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Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses

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Summary

The two major lineages of classical dendritic cells (cDCs) express and require either IRF8 or IRF4 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 challenge (HDM), without affecting cytotoxic T lymphocyte (CTL), Th1 and 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 cDCs 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 basis for specification and commitment of cDC

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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 (Kashiwada et al., 2011) and ID2 (Hacker et al., 2003; Spits et al., 2000). Selective loss of $CD8\alpha^+$ 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*, *Irf2* and *Irf4* (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 (Bajana et al., 2012) and expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules by cDCs (Vander 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). In hematopoietic cells, *Klf4* is expressed on myeloid cells, is required for monocyte development (Feinberg et al., 2007; Alder et al., 2008; Kurotaki et al., 2013) as well as for *in vitro* M2 macrophage polarization (Feinberg et al., 2007; Kurotaki et al., 2013; Terry and Miller, 2014). *Klf4* conditional deficient mice have reduced $CD11b^+$ cDCs in spleen, however the nature of the defect was not further analyzed with respect to cDC subsets or function (Park et al., 2012). Here we showed that *Klf4* is required within IRF4-expressing cDC subsets for normal priming of Th2 cell responses. Our results indicated that

the IRF4-expressing cDC lineage is functionally heterogeneous, with *Klf4* promoting a DC transcriptional program controlling Th2 cell responses.

Results

Conditional deletion of *Klf4* alters development of IRF4-expressing pre-cDCs

Klf4 expression was transiently up-regulated at the bone marrow (BM) pre-cDC stage, while *Irf8* was induced in common DC progenitors (CDPs) (Liu et al., 2009) (Figure 1A). *Klf4* expression within mature splenic cDC subsets was reduced compared to *Irf8* and *Irf4* (Figure 1B). We crossed the *Klf4*$^{fl/fl}$ allele (Katz et al., 2002) onto *Vav1-icre*, *Itgax-cre* and *Lyz2-cre* deleter strains (Caton et al., 2007; de Boer et al., 2003; Clausen 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^{hi} monocyte development (Figure S1B–C), as previously reported (Feinberg et al., 2007). Neither *Lyz2-cre*- nor *Itgax*-cre-mediated deletion of *Klf4* impaired Ly6C^{hi} 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* (Figure S1B–C). *Klf4* deletion by *Vav1-icre* reduced the expression of IRF4 on pre-cDC (Figure S1E) and impaired development of SiglecH^{-} pre-cDCs (Figure 1C–E), which also had reduced IRF4 expression (Figure S1E). Macrophage and DC precursors (MDPs) and CDPs were unaltered in *Klf4*$^{fl/fl}$ *Vav1-icre* mice (Figure 1C–E, S1I). CD11c is induced at the pre-cDC stage (Naik, 2010; Liu et al., 2007) and comparison of *Klf4*$^{fl/fl}$ *Itgax-cre* and *cre*-negative progenitors showed few changes in gene expression in CDPs, but increased differences in pre-cDCs (Figure S1F–I). Deletion of *Klf4* by *Vav1-icre* or *Itgax-cre* reduced IRF4 expression in progenitors, but still 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* (Figure 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 (Figure 2D, S2G), mostly compromising Sirp-α^{+} CD24^{-} cDCs (Figure 2D and Figure S2G). Approximately a 50% reduction of CD11b^{+} cDCs occurred across all lymphoid and peripheral tissues examined (Figure 2, and Figure S2). In skin draining lymph nodes (sLN), one subset within the migratory cDCs was completely absent (Figure 2A,B, and S2B). In skin, Langerhans cells (LC) 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*-depedendent 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 (Malissen et al., 2014; Henri et al., 2010) and characterized as CD207^{-} CD11b^{-} Sirp-α^{+} CX3CR1^{+} (Figure 2B, S2A and B 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 (Figure E and Figure S2J,K)), which comprised 20% of cDCs (Figure S2J,K), 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 (Figure S2l,M and data not shown). In the liver, *Klf4*-deficiency reduced the frequency of Sirp-α$^+$ CD24$^-$ cDCs, which also expressed Mgl-2 (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* (Figure S2E,F Figure 2F and Figure 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 (Figure 2,E,S2 and data not shown). Expression levels of IRF4 and IRF8 were analyzed on *Klf4*$^{f/f}$ cre-negative mice (Figure 2C 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^{hi} 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 (Figure 3A, Figure S3A), were not affected by *Lyz2-cre*-mediated *Klf4* deletion, and were present in *Csf2rb*$^{-/-}$ mice (Figure 3B,C). 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 *Flt3l*$^{-/-}$ and *Irf4*$^{-/-}$ mice (Figure 3B,C), 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 *Flt3l*$^{-/-}$ mice (Figure 3B,C), nor the development of LCs, which were severely reduced in *Il34*$^{-/-}$ 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 (Figure 3D, Figure S3G). In *Zbtb46*^{gfp} mice, the *Klf4*-dependent CD11b$^-$ cells expressed GFP at levels similar to migratory CD11b$^+$ cDCs and resident cDCs in sLN (Figure 3E, and Figure S3H–J). 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 *Klf4*$^{fl/fl}$*Itgax-cre* and *Zbtb46*^{gfp} BM (Figure 3F, and Figure S3B–D). 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*$^{fl/fl}$ BM. We also excluded an indirect action of *Klf4* deficiency on other hematopoietic lineages in regulating development of migratory CD11b^{-} cDCs, since this subset was positive for GFP expression in chimeras reconstituted with a 10:1 mixture of *Itgax-cre Klf4*$^{fl/fl}$ BM and *Zbtb46*^{gfp} BM (Satpathy et al., 2012a) (Figure S3C, D). As a control, we observed that similar ratios of GFP-positive and -negative cells that developed within the CD24^{+} and CD11b^{+} migratory DC subsets (Figure S3D). Collectively, these results argue for a cell-intrinsic requirement for *Klf4* acting o*n* 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 (Figure 4A–C). *Itgax-cre Klf4*$^{fl/fl}$ and *Vav1-icre Klf4*$^{fl/fl}$ mice showed reduced percentage and absolute numbers of FITC^{+} cells present in sLNs following FITC painting (Figure 3A,B 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 (Figure 4B,C, Figure S4B). In the absence of migratory CD11b^{-} cDCs, the relative contribution to antigen transport by CD24^{+} cDCs was increased from 10% to roughly 40% (Figure 4B,C and Figure S4B). In contrast to *Irf4*$^{-/-}$ mice (Bajana et al., 2012; Gao et al., 2013), deletion of *Klf4* did not affect antigen transport, as *Klf4*-independent migratory CD11b^{+} cDCs subset transported substantial antigen from skin to sLNs, in both *Itgax-cre Klf4*$^{fl/fl}$ and *Vav1-icre Klf4*$^{fl/fl}$ mice (Figure 4B,C and S4B).

Deletion of *Klf4* in cDCs selectively impairs Th2 cell responses to pathogens

IRF4-expressing DCs produce of 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* (c*Notch2*$^{-/-}$). These mice lost 10–15% of their body weight and succumbed to infection around day 12 (Figure 5A, B). In contrast, *Itgax*-cre *Klf4*$^{fl/fl}$ mice did not lose weight and survived infection similar to wild type *Klf4*$^{fl/fl}$ *cre*-negative control mice, suggesting normal *in vivo* activation of ILC3 and Th17 cell responses. While c*Notch2*$^{-/-}$ mice have severely reduced splenic ESAM^{+} cDCs, *Klf4* deficiency did not compromise development of this subset (Figure S5A–C).

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 survive beyond 100 days after challenge (Figure 5C).

Since *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 inTh17 or Th2 cells compared to cre-negative controls (Figure 5D, E), but *Batf*$^{-/-}$ *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 either GATA3 (Figure 5D, E). Thus, deletion of *Klf4* in T cells by *Vav-icre* leaves Th2 cell polarization intact, suggesting that susceptibility to *S. mansoni* infection occurred through a mechanism other than a 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 (Figure S5D–G). Moreover, switching of germinal center B cells from surface immunoglobulin IgM to IgG1 after SEA-immunization was reduced in *Klf4*$^{fl/fl}$ *Itgax*-cre mice relative to *cre*-negative control mice (Figure S7H, I).

To test if *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) (Mashayekhi 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 (Figure S5J,K). 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) seven days after infection (Figure S5L,M). 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, since 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). Since 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 (Figure 6A, B). 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 of *Klf4* by *Vav1-icre* altered the expression of relatively few genes, but among these, IRF4 was severely down-regulated as measured by microarray expression and intracellular staining. Reduction of IRF8 and induction of IRF4 occurs at the SiglecH^{-} pre-cDCs stage, a stage that was severely reduced in *Vav1-icre* *Klf4*$^{fl/fl}$ mice. However, IRF4 expression was not altered in mature cDCs or in Flt3L 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-epxressing cDCs. The subset most clearly dependent on *Klf4* was the previously described migratory subset of CD11b^{-}CD24^{-} (DN) cDCs in sLN. This subset had highest expression levels 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 et al., 2014) which could explaining 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-α^+ CD24$^+$ Mgl-2$^+$. Conceivably, *Klf4*-dependent Sirp-α^+ CD24$^+$ lung cDCs might mediate Th2 cell responses to house dust mite, while *Klf4*-independent Sirp-α^+ CD24$^-$ cDCs mediate Th17 cell immunity to fungal infections. Alternately, *Klf4* may 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, 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 (ILC2) in the lung (Halim et al., 2014; Licona-Limon et al., 2013; Halim et al., 2012) and of eosinophils in the intestine (Chu et al., 2014). It will be important to determine if *Klf4*-dependent cDCs utilize these cells, since 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), *Itgax*-cre (B6.Cg-Tg$^{(Itgax\text{-}cre)1\text{-}1Reiz/J}$) (Caton et al., 2007), *Vav1-icre* (B6.Cg-Tg$^{(Vav1\text{-}cre)A2Kio/J}$) (Harker et al., 2002), *Lyz2-cre* (B6.129P2-Lyz2$^{tm1(cre)Ifo/J}$) (Clausen et al., 1999), *Cx3cr1*^{gfp} (B6.129P-Cx3cr1tm1Litt/J) (Jung et al., 2000). *Irf4*$^{-/-}$ were generated in our facility by backcrossing *Irf4*$^{fl/fl}$ mice (B6.129S1-Irf4tm1Rdf/J) to the cytomegalovirus (CMV) promoter expressing CMV-cre mice (B6.C-Tg(CMV-cre)1Cgn/J), both purchased from Jackson Laboratories. *Csf2rb*$^{-/-}$ (B6.129S1-Csf2rb$^{tm1Cgb/J}$) and *Flt3L*$^{-/-}$ (C57BL/6-flt3L^{tm1Imx}) were obtained from Taconic. *Irf8*$^{-/-}$ mice were obtained from the European Mutant Mouse Archive and maintained on the C57BL/6 background. *Notch2*$^{fl/fl}$*Itgax*-cre were generated and maintained as described (Satpathy et al., 2013). *Klf4*$^{fl/fl}$ mice were obtained from MMRC (MMRRC line 29877) and backcrossed to *Itgax*-cre, *Vav1-icre*, *Lyz2-cre*. *Zbtb46*^{gfp} were generated as

previously described (Satpathy et al., 2012a). *Batf3*$^{-/-}$ were generated as previously described (Hildner et al., 2008). *IL4*$^{-/-}$(B6.129P2-Il4$^{tm1Cgn/J}$) (Kopf et al., 1993) mice were obtained from EJ Pearce (Pearce et al., 1996). For BM chimera experiments, the CD45.1+ B6.SJL (B6.SJL-PtprcaPepcb/BoyJ) mice were purchased from Jackson Laboratories. Unless otherwise indicated, experiments used sex and age matched littermates between 6 to12 weeks of age.

Infection models

All animal model studies described were done in accordance with institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis.

For *Citrobacter rodentium* infection Mice were orally inoculated with 2×10^9 colony-forming units of *C. rodentium*, strain DBS100 (American Type Culture Collection) as described (Lee et al., 2012). Survival and weight loss were monitored over 30 days. Mice used for *C. rodentium* experiments weighed less than 22 g.

For *Toxoplasma gondii* infection, the type II Prugniaud strain of T. gondii expressing a transgene encoding firefly luciferase and GFP (PRU-FLuc-GFP) provided by J. (Saeij et al., 2005) was used. The parasites were grown in human foreskin fibroblasts cultures as described (Mashayekhi et al., 2011) For infection, freshly egressed parasites were filtered, counted, and 100 tachyzoites per mouse were injected intraperitoneally. Survival of mice was monitored over 30 days; parasite burden was measured every 2 days as described below.

Snails infected with *S. mansoni* (strain NMRI, NR-21962) were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, NIAID NIH. Mice were each infected by percutaneous exposure to 100 cercariae. *S, mansoni* Egg Antigen (SEA) was made from parasite eggs isolated from the livers of infected mice, as previously described (MacDonald et al., 2001). Mice were each immunized with 50 μg of SEA s.c. into a rear footpad. For Herpes simplex virus 1 (HSV-1) infection mice were infected with the KOS strain subcutaneously (s.c.) with 1.5×10^5 plaque-forming units (p.f.u.) 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 h in the presence of brefeldin A at 1 μg ml −1 and analyzed by FACS for intracellular IFN-γ and TNF-α production as described later.

Similarly, one week after SEA injection, 3×10^5 cells harvested from popliteal lymph nodes were collected and restimulated under different conditions: SEA (20μg/ml), plate-bound anti-CD3, PMA and Ionomycin (50ng/ml and 1μg/ml) or media alone. For detection of IL-4, 2.5μg /ml of anti-IL-4R antibody (M1) was added to the culture. Supernatants were collected after 72h and analyzed using the BD CBA mouse Th1/Th2 Kit™ (BD Biosciences), and data were analyzed with FCAP Array software (Soft Flow, Inc.).

House dust mite (HDM) induced asthma

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

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, Inc.). P values less than 0.05 were considered significant. *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; ****P < 0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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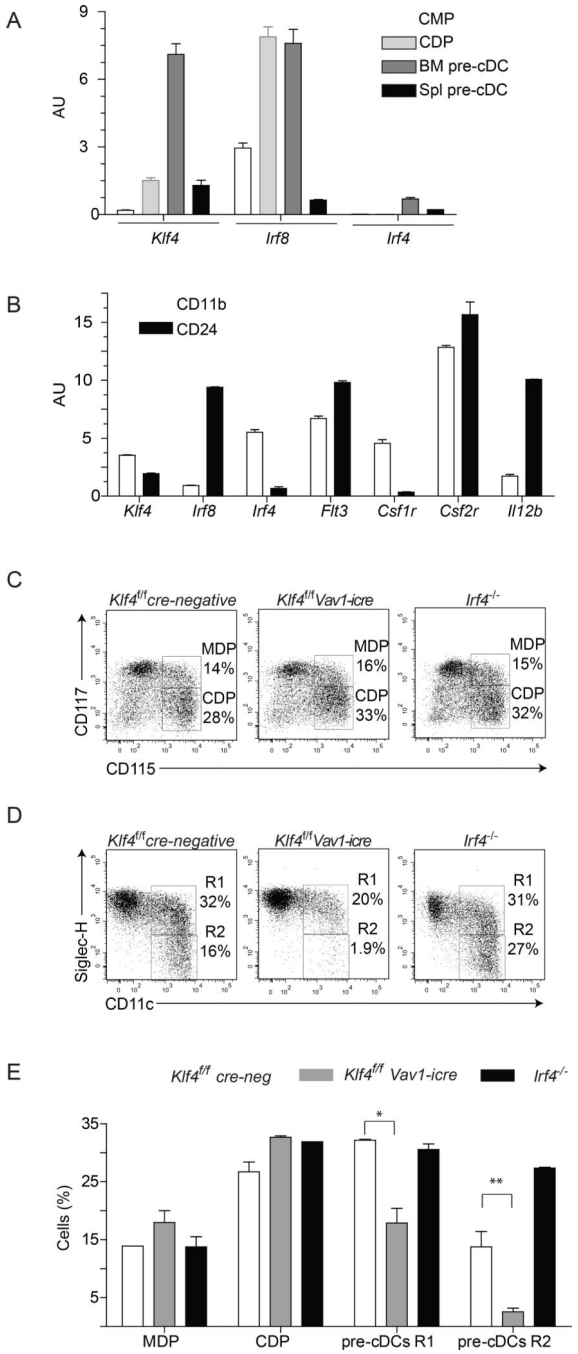

Figure 1. KLF-4 deletion impairs development of bone-marrow pre-cDC progenitors

(A) Relative expression of *Klf4*, *Irf8*, and *Irf4* determined by microarray analysis is shown for the indicated stages of DC progenitors. (B) Relative expression determined by microarray analysis of the indicated genes is shown for splenic CD24^{+} Sirp-α^{-} CD11b^{-} (CD24) and CD24^{-} Sirp-α^{+} CD11b^{+} (CD11b) DCs. (C,D) DC progenitors in BM were analyzed from wild type mice (WT), *Klf4*$^{fl/fl}$ (*cre*-neg) or *Klf4*$^{fl/fl}$ mice crossed onto *Vav1-icre* (Harker et al., 2002), or *Irf4*$^{fl/fl}$ mice crossed into B6.C-Tg(CMV-cre)1Cgn/J (The Jackson Laboratory) backgrounds. CDP, MDP and pre-cDC were gated as described (Liu et

al., 2009) in the Methods and analyzed for the indicated markers. (E) The percent of the progenitors from the indicated genotype analyzed in (C,D) are shown. The experiment was repeated 4 times and 2 mice per genotype were analyzed Error bars, ± s.d., n = 5, Student's t-test. *P < 0.05; ***P < 0.001; NS, P > 0.05. See also Supplemental Figure S1

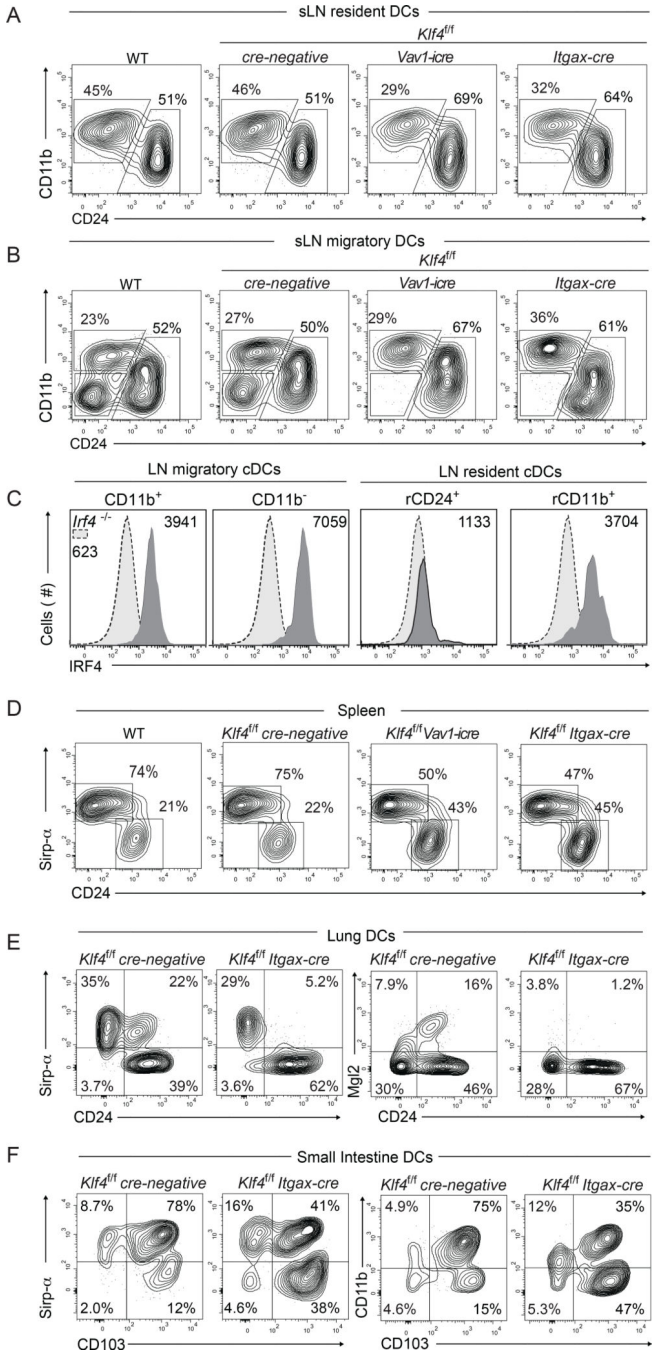

Figure 2. A discrete subset of migratory cDCs in skin-draining lymph nodes requires *Klf4*

(A,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 Figure 2A,B for resident and migratory DCs isolated from $Klf4^{f/f}$ 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} $CD11c^{hi}$) 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 3 independent experiments using at least 2 mice per genotype. See also Supplemental Figure S2

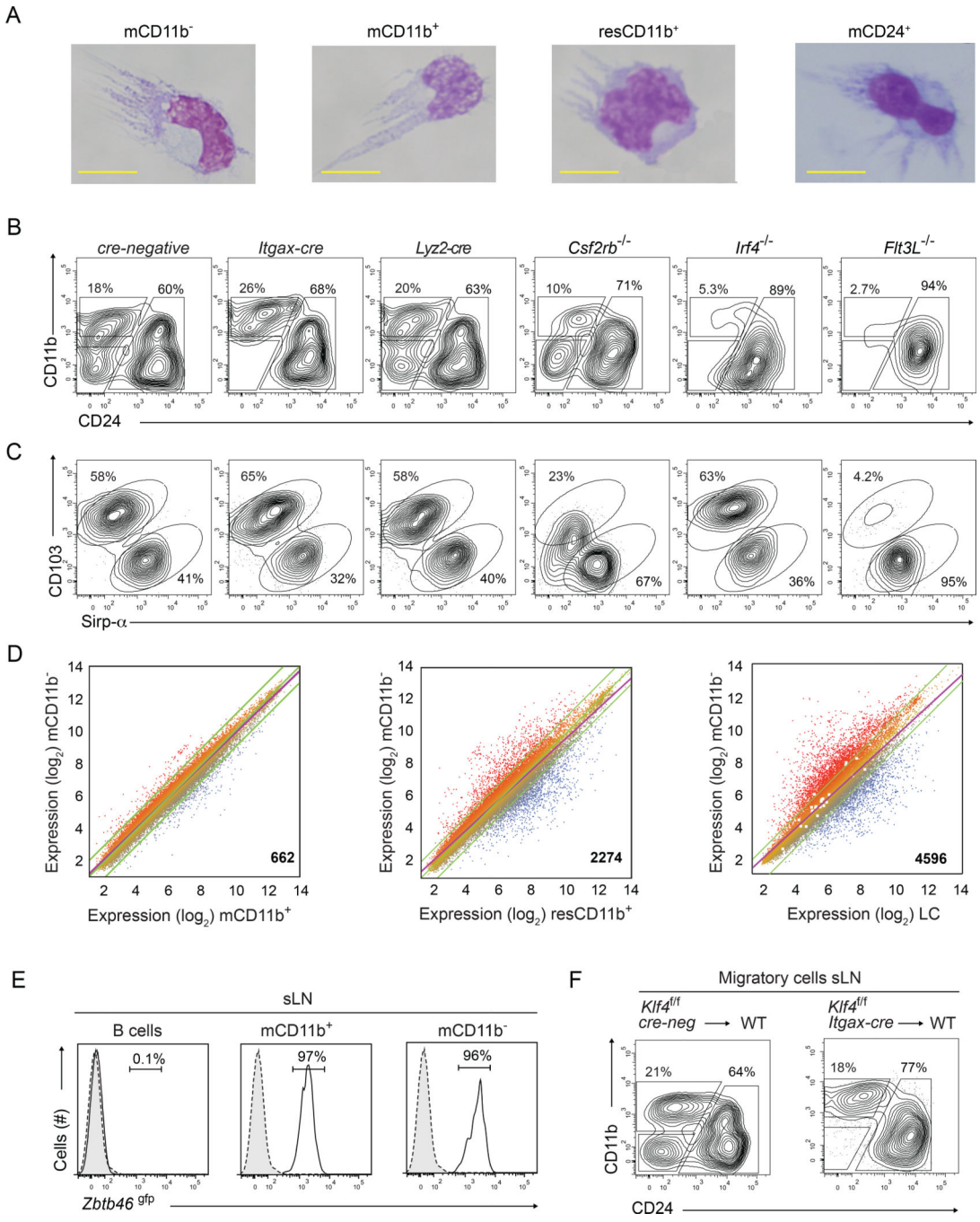

Figure 3. *Klf4*-dependent CD11b⁻ cDCs require IRF4 and FLT3L, express *Zbtb46*^{gfp} and most closely resemble CD11b⁺ cDCs

(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 2 independent experiments, cells from 2 mice were pooled and at least 10 cells were evaluated per subset. (B,C) Representative two-color histograms for CD11b, CD24 (B) and CD103, Sirp-α (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 2 independent experiments and at least 3 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. (E) Shown are single-color histograms for *Zbtb46*^{gfp} expression for B cells, mCD11b^{+} or mCD11b^{-} cDCs from sLN of *Zbtb46*$^{+/gfp}$ mice (Satpathy et al., 2012a). Numbers are the percent of live cells within the indicated gate. (F) Chimeras 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 4 mice per group were analyzed. See also Supplemental Figure S3

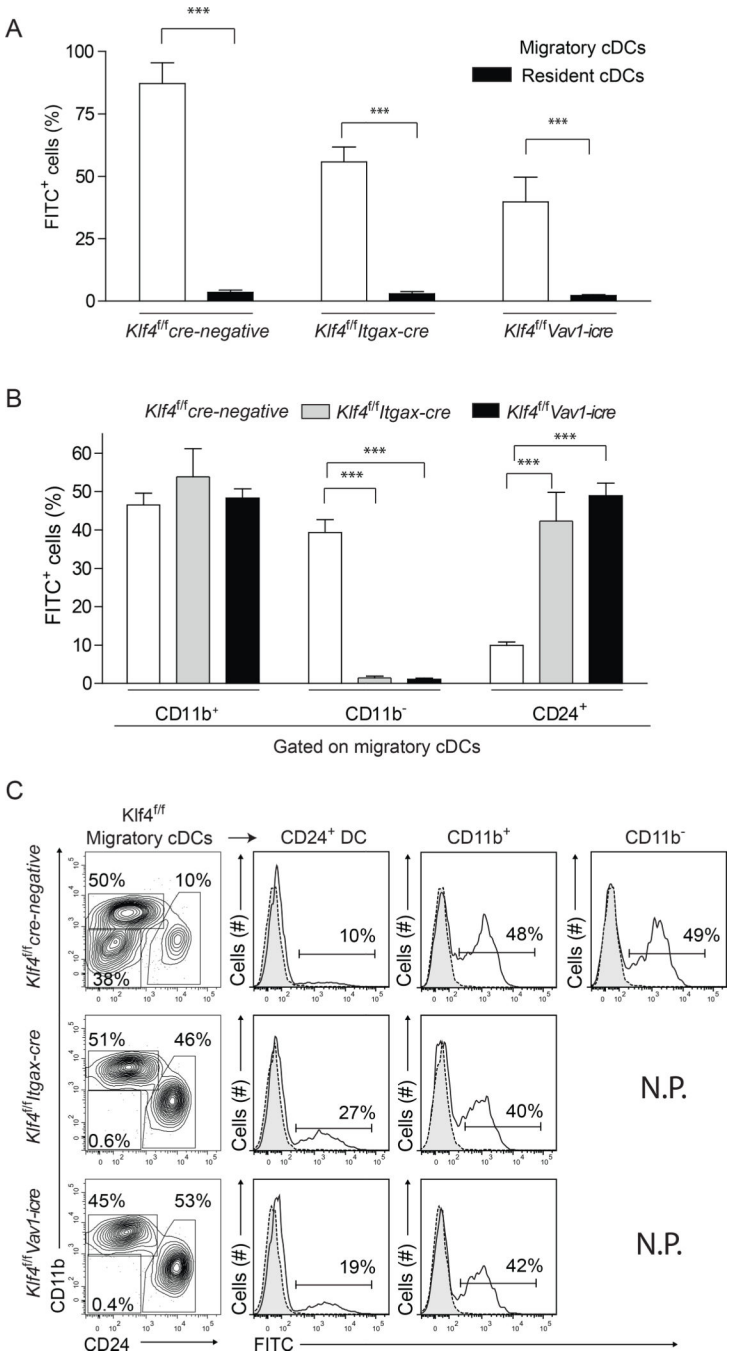

Figure 4. *Klf4*-dependent CD11b^{-} cDCs account for substantial antigen transport from skin to lymph nodes

(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 h after FITC painting, as a percentage of total FITC^{+} sLN cells. Error bars, ± s.d., n = 6, Student's t-test. *P < 0.05; ***P < 0.001; NS, P > 0.05. (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 h after FITC painting. (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 3 times and 2 mice per group were used. See also Supplemental Figure S4

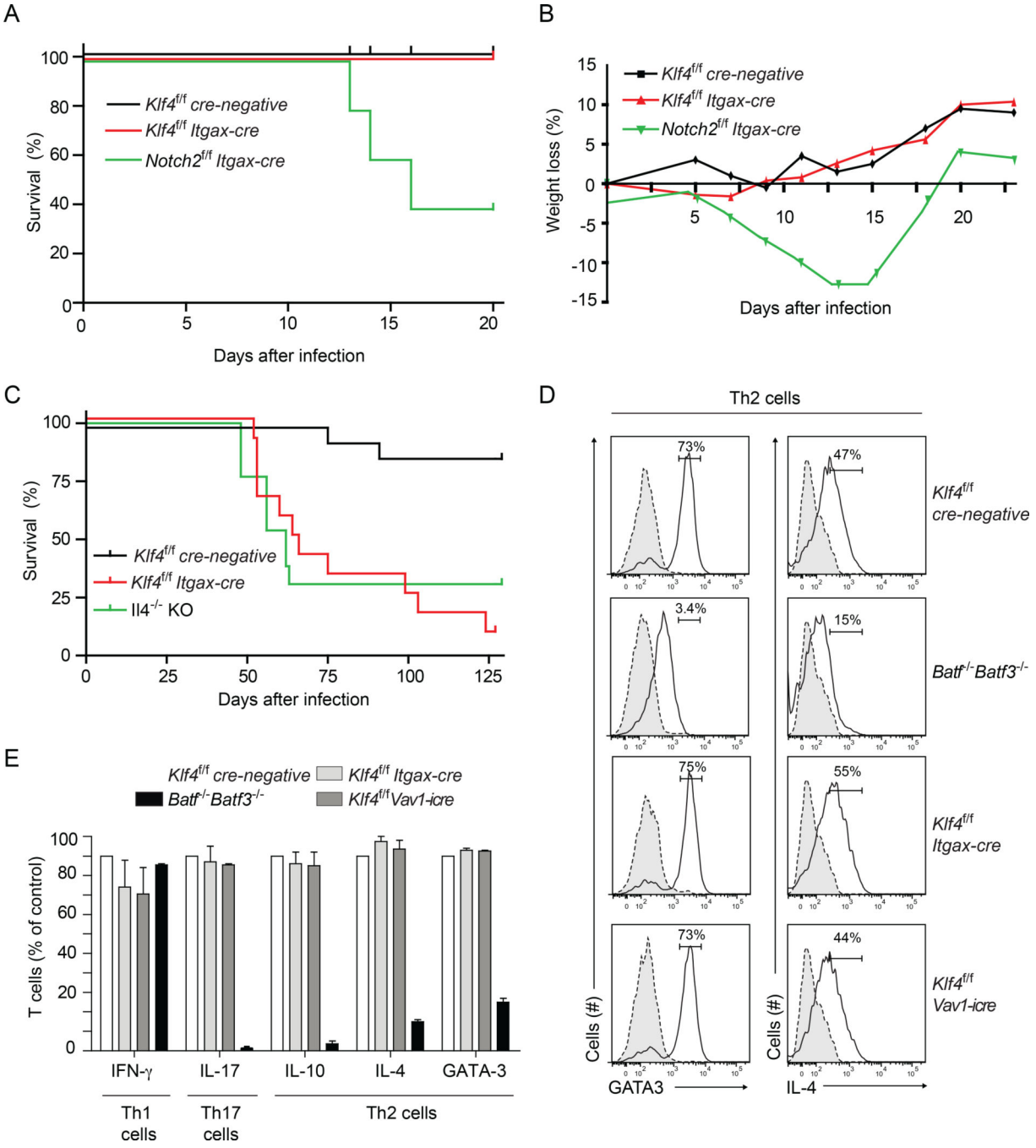

Figure 5. Conditional deletion of *Klf4* in DCs selectively impairs Th2 cell responses to *S. mansoni*

(A, B) Survival (A) and weight loss (B) of mice following oral inoculation of *C. rodentium* (2×10^9 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*$^{c-/-}$) or *Klf4*$^{fl/fl}$ *cre*-negative (*cre*-neg) (n=10) as indicated Experiment was repeated 2 times. (C) Survival of mice following 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 3 times. (D) Shown are histograms

for intracellular GATA3 and IL-4 staining for $CD4^+$ T cells isolated from mice of the indicated gentoypes 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 *Batf*$^{-/-}$*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 4 times. Error bars, ± s.d., n = 8, Student's t-test. *P < 0.05; ***P < 0.001; NS, P > 0.05. See also Supplemental Figure S5

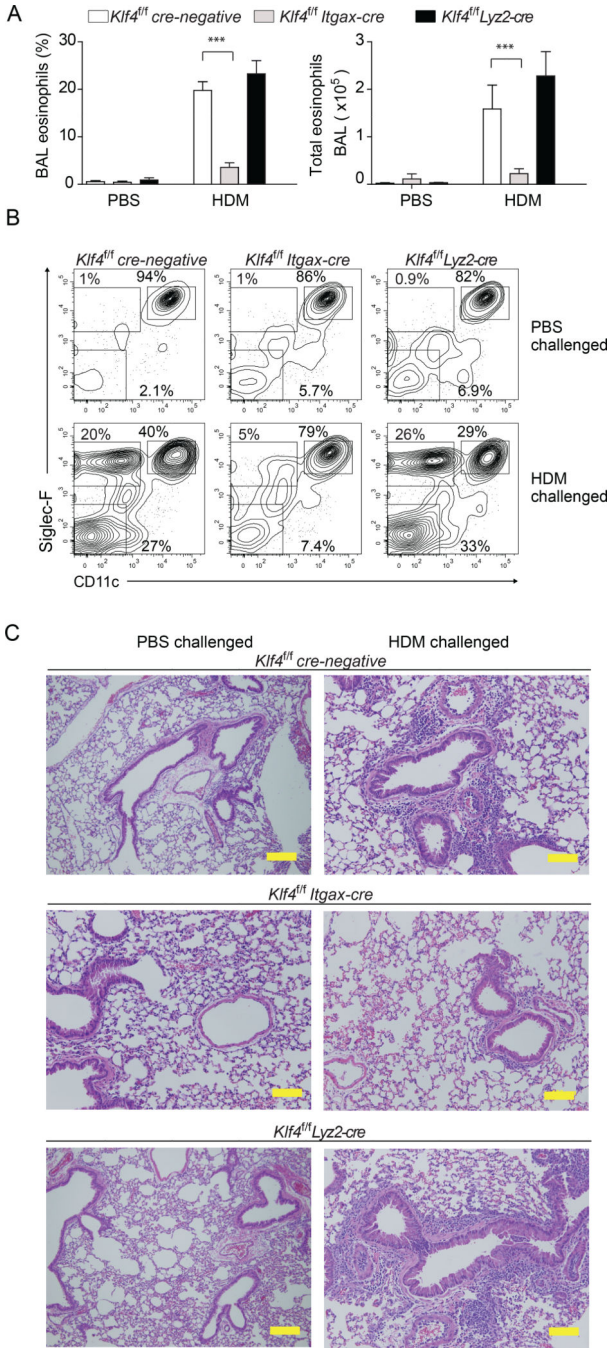

Figure 6. Conditional deletion of *Klf4* in DCs prevents development of HDM-induced allergic inflammation

(A) HDM sensitized mice of the indicated genotypes were challenged intra-nasally 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 $Klf4^{fl/fl}$ crossed to *Itgax*-cre (n=8) or *Lyz2-cre* (n=8) or a *cre*-negative background (n=8) 3 mice per genotype used per experiment. Experiment was repeated 3 times. Error bars, ± s.d., n = 8, Student's t-test. *P < 0.05; ***P < 0.001; NS, P > 0.05. (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 bar indicates 200μm.