**

The GEO accession number for the microarray data reported in this paper is GSE68590.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.04.017.

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