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Homeostatic NF-B Signaling in Steady-State Migratory Dendritic Cells Regulates Immune Homeostasis and Tolerance

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Article

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Graphical Abstract
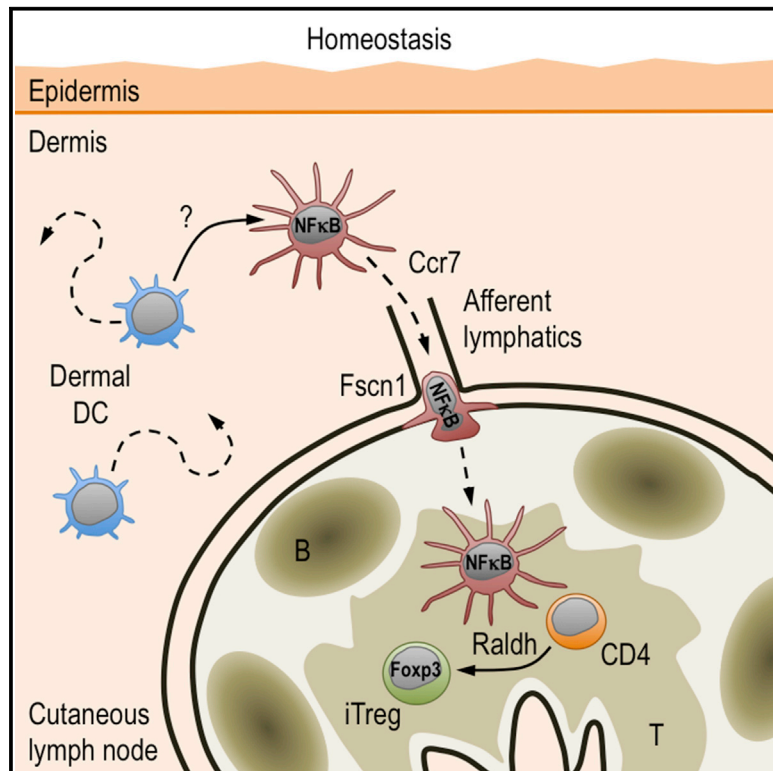
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In Brief

Baratin et al. uncover a new role for NF-κB in tolerance. They show that NF-κB regulates migratory dendritic cells (DCs), which are required to maintain tolerance to tissue antigens. When they block NF-κB activation in DCs, these cells no longer migrate to draining lymph nodes, leading to impaired tolerance to tissue antigens.

Highlights

- A NF-κB-regulated gene network in steady-state migratory NLT-DCs

- Spontaneous autoimmunity from targeted deletion of IKKβ in DCs

- IKKβ is required for steady-state migratory NLT-DC accumulation

- IKKβ in DCs regulates tolerance to endogenous tissue antigens

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Homeostatic NF-κB Signaling in Steady-State Migratory Dendritic Cells Regulates Immune Homeostasis and Tolerance

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SUMMARY

Migratory non-lymphoid tissue dendritic cells (NLT-DCs) transport antigens to lymph nodes (LNs) and are required for protective immune responses in the context of inflammation and to promote tolerance to self-antigens in steady-state. However, the molecular mechanisms that elicit steady-state NLT-DC maturation and migration are unknown. By comparing the transcriptome of NLT-DCs in the skin with their migratory counterparts in draining LNs, we have identified a novel NF-κB-regulated gene network specific to migratory DCs. We show that targeted deletion of IKKβ in DCs, a major activator of NF-κB, prevents NLT-DC accumulation in LNs and compromises regulatory T cell conversion in vivo. This was associated with impaired tolerance and autoimmunity. NF-κB is generally considered the prototypical pro-inflammatory transcription factor, but this study describes a role for NF-κB signaling in DCs for immune homeostasis and tolerance that could have implications in autoimmune diseases and immunity.

INTRODUCTION

Our skin and mucosal tissues, such as lung and intestine, are continually exposed to foreign antigens and microorganisms in our diet and the environment. Maintenance of tissue homeostasis is dependent upon the immune system's ability to remain tolerant of these antigens and commensal microflora while retaining the ability to mount the appropriate immune responses to pathogenic microorganisms upon infection or injury. Defects in this delicate balance can lead to the development of chronic tissue inflammation and autoimmune diseases.

Dendritic cells (DCs) are key regulators of both protective immune responses and tolerance to self-antigens (Steinman et al.,

2003). In steady state, two main groups of DCs can be distinguished according to their location and migratory capacity. Resident lymphoid-tissue (LT)-DCs include plasmacytoid DCs (pDCs) and CD8α^{+} and CD11b^{+} conventional DCs (cDCs). Non-lymphoid tissue (NLT)-DCs, found in the parenchyma of tissues, also include CD8α-type and CD11b^{+} cDC subsets. However, in steady-state a fraction of NLT-DCs migrate through the lymphatics to draining lymph nodes (LNs), where they are classified as migratory NLT-DCs (migDCs). NLT-DCs in mucosal tissues and skin regulate both immune tolerance and the response to invading pathogens by virtue of their ability to migrate to draining LNs and transport antigens. In steady-state, this function promotes the expansion of inducible regulatory T (iTreg) cells, required for tolerance to self-antigens (Azukizawa et al., 2011; Guilliams et al., 2010). However, in the context of inflammation, these cells can transport self or foreign antigens and promote pathogenic or protective T cell responses, respectively (Bedoui et al., 2009; Henri et al., 2010).

The molecular mechanisms that regulate NLT-DC migration in steady state are poorly understood. Accumulation of migDCs in draining LNs is dependent on expression of the chemokine receptor CCR7 and its ligands CCL21 and CCL19, which are highly expressed in lymphoid tissues and present all along the lymphatic endothelium (Ohl et al., 2004). It has been proposed that NLT-DCs undergo a "limited" maturation program that results in upregulated expression of CCR7 and subsequent migration in steady-state (Lutz and Schuler, 2002). The functional maturation of DCs has been widely studied in the context of microbial stimuli or pro-inflammatory cytokines, which promote morphological, phenotypic, and functional changes required for the effective priming and activation of naive T cells. Migratory NLT-DCs undergo similar morphological and phenotypic changes, including increased expression of MHC class II and CD40. But in contrast with DC maturation during inflammation, the steady-state maturation of NLT-DCs is associated with induction of tolerance rather than T cell priming and activation. However, the signaling pathways that regulate steady-state NLT-DC maturation remain an enigma.

Comparing the transcriptional program of resident NLT-DCs to their migratory LN counterparts, we have identified a novel NF-κB-regulated gene network specific to steady-state migratory DCs that includes genes previously shown to regulate migration and Treg cell conversion. Although the NF-κB pathway is thought to play a prominent role in DC activation in the context of microbial stimuli and pro-inflammatory cytokines (Dev et al., 2011), the role of NF-κB in homeostatic DC maturation had not been addressed. We therefore generated mice with a targeted deletion of IκB kinase β (IKKβ) in the DC lineage—a critical regulator of NF-κB activation. We found that IKKβ deletion in DCs results in dis-regulated immune homeostasis and spontaneous autoimmunity. Moreover our studies show that homeostatic NF-κB signaling is required for the steady-state accumulation of migratory DCs in draining LNs and maintenance of immune tolerance to tissue antigens. This represents a new and unexpected role for NF-κB in immune homeostasis and tolerance that could have important implications in host defense and autoimmune diseases.

RESULTS

Steady-State Migratory NLT-DCs Activate a NF-κB-Regulated Gene Network

Migratory NLT-DCs (migDCs) are critical for both T cell priming in the context of inflammation and tolerance to innocuous tissue antigens in steady-state. But the molecular pathways that regulate NLT-DC maturation and migration are unknown. Skin contains two major DC populations, epidermal Langerhans cells (LCs) and dermal DCs (dDCs), the latter of which can be divided into three subsets (CD11b^{lo}CD207$^-$, CD11b^{lo}CD207$^+$, and CD11b^{hi}CD207$^-$) that represent more than 60% of all dDCs (Henri et al., 2010). Migratory counterparts of all four populations are found in the cutaneous LNs (CLNs) draining the skin.

To investigate the genes and signaling pathways that are specifically associated with steady-state migDCs from the skin, we examined the global gene expression profiles of skin-resident CD11b^{hi}CD207$^-$CD64$^-$ dDCs and their migratory counterparts in the CLNs from published microarray data (Tamoutounour et al., 2013). We found several hundred genes that were differentially expressed between migDCs in the CLNs and their resident counterparts in the dermis (Figures 1A and S1). Ingenuity Pathway Analysis (IPA) revealed that this set of genes was significantly enriched for annotations pertaining to "DC maturation" and for targets of the "NF-κB complex" (Figure 1A). Moreover, by using distant regulatory elements (DiRE) analysis, we found that NF-κB was the transcription factor most significantly associated with the migDC-specific gene signature (Figure 1B). The over-representation of NF-κB binding motifs in these genes was also confirmed by Clover (cis-element overrepresentation) analysis with a p value of 10^{-3} (Figure 1C). The vast majority of these genes were validated as bona fide NF-κB target genes in DCs by previous ChIP-seq analysis of RelA binding sites during TLR-induced DC maturation (Figure 1D; Garber et al., 2012). To further analyze the commonalities between the strong NF-κB signature in DCs during TLR-induced maturation and the NF-κB-associated gene signature in steady-state migDCs, we compared the fold changes in expression of all validated

RELA-target genes from TLR-induced DC maturation (Garber et al., 2012) and predicted NF-κB targets induced in steady-state migDCs, compared to their tissue-resident counterparts. This comparison revealed a strong association between TLR-induced DC maturation and high expression of canonical NF-κB target genes, such as *Il1b*, *Il1a*, *Il6*, *Il12a*, *Il12b*, and *Tnfsf1a* (Figure 1E). In contrast, these genes were not highly expressed, or even repressed, in steady-state migDCs. Reciprocally, many of the predicted NF-κB target genes that were induced specifically in migDCs (*Fscn1*, *Il15ra*, *Aldh1a2*, and *Il4i1*) were not regulated during LPS-induced DC maturation, and several genes were in fact strongly repressed in LPS-activated DCs (*Sox4*, *Idh1*). This indicates a NF-κB-regulated gene network in migDCs that is likely to have unique upstream regulators.

These analyses suggest that NF-κB activation might control the steady-state maturation of NLT-DCs through regulation of a novel gene network that is distinct from its function in DC maturation during inflammation or infection.

Targeted Deletion of IKKβ in DCs Leads to Spontaneous Autoimmunity

To examine the role of NF-κB signaling in NLT-DCs, we generated mice with a targeted deletion of IKKβ (*Ikbkb*) in the DC lineage. *Ikbkb*$^{F/F}$ mice were crossed with Tg(*Itgax*-Cre) mice, which express Cre recombinase from the CD11c (*Itgax*) promoter (Caton et al., 2007). Homozygous *Ikbkb*$^{ΔItgax/ΔItgax}$ (*Ikbkb*^{ΔItgax}) mice were born in normal Mendelian ratios and we confirmed efficient deletion of IKKβ expression specifically in DCs by analysis of both mRNA and protein expression (Figure S2). From adulthood, *Ikbkb*^{ΔItgax} mice presented with frequent LN adenopathy and splenomegaly (Figures 2A and 3A). HE staining and immunohistochemical (IHC) analysis of spleens from *Ikbkb*^{ΔItgax} mice showed a dramatic increase in B cell follicle germinal centers (GCs), marked by PNA staining, compared to littermate controls (Figure 2B), reflecting an increase in antigen-induced B cell proliferation. In addition, there was a striking increase in the infiltration of DCs into the T cell zone of B cell follicles in *Ikbkb*^{ΔItgax} mice (Figure 2C), suggesting increased DC-mediated T cell activation. Polyclonal B cell activation was associated with the presence of anti-nuclear antibodies (ANA) in the serum of *Ikbkb*^{ΔItgax} mice (Figure 2D), a common indicator of autoimmune disease. Another frequent manifestation of autoimmune disease is the deposition of immune complexes in glomeruli of the kidney, which subsequently trigger inflammation and glomerular nephritis. IHC analysis also revealed glomerular antibody deposition (IgG) in the kidneys of *Ikbkb*^{ΔItgax} mice, which was absent in littermate controls (Figure 2C). Collectively, these data suggest that inhibition of steady-state NF-κB signaling in DCs results in spontaneous autoimmunity.

IKKβ in DCs Regulates Immune Homeostasis

To further characterize the autoimmune phenotype in *Ikbkb*^{ΔItgax} mice, we performed flow cytometry analysis on LN and spleen cell suspensions. This revealed a significant increase in proportions of neutrophils (PMNs), Ly6C^{hi} "inflammatory" monocytes, and B cells compared to *Ikbkb*$^{F/F}$ control mice (Figure 3B). In contrast, the proportions of CD8$^+$ and CD4$^+$ T cells were significantly reduced in LNs (Figure 3B). Resident LT-DCs play an important role in maintaining immune homeostasis (Birnberg
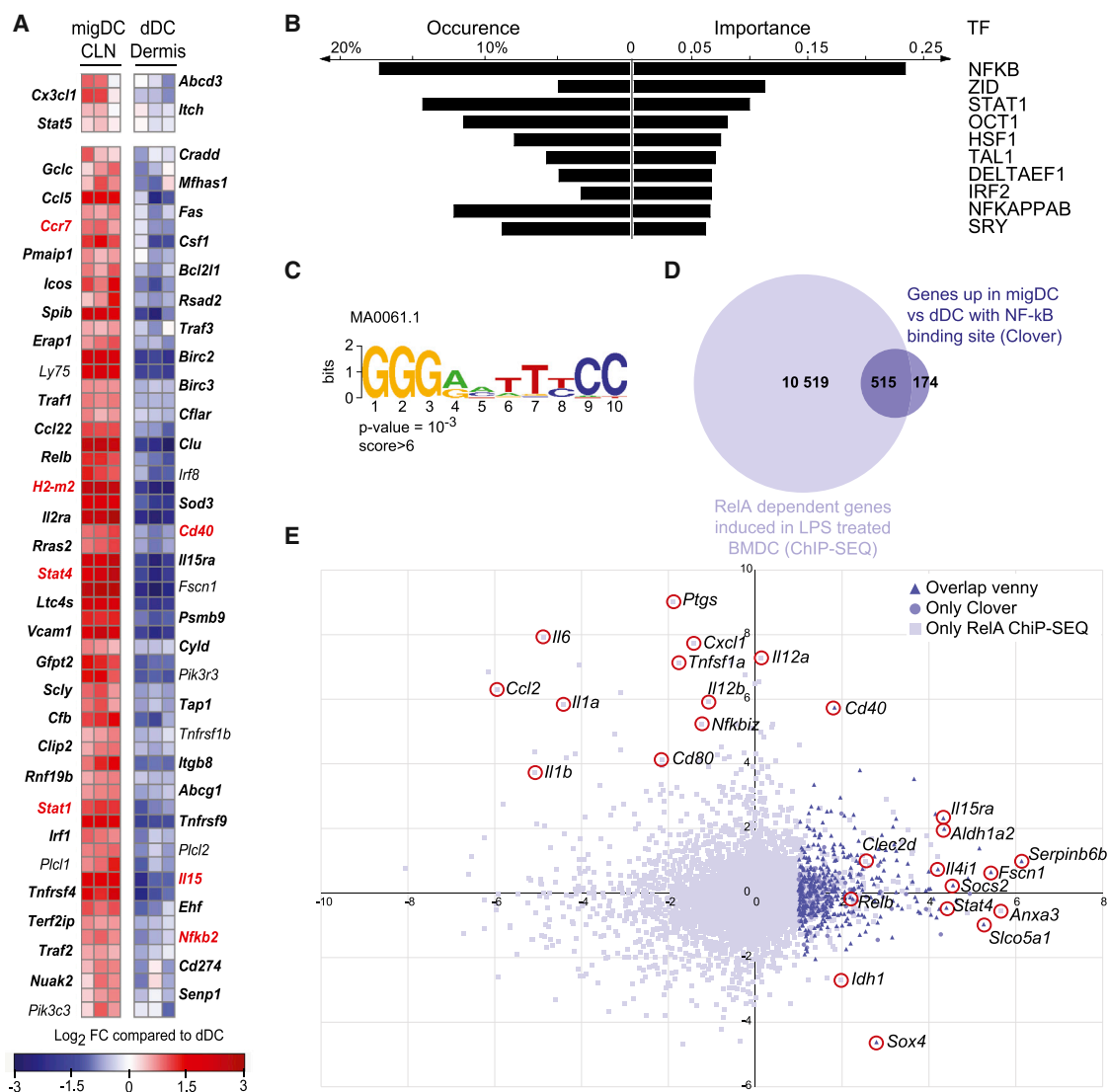
Figure 1. A NF-κB-Regulated Gene Network in Steady-State Migratory DCs

(A) Heat map showing the expression pattern of upregulated genes in migDCs from CLNs compared to dermal DCs (dDCs). Relative expression values are represented for the genes of the "DC maturation" pathway (black) and the upstream regulator "NF-κB complex" (black bold), based on IPA. Genes that are common to both analyses are in bold red.

(B) Diagram showing the top 10 transcription factor motifs enriched in migDC-upregulated genes. The occurrence represents percent of genes with a conserved binding site for the TRANSFAC transcription factors. The importance is the product between the occurrence and the weight assigned to each transcription factor.

(C) The consensus NF-κB binding motif (MA0061.1) was used for Clover analysis that revealed a significant enrichment in genes upregulated in migDCs; p value and score are indicated.

(D) Venn diagram showing the overlap between the migDC-upregulated genes with RelA target genes identified in LPS-stimulated DCs by ChIP-seq analyses (Garber et al., 2012).

(E) Scatter plot comparing fold changes (log2) in the expression of bona fide NF-κB target genes (based on RELA binding) and putative NF-κB target genes in steady-state migDCs, as shown in (D). LPS-stimulated DCs (y axis) and steady-state migDCs (x axis). Examples of canonical pro-inflammatory NF-κB target genes upregulated in LPS-stimulated DCs and newly identified NF-κB target genes specifically regulated in migDCs are highlighted by red circles.

et al., 2008), but we found no significant differences in numbers of LT-DCs in LNs and spleen from $Ikbkb^{\Delta Itgax}$ mice (Figure 3C). The proportions of $CD11b^+$ and $CD8^+$ LT-DC subsets was also not altered in $Ikbkb^{\Delta Itgax}$ mice, but there was a decrease in the frequency of $CD4^+$ DCs among the $CD11b^+$ subset and a reciprocal increase in $CD4^- CD11b^+$ DCs (Figure S3). These data showed that IKKβ signaling was not required LT-DC development or survival.

CD11c is expressed throughout the DC lineage, including monocyte-derived DCs (moDCs), but also by some macrophage populations (Miller et al., 2012). To assess the role of IKKβ more specifically in cDCs, we compared $Ikbkb^{\Delta Itgax}$ mice with $Ikbkb^{\Delta Lyz2}$ mice, where IKKβ is deleted in lysozyme M (Lyz2)-expressing myeloid cells, including macrophages, PMNs, and moDCs, but is not highly expressed in cDCs (Figure S2). Although $Ikbkb^{\Delta Lyz2}$ mice also showed some degree of splenomegaly,
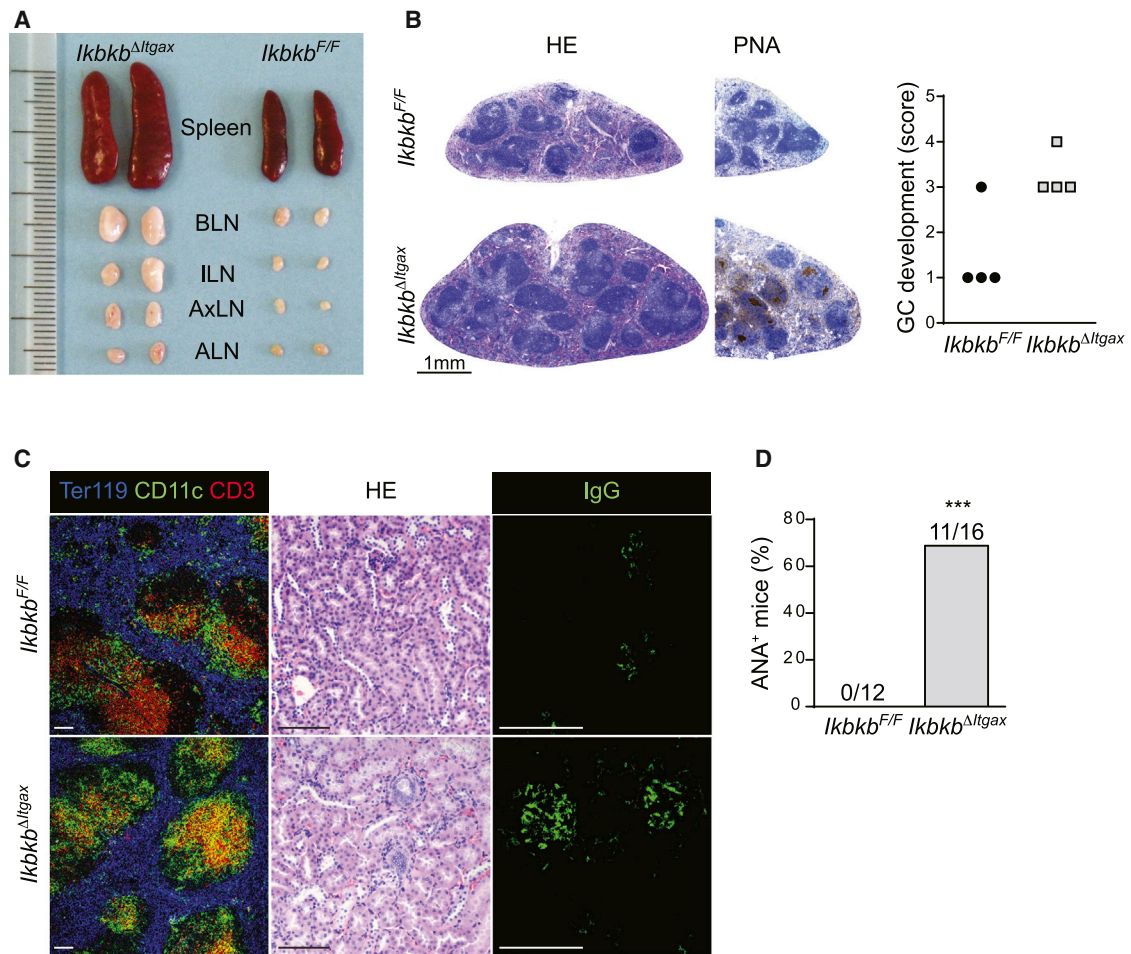
Figure 2. IKKβ Deficiency in DCs Leads to Spontaneous Autoimmunity
(A) Spleen and CLNs of $Ikbkb^{F/F}$ and $Ikbkb^{\Delta Itgax}$ mice. Abbreviations are as follows: BLN, brachial LN; ILN, inguinal LN; AxLN, axillary LN; ALN, auricular LN.
(B) Spleen sections were analyzed by HE and PNA histochemistry, GC development was scored in n = 4 mice.
(C) Spleen sections were analyzed for Ter119, CD11c, and CD3 staining by IHC (left), kidney sections were analyzed by HE (middle), and IgG deposition by IHC (right); scale bars represent 100 μm.
(D) Anti-nuclear autoantibodies (ANA) were measured in serum from $Ikbkb^{F/F}$ and $Ikbkb^{\Delta Itgax}$ mice. Statistical analysis was performed with a Fischer exact test.

compared to littermate controls, they did not present with LN adenopathy or increased LN cellularity (Figure 3A). Furthermore, flow cytometry analysis did not reveal any significant changes in the proportions of B cells or T cells in LN and spleen from $Ikbkb^{\Delta Lyz2}$ mice in contrast to $Ikbkb^{\Delta Itgax}$ mice (Figure 3B). There was a slight increase in the proportions of PMNs and Ly6C$^+$ monocytes in LN and spleen from $Ikbkb^{\Delta Lyz2}$ mice (Figure 3B).

These data indicate that IKKβ signaling in cDCs is not required for LT-DC development or survival in steady-state but has an important role in maintaining immune homeostasis.

IKKβ in DCs Regulates Treg Cell Accumulation in CLNs

DCs contribute to the maintenance of immune tolerance by regulating the accumulation of Treg cells. There are two major populations of Treg cells in mice: natural Treg (nTreg) cells produced in the thymus and iTreg cells generated in peripheral lymphoid organs. Thymus-dependent nTreg cells provide central tolerance to self-antigens expressed by thymic medullary

epithelial cells (mTECs), and there might also be a role for thymic DCs in nTreg cell selection through the cross-presentation of mTEC-associated antigens. In the periphery, naive T cells can be converted to iTreg cells by DC-mediated antigen presentation (Steinman et al., 2003). To assess the role of IKKβ in DCs for Treg cell accumulation, we measured the number of CD25$^+$FOXP3$^+$CD4$^+$ Treg cells in thymus, spleen, and CLNs of $Ikbkb^{\Delta Itgax}$ and littermate $Ikbkb^{F/F}$ control mice. There was a significant reduction in the proportion of Treg cells in CLNs from $Ikbkb^{\Delta Itgax}$ mice compared to littermate controls (Figure 3D), but the proportions of Treg cells in the spleen and thymus were not affected (Figure 3D). However, the frequency of L-selectin (CD62L)-expressing Treg cells was significantly reduced in spleen, whereas NRP1$^+$ (neuropillin) thymus-dependent nTreg cells were unaffected (Figure S3). This suggests that IKKβ in DCs contributes to iTreg cell conversion and activation in the periphery and particularly in CLNs that acquire antigens from the skin.
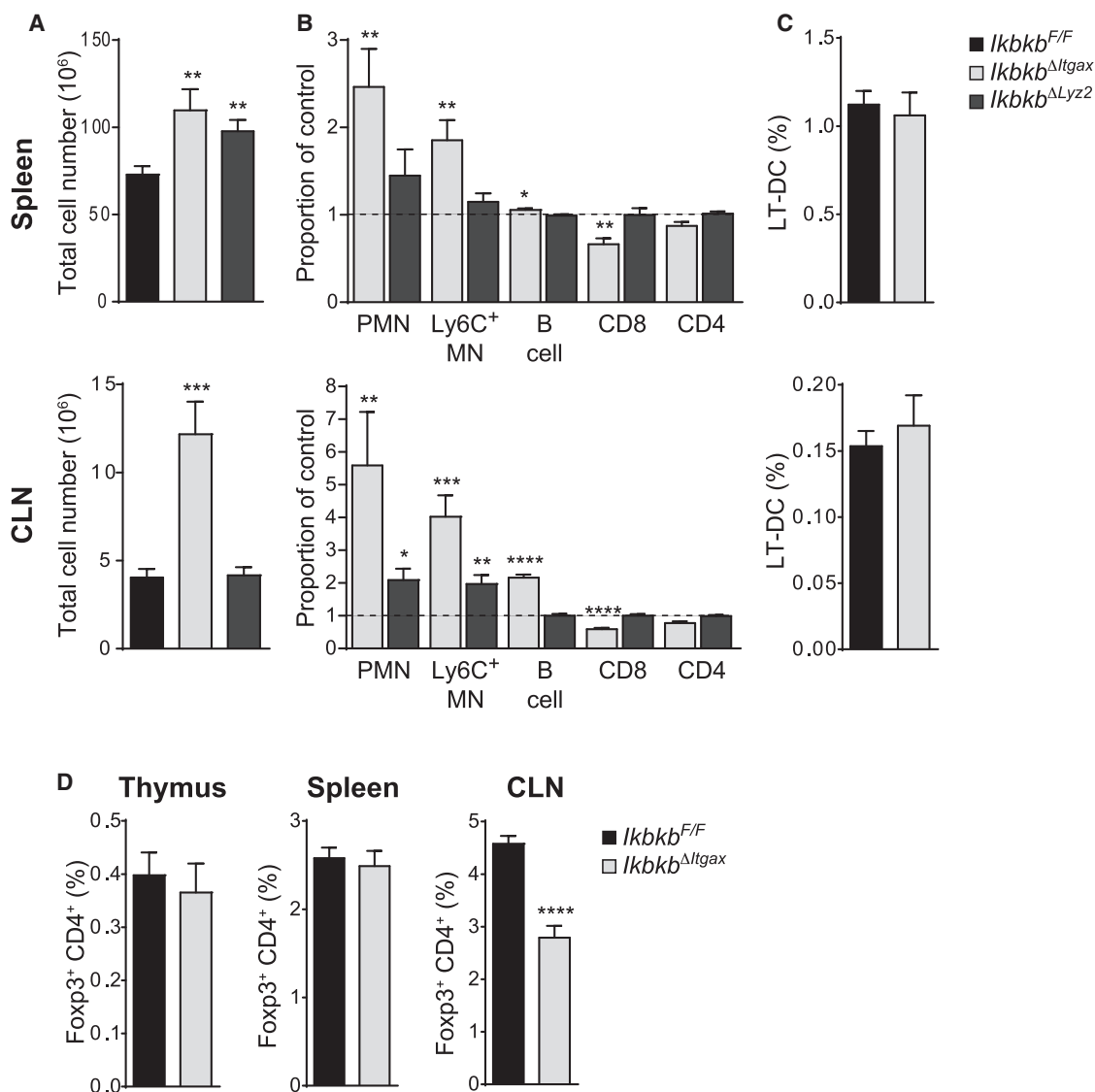
Figure 3. Targeted Deletion of IKKβ in DCs Disrupts Immune Homeostasis

Flow cytometry analysis of spleen and CLNs from *Ikbkb*[F/F], *Ikbkb*[ΔItgax], and *Ikbkb*[ΔLyz2] mice.

(A–C) Total cell number (A), proportions of immune cells represented as the ratio between *Ikbkb*[F/F] and *Ikbkb*[ΔItgax] mice (B), and percentage of resident LT-DCs (C).

(D) Proportions of Foxp3^{+}CD4^{+} cells in thymus, spleen, and CLN.

Data are shown as mean ± SEM; graphs represent pooled data from at least three independent experiments. Statistical analysis was performed with Mann-Whitney test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

IKKβ Is Required for Steady-State NLT-DC Accumulation in the CLNs

The generation of iTreg cells in CLNs is dependent on migratory NLT-DCs (migDCs) from the dermis that transport antigens to the CLNs in steady-state (Azukizawa et al., 2011; Guilliams et al., 2010). These migDCs can be distinguished from resident LT-DCs on the basis of their MHCII^{hi}CD11c^{int}CCR7^{+} phenotype. We measured the accumulation of migDCs in the CLNs from *Ikbkb*[ΔItgax] mice and littermate controls by flow cytometry. *Ikbkb*[ΔItgax] mice showed a dramatic reduction in accumulation of migDCs, whereas the proportion of resident LT-DCs was unaffected (Figures 4A and 3C). The specificity of the analysis for migDCs was confirmed with CLNs from CCR7-deficient mice

(*Ccr7*^{−/−}), which lack migDCs in draining LNs (Figure 4A; Ohl et al., 2004). We also performed IHC analysis on LN sections from *Ikbkb*[ΔItgax] mice; this analysis confirmed the lack of accumulation of CD207^{+} skin-derived DCs in the T cell zone of the LN (Figure 4B). These data clearly showed that IKKβ expression in DCs is required for the steady-state accumulation of migDCs in the CLNs.

To evaluate the role of IKKβ in NLT-DC development and homeostasis, we analyzed the distribution of NLT-DCs in the epidermis (LCs) and dermis (dDCs). IHC analysis showed no change in LC number or distribution in epidermis from *Ikbkb*[ΔItgax] mice (Figure 4B). FACS analysis of dermal cell suspensions also showed no significant differences in the frequency of dDCs in
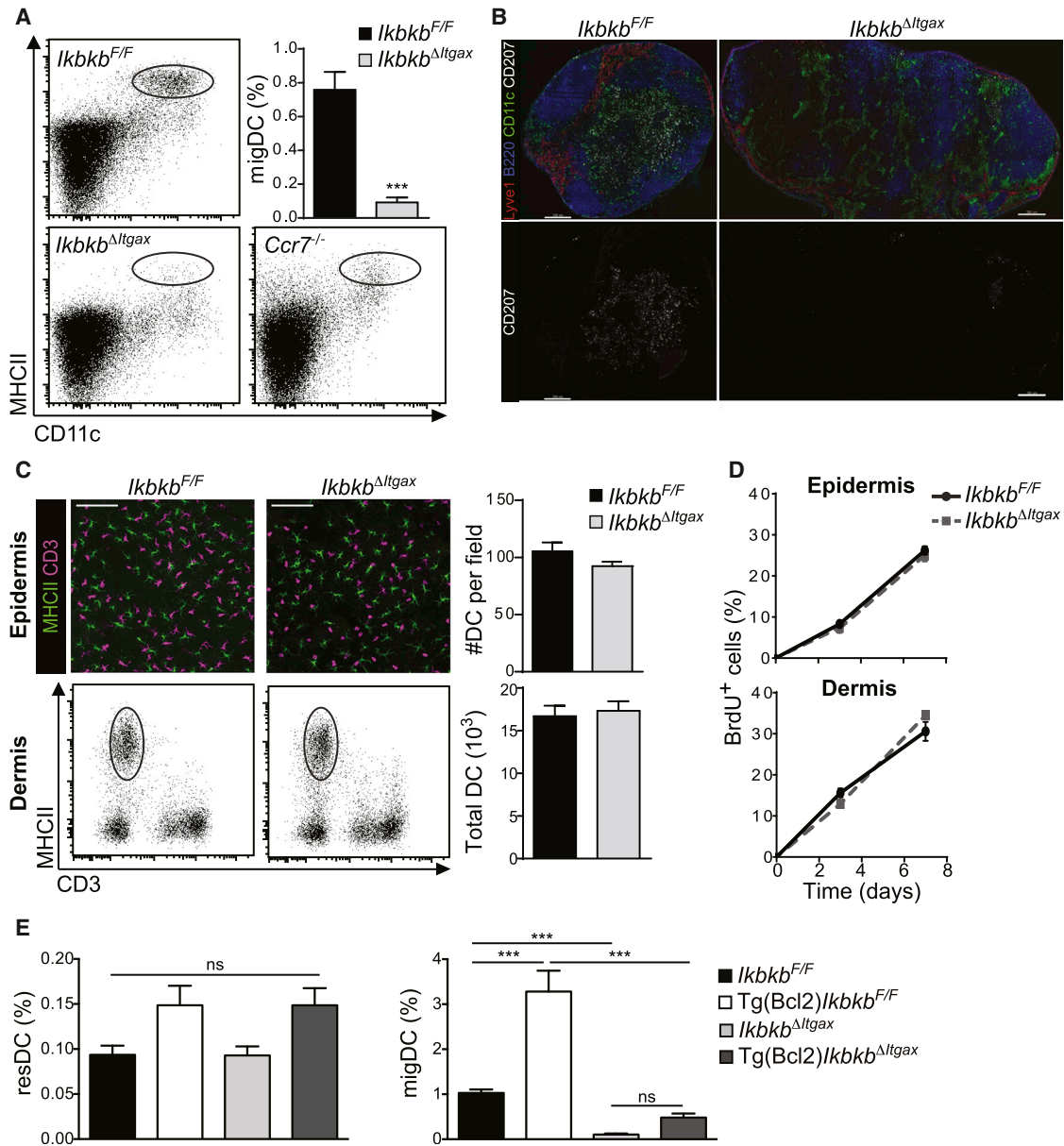
Figure 4. IKKβ Is Required for Accumulation of migDCs in Steady-State

(A) Flow cytometry analysis of CLNs from *Ikbkb*[F/F], *Ikbkb*[ΔItgax], and *Ccr7*^{−/−} mice. Resident LT-DCs and migDCs were distinguished by MHCII and CD11c staining, representative FACS plots are shown, and the graph represents pooled data from three independent experiments. Statistical analysis was performed with Mann-Whitney test; ***p < 0.001.

(B) Sections of intact CLNs were stained for CD207, LYVE1, B220, and CD11c and analyzed by confocal microscopy. Representative images are shown; scale bars indicate 200 μM.

(C) Ear epidermal sheets were stained for MHCII and CD3 and epidermal DCs counted from at least three fields (n = 6). Lower panels: Dermal cell suspensions were prepared and stained for MHCII, CD45.2, and CD3. Plots represent CD45.2 gated cells, the total number of DCs/ear from three independent experiments are represented in the graph.

(D) BrdU was administered i.p. daily for 7 consecutive days. On day 3 and 7, mice were sacrificed, dermal and epidermal cell suspensions from the ears were prepared, cells were stained as described in (C), and BrdU[+] incorporation by DCs was determined. Graphs represent two pooled independent experiments of n = 3.

(E) Tg(*Itgax-Bcl2*) mice were crossed with *Ikbkb*[ΔItgax] mice to generate mice overexpressing BCL2 in DCs (Tg(*Bcl2*)) and proportions of resDCs and migDCs in CLNs were analyzed as described in (A); data are shown as mean ± SEM of n = 5 and are representative of at least 2 independent experiments. Statistical analysis was performed with Mann-Whitney test; ***p < 0.001.

Ikbkb[ΔItgax] mice (Figure 4B). Moreover, the different dDC subsets were present in similar proportions as littermate control mice (Figure S4). These data showed that IKKβ was not required for

the development or accumulation of NLT-DCs in the skin. Previous studies have estimated the half-life of NLT-DCs in the dermis to be between 3 and 5 days, being constantly replenished from

circulating common dendritic cell progenitors (CDPs) that develop in the bone marrow (Poulin et al., 2007). On the other hand, epidermal LCs are maintained by self-renewal in the tissue with little contribution from blood-borne progenitors (Poulin et al., 2007). To assess the role of IKKβ in NLT-DC turnover, we used in vivo BrdU labeling. Epidermal LCs and dermal DCs from *Ikbkb*$^{\Delta Itgax}$ mice showed no difference in BrdU incorporation compared with littermate controls during a 7 day labeling period (Figure 4C), indicating that IKKβ deletion does not affect the proliferation or turnover of skin DCs in steady-state.

The experiments described above clearly showed that IKKβ deletion in DCs did not affect the homeostasis of NLT-DCs in the skin, yet there was a dramatic absence of migDCs in CLNs. This presented the possibility that IKKβ might regulate migDC survival in the draining LN after maturation and migration from the skin. To address this question, we crossed *Ikbkb*$^{\Delta Itgax}$ mice to transgenic mice that expressed the anti-apoptotic protein Bcl2 specifically in the DC lineage (Tg(*Itgax-Bcl2*)) (Gautier et al., 2009). The proportions of DCs in the dermis were not significantly altered in *Ikbkb*$^{F/F}$ or *Ikbkb*$^{\Delta Itgax}$ mice expressing the Bcl2 transgene (Figure 4E). However, although there was a significant increase in the accumulation of migDCs in CLNs from *Ikbkb*$^{F/F}$ mice expressing the Bcl2 transgene, this did not rescue the defect in migDC accumulation in *Ikbkb*$^{\Delta Itgax}$ mice (Figure 4E). These studies demonstrate that the accumulation of migDCs in steady state is limited by apoptotic cell death; however, rescuing cell survival is not sufficient to restore migDC accumulation in the absence of IKKβ signaling, suggesting that IKKβ activation in steady-state migDCs drives the maturation and migration of NLT-DCs from the skin and not their persistence in the CLNs.

IKKβ Regulates Spontaneous Maturation of NLT-DCs in the Skin

Steady-state NLT-DC maturation is associated with the upregulation of major histocompatibility complex II (MHCII) and co-stimulatory molecules such as CD40 on migratory DCs. Culture of intact skin explants ex vivo results in spontaneous DC maturation and the acquisition of migratory properties that can be measured by DC emigration into the culture medium. The number of DCs that emigrated from skin explants of *Ikbkb*$^{\Delta Itgax}$ mice was significantly reduced compared to littermate controls (Figure 5A). Emigrated DCs from *Ikbkb*$^{\Delta Itgax}$ mice showed no difference in Annexin V staining, suggesting that the reduced emigration was not due to effects on cell survival (Figure 5B). Culture of intact skin explants also results in accumulation of DCs along dermal lymphatic vessels in a CCR7-dependent manner, forming so-called dermal cord structures (Ohl et al., 2004; Weinlich et al., 1998); however, explants from *Ikbkb*$^{\Delta Itgax}$ mice showed no defect in dermal cord formation compared to skin from littermate control mice (Figure 5C). In contrast, those structures were absent from the dermis of skin explants from *Ccr7*$^{-/-}$ mice (Figure 5D), as previously described by Ohl et al. (2004), suggesting that IKKβ is not required for CCR7-mediated sensing of chemokines expressed by lymphatic endothelium. This is supported by the increased accumulation of LT-DCs in the T cell zones of splenic B cell follicles in *Ikbkb*$^{\Delta Itgax}$ mice (Figure 2C), which is also CCR7 dependent (Ohl et al., 2004).

To further examine the role of IKKβ in spontaneous maturation of skin DCs, we isolated epidermal and dermal DCs from *Ikbkb*$^{\Delta Itgax}$ mice and littermate controls and measured their phenotype ex vivo. The isolation and culture of skin DCs at 37°C, without exogenous stimulation, was sufficient to induce the upregulation of typical markers of maturation including surface expression of MHCII, CD40, and CCR7, as well as intracellular expression of the actin bundling protein FASCIN (*Fscn1*)—which has been shown to be required for migration of mature DCs (Yamakita et al., 2011) and to be a putative NF-κB-regulated gene in steady-state migDCs (Figure 1A). IKKβ-deficient DCs showed no difference in upregulation of MHCII or survival upon culture ex vivo (Figure 5E) but showed reduced expression of CD40, CCR7, and FASCIN compared to wild-type cells (Figure 5E), demonstrating that IKKβ contributes to the intrinsic spontaneous maturation of isolated skin DCs. Furthermore, addition of specific small molecule inhibitor of IKKβ (Zhang et al., 2014) blocked the spontaneous maturation of isolated dermal DCs in this context (Figure S5).

Finally, to confirm the IKKβ-dependent expression of putative NF-κB target genes upregulated in steady-state migDCs, we performed quantitative PCR analysis of mRNA expression in isolated dermal DCs before and after spontaneous maturation. We confirmed the IKKβ-dependent induction of several predicted NF-κB target genes during spontaneous maturation of dermal DCs, including *Fscn1* and *Aldh1a2*, but not the canonical pro-inflammatory target gene *Tnfsf1a*, which was in fact downregulated during spontaneous maturation (Figure 5F).

These studies demonstrate that IKKβ activation regulates the spontaneous maturation of skin DCs ex vivo and the upregulation of genes specifically associated with steady-state migDCs, including genes required for migration and Treg cell conversion.

Steady-State NLT-DC Migration Does Not Depend on Canonical NF-κB Stimuli

The signaling pathways that regulate steady-state NLT-DC maturation are unknown. The canonical NF-κB pathway is most widely studied in the context of TNF-α, IL-1β, and TLR signaling (Dev et al., 2011), so to determine the role of these pathways in homeostatic NLT-DC migration, we analyzed CLNs from *Myd88*$^{-/-}$ *Trif*$^{-/-}$ mice, deficient in all TLR-signaling pathways and signaling through the IL-1 receptor, and from mice deficient in both TNF-α receptors (*Tnfsr1*$^{-/-}$*Tnfsfr2*$^{-/-}$). We found no defects in the accumulation of migDCs in either of these strains (Figure S6). This is in keeping with our analysis of the transcriptional signature of steady-state migDCs, which indicates that the NF-κB-regulated gene network in migDCs is distinct from the network of NF-κB-regulated genes during TLR-induced DC maturation (Figure 1E). In addition, Ingenuity Pathway Analysis (IPA) with the genes differentially expressed by migDCs compared to their tissue-resident counterparts showed a negative enrichment for known upstream regulators of pro-inflammatory NF-κB signaling (Figure S1), indicating that the "canonical" NF-κB pathway is in fact downregulated in these cells.

The alternative NF-κB pathway, leading to activation of RELB p52 heterodimers, has also been suggested to play an important role in DC maturation (Azukizawa et al., 2011; Shih et al., 2012). The alternative pathway is normally dependent on IKKα activation and not IKKβ, but a recent study showed that IKKβ can

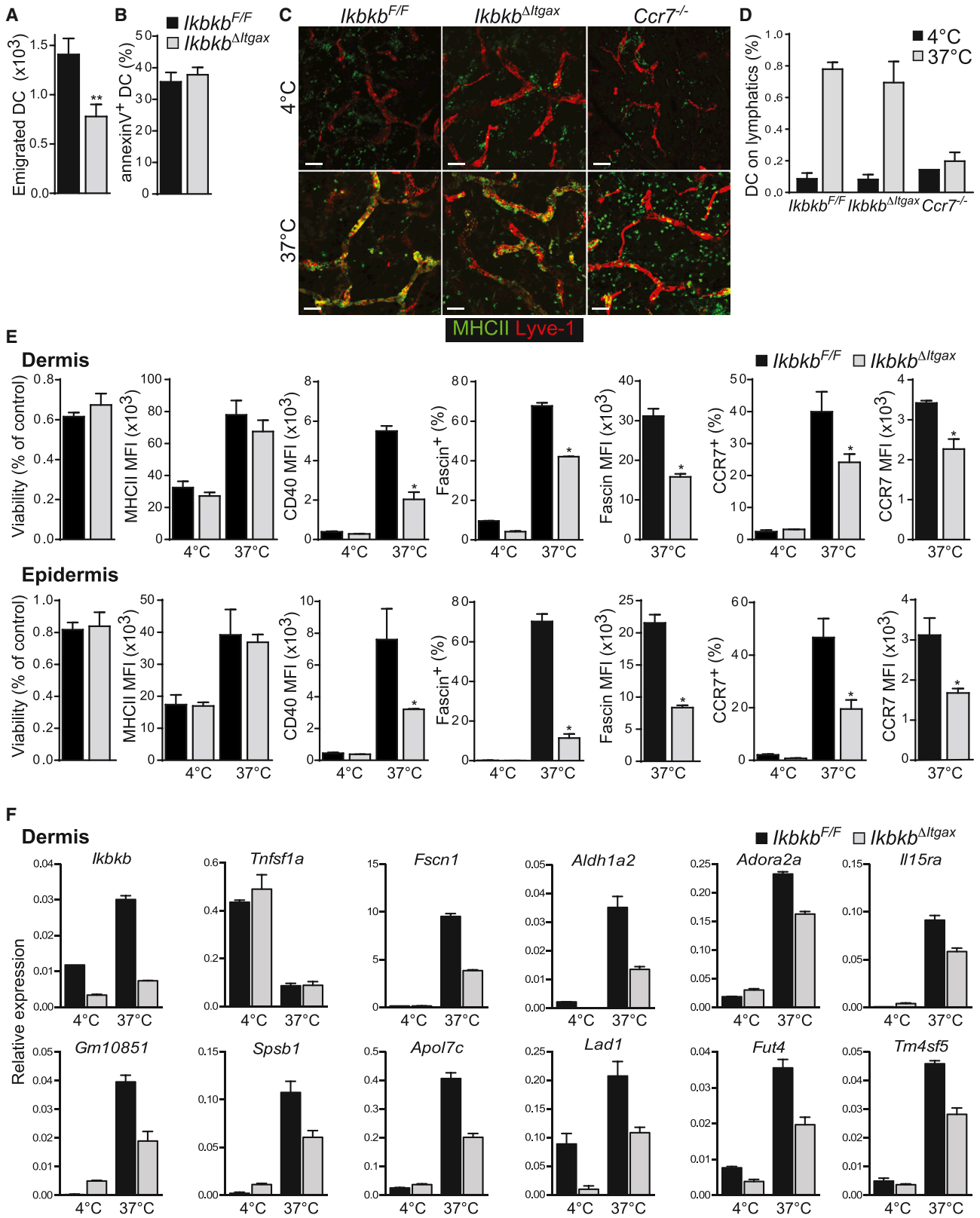**Figure 5. IKKβ Regulates Spontaneous Skin NLT-DC Maturation**
(A and B) Ear skin flaps from Ikbkb[F/F] and Ikbkb[ΔItgax] mice were floated on tissue culture medium for 72 hr and DC emigration was analyzed by flow cytometry; (A) represents total emigrated DCs per ear and (B) the percentage of Annexin V^{+} cells among emigrated DCs.

(legend continued on next page)

regulate activation of RELB in DCs after lymphotoxin β (LTβ) stimulation (Shih et al., 2012). We found no defect in accumulation of migDCs in the CLN of IKKα^AA knock-in mice (Mancino et al., 2013), which express an inactive mutant of IKKα (Figure S6). Furthermore, blockade of LTβR signaling through the administration of LTβR-Ig fusion protein had no impact on migDC accumulation in CLNs (Figure S6). These data indicate that the alternative NF-κB pathway and LTβR signaling through either IKKα or IKKβ is not required for the steady-state migration of NLT-DCs in the skin.

IKKβ Regulates Dermal NLT-DC Migration and Antigen Trafficking to CLNs

Migratory dermal NLT-DCs transport antigens from the skin to the CLNs for subsequent presentation to naive T cells. To confirm the role of IKKβ in NLT-DC migration to the CLNs, we applied FITC epicutaneously to the ear skin of Ikbkb^F/F and Ikbkb^ΔItgax mice and measured the accumulation of FITC-labeled migDCs in the CLNs. Previous studies have established that all FITC^+ DCs in the CLNs via this procedure represent migratory dDCs or LCs (Ohl et al., 2004). As expected, we observed a profound defect in accumulation of FITC^+ DCs in the CLNs from Ikbkb^ΔItgax mice compared to littermate controls (Figure 6A). Because FITC acts as a hapten, forming adducts with endogenous proteins, these data suggest that IKKβ deficiency in NLT-DCs would result in impaired trafficking of antigens from the skin to the CLNs. To test the role of IKKβ in NLT-DC-mediated antigen presentation and T cell priming in the CLNs, we applied the TLR7 agonist R848, as an adjuvant, in combination with MHC I-restricted ovalbumin peptide (OVAp) to the ear skin of Ikbkb^ΔItgax and littermate control mice; we then measured the expansion of OVAp-specific CD8^+ T cells in the CLNs by FACS by using OVAp-specific tetramers. There was a significant reduction in antigen-specific CD8^+ T cell expansion in CLNs from Ikbkb^ΔItgax mice compared to littermate controls (Figure 6B), demonstrating that impaired NLT-DC migration in Ikbkb^ΔItgax mice prevented the transport and presentation of an exogenous antigen applied to the skin. In addition, the significant increase in NLT-DC migration promoted by R848 application was impaired in Ikbkb^ΔItgax mice (Figure 6C), suggesting that IKKβ is required for NLT-DC migration in both steady-state and inflammatory conditions.

T cell priming upon sub-cutaneous (s.c.) immunization with the commonly used adjuvant CFA is dependent on recruitment of monocyte-derived DCs (moDCs) into draining LNs through high endothelial venules (HEVs) (Nakano et al., 2009)—as opposed to migration of NLT-DCs through lymphatics. We did not observe any defect in the accumulation of OVAp-specific CD8^+ T cells in the CLNs of Ikbkb^ΔItgax mice after s.c. immunization with CFA and OVA, and moDC accumulation in CLNs was not affected in Ikbkb^ΔItgax mice (data not shown). These data suggest that IKKβ is not required for the recruitment of moDCs or subsequent T cell priming in inflammatory conditions, but specifically regulates the maturation and migration of NLT-DCs.

IKKβ in DCs Promotes Tolerance to an Endogenous Tissue Antigen

In steady state, migratory NLT-DCs are required for conversion of naive CD4^+ T cells to FOXP3^+ Treg (iTreg) cells and tolerance to self-antigens (Azukizawa et al., 2011; Guilliams et al., 2010). Ikbkb^ΔItgax mice showed a profound absence of steady-state migDCs and significantly reduced accumulation of FOXP3^+ Treg cells (Figures 4A and 3D). The data described above show that IKKβ signaling in NLT-DCs is required for the transport and presentation of exogenous antigens from the skin in the draining LNs. To evaluate the role of IKKβ in skin NLT-DCs for tolerance to an endogenous self-antigen, we used K5-mOVA transgenic mice (Tg(K5-mOVA)), which express membrane-bound OVA in epidermal keratinocytes (Azukizawa et al., 2003). Previous studies with K5-mOVA mice have shown that migratory NLT-DCs are required for the transport and presentation of OVA antigens from the skin to naive T cells in the CLNs. In the case of CD8α-type migDCs, this was required for cross-presentation of OVA-peptide to autoreactive CD8^+ T cells and their subsequent deletion (Waithman et al., 2007). However, in the case of CD11b^+ migDCs, this was required for the conversion of OVA-specific naive CD4^+ T cells to iTreg cells (Azukizawa et al., 2011; Guilliams et al., 2010). To test the role of IKKβ in this process, we generated K5-mOVA mice with a targeted deletion of IKKβ in DCs (Tg(K5-mOVA) Ikbkb^ΔItgax). First, we confirmed the absence of migDCs in CLNs from Tg(K5-mOVA) Ikbkb^ΔItgax mice compared to Tg(K5-mOVA) Ikbkb^F/F littermate controls (Figure 7A). This was associated with a significant reduction in the accumulation of CD25^+FOXP3^+ Treg cells in the CLNs of Tg(K5-mOVA) Ikbkb^ΔItgax mice (Figure 7A). To test the role of IKKβ in migDCs for deletion of autoreactive CD8^+ T cells, we adoptively transferred purified OVA-specific CD8^+ T cells (OT-I) to cohorts of Tg(K5-mOVA) Ikbkb^ΔItgax and Tg(K5-mOVA) Ikbkb^F/F mice and monitored the deletion of OT-I cells in the blood and CLNs by FACS, using specific tetramers. All control mice deleted adoptively transferred OT-I cells within 2 weeks (Figure 7B); however, OT-I cells expanded in a significant proportion of mice with IKKβ deletion in DCs (Figure 7B),

(C and D) Ear skin flaps were floated on tissue culture medium for 72 hr before dermis was separated from epidermis and stained for MHCII and LYVE1. Quantitative analysis was performed with ImageJ software and data are plotted as proportion of MHCII^+ cells co-localized to Lyve1^+ lymphatics. Scale bars represent 100 μm. Graphs represent pooled data from at least three independent experiments. Statistical analysis was performed with Mann-Whitney test; **p < 0.01, ***p < 0.001.
(E) Epidermal and dermal cell suspensions were prepared and cultured overnight at 37°C. DCs were counted and stained for viability, MHCII, CD40, CCR7, and Fascin expression by flow cytometry. The first graphs represent the ratio of viable DCs upon culture at 37°C compared to cells kept at 4°C. MHCII, CD40, CCR7, and Fascin expression by epidermal and dermal DCs are shown at 4°C and after culture at 37°C. MHCII and CD40 expression are represented as MFI only, because they were uniformly expressed by DCs. CCR7 and Fascin expression are expressed as proportion of positive cells among DCs and MFI of positive cells at 37°C. Data are shown as mean ± SEM and are representative of three independent experiments. Statistical analysis was performed with Mann-Whitney test; *p < 0.05.
(F) Dermal cell suspensions were prepared as described in (E); DCs were sorted and mRNA expression analyzed by quantitative RT-PCR (qRT-PCR). Relative expression of specific genes in dermal DCs at 4°C and 37°C is indicated normalized to cyclophillin (CPH) expression. Data represent mean ± SEM of three replicates from pooled cells of n = 3 mice and are representative of two independent experiments.
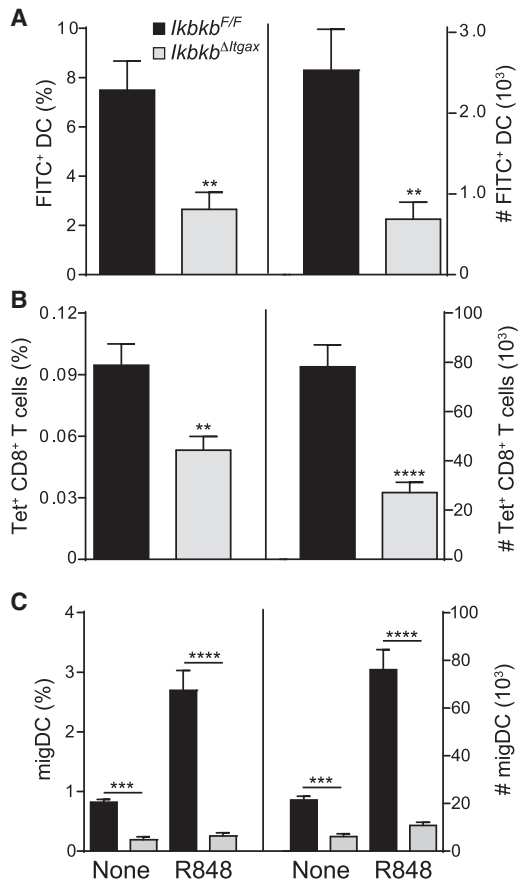
Figure 6. IKKβ Is Required for Dermal NLT-DC Migration and Antigen Trafficking to CLNs

(A) FITC was applied epicutaneously to the ear skin of *Ikbkb*[F/F] and *Ikbkb*[ΔItgax] mice, and 3 days later the accumulation of FITC-labeled DCs in draining ALNs was measured by flow cytometry. Representative data showing the percentage and the number of FITC+ DCs are plotted from three independent experiments.

(B) Mice were immunized by topical application of R848 on the ear in combination with MHC I-restricted ovalbumin peptide (OVAp). After 7 days, the expansion of OVAp-specific CD8+ T cells was measured in the ALN by cytometry using tetramers. Graphs represent pooled data from three independent experiments and show the percentage and the number of OVAp-specific CD8+ T cells in ALNs.

(C) R848 was applied to the ears and accumulation of migDCs in draining ALNs after 2 days was evaluated by flow cytometry. Representative data showing the percentage and the number of migDCs from three independent experiments are shown.

Statistical analysis was performed with Mann-Whitney test; **p < 0.01, ***p < 0.001, ****p < 0.0001.
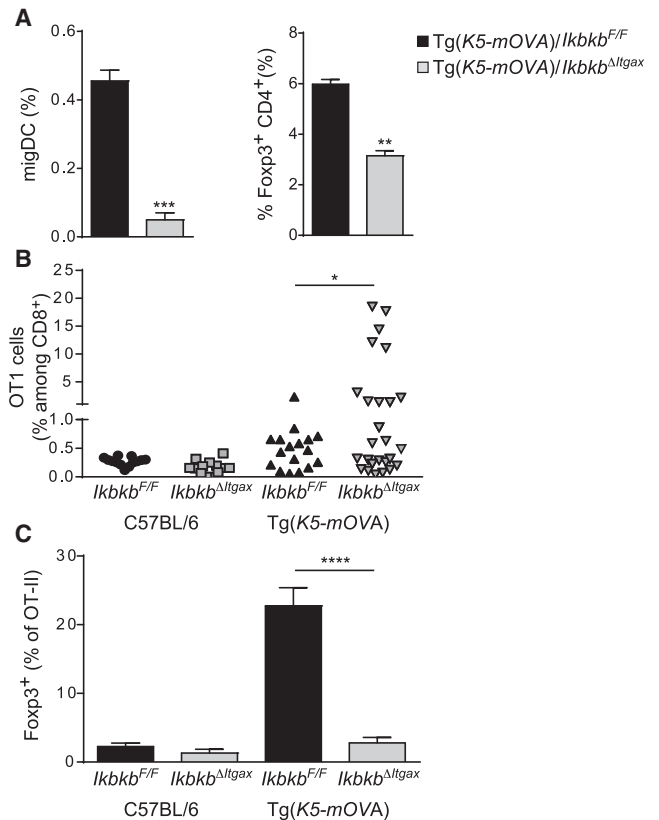

Figure 7. IKKβ in DCs Regulates Tolerance to an Endogenous Cell-Associated Antigen in the Skin

(A) The proportions of migDCs and Foxp3+ Treg cells in CLNs from Tg(*K5-mOVA*)/*Ikbkb*[ΔItgax] mice and Tg(*K5-mOVA*)/*Ikbkb*[F/F] littermates was evaluated by flow cytometry. Graphs represent pooled data from three independent experiments.

(B) Purified OVA-specific CD8 T cells (OT-I) were transferred to Tg(*K5-mOVA*)/*Ikbkb*[ΔItgax] and Tg(*K5-mOVA*)/*Ikbkb*[F/F] mice and the proportion of activated OT-I cells measured in peripheral blood after 11 days by flow cytometry using tetramers. Data from four independent experiments are represented on the graph. Each point corresponds to one mouse.

(C) Purified naive CD25− CD4+ OT-II cells were adoptively transferred to Tg(*K5-mOVA*)/*Ikbkb*[ΔItgax] and Tg(*K5-mOVA*)/*Ikbkb*[F/F] mice, and after 13 days the proportion of CD25+FOXP3+ OT-II cells among CD4+ cells in the CLN was evaluated by flow cytometry.

Data are representative of three independent experiments. Statistical analyses in (A) and (C) were performed with Mann-Whitney test; **p < 0.01, ***p < 0.001, ****p < 0.0001. Statistical analysis in (B) was performed by Fischer exact test; *p < 0.05.

indicating impaired deletional tolerance. In fact, the expansion of adoptively transferred autoreactive CD8+ T cells in Tg(*K5-mOVA*) *Ikbkb*[ΔItgax] mice frequently led to inflammatory lesions in the skin as a result of the activation of autoreactive CD8+ T cells (data not shown).

To evaluate the role of IKKβ-dependent migDCs in the conversion of naive OVA-specific CD4+ T cells to iTreg cells, we adoptively transferred purified naive CD25− CD4+ OT-II cells to cohorts of Tg(*K5-mOVA*) *Ikbkb*[ΔItgax] and Tg(*K5-mOVA*) *Ikbkb*[F/F] mice and measured the accumulation of CD25+FOXP3+ OT-II

cells in the CLNs. In these experiments, Tg(*K5-mOVA*) *Ikbkb*[ΔItgax] mice showed a profound impairment in accumulation of OT-II iTreg cells compared to littermate controls (Figure 7C), demonstrating that IKKβ-dependent migDCs were required for OVA-specific iTreg cell conversion in CLNs.

Collectively, these data demonstrate that IKKβ-dependent accumulation of migDCs in the CLNs in steady state plays an important contribution to tolerance toward endogenously expressed cell-associated antigens by regulating both iTreg cell conversion, through MHCII-mediated presentation to naive CD4+ T cells, and deletional tolerance, through cross-presentation of cell-associated antigens to CD8+ T cells.

DISCUSSION

The homeostatic maturation of NLT-DCs and their subsequent migration from the tissue parenchyma to the draining LNs is critical to maintain tolerance to innocuous tissue antigens. Triggering of pattern recognition receptors (PRRs), such as TLRs, on DCs is well known to induce a process of functional maturation that is required for the priming of naive T cells. Similar changes are associated with steady-state migDCs compared to their tissue-resident counterparts, but results in tolerogenic activity rather than T cell priming. However, the signaling pathways that regulate the maturation and accumulation of migDCs in steady state have remained an enigma. Here we have shown that NF-κB signaling is required for the steady-state maturation and migration of NLT-DCs and we demonstrate that IKKβ deletion in DCs was sufficient to prevent the steady-state accumulation of migDCs from the skin. Deletion of IKKβ did not affect DC development or survival but was critically required for maturation and migration of NLT-DCs in the skin. Furthermore, the IKKβ-dependent accumulation of migDCs played an important role in tolerance toward endogenously expressed cell-associated antigens by regulating both iTreg cell conversion, through MHC II-mediated presentation to naive CD4$^+$ T cells, and deletional tolerance, through cross-presentation of cell-associated antigens to CD8$^+$ T cells.

Our analysis of the specific gene expression signature in steady-state migDCs reveals a strong enrichment for NF-κB-regulated genes. However, canonical NF-κB target genes that are highly expressed upon TLR-induced DC maturation were not upregulated or were even repressed, indicating a novel NF-κB-regulated gene network in migDCs that is likely to depend on unique upstream regulators. In keeping with this hypothesis, we showed that mice deficient in TLR, IL-1β, or TNF-α signaling had no defects in accumulation of migDCs in steady state. This is in accordance with a previous study that showed no alterations in migDC accumulation or maturation in germ-free mice and mice deficient in TLR signaling (Wilson et al., 2008). The NF-κB-regulated gene network in steady-state migDCs from the skin included genes previously linked with DC migration (*Fscn1*) and Treg cell conversion (*Aldh1a2*), both functions that were impaired in vivo upon deletion of IKKβ in DCs, as well as several genes with no known function in DCs.

One possible explanation for the distinct networks of NF-κB-regulated genes during steady-state (tolerogenic) and Toll-like receptor (TLR)-induced (immunogenic) DC maturation might be the context-specific activation or expression of transcriptional co-regulators. For example, the transcription factor IRF4 was recently shown to be required for development of CD11b$^+$ NLT-DCs in both skin and intestine and the steady-state accumulation of migDCs in CLNs and MLNs, respectively (Persson et al., 2013; Schlitzer et al., 2013). IRF4 and NF-κB are known to interact in B cells and co-operatively regulate specific genes. We have also found significant enrichment for IRF binding sites in genes upregulated in steady-state migDCs (Dalod et al., 2014), suggesting that IRF4 and NF-κB might co-operatively regulate steady-state NLT-DC maturation.

The homeostatic signals that drive NLT-DC maturation and the accumulation of migratory DCs are completely unknown. In fact, it isn't even clear whether this is an intrinsically stochastic process or one driven by instructive signals in the tissue micro-environment. Factors that have been suggested to play a role in NLT-DC homeostasis and maturation include CSF2 (GM-CSF) and TGF-β. CSF2 was shown to promote the survival of NLT-DCs and thus affect the accumulation of migDCs in draining LNs (Greter et al., 2012), but we found no role for IKKβ in NLT-DC survival, and furthermore, blocking CSF2 failed to prevent the spontaneous maturation of dermal DCs ex vivo, which was abrogated by inhibition of IKKβ (Figure S5). TGF-β has been linked with tolerogenic functions in DCs (Travis et al., 2007) and plays a critical role in the homeostasis of LCs in the epidermis (Kel et al., 2010). However, we also found that TGF-β neutralization did not affect spontaneous maturation of dermal DCs ex vivo in contrast to IKKβ inhibition (Figure S5).

Several previous studies have suggested that WNT-β-catenin signaling in DCs is important for tolerogenic function (Jiang et al., 2007; Manicassamy et al., 2010). Targeted deletion of β-catenin in DCs led to impaired Treg cell conversion by DCs in the intestine and disruption of immune homeostasis (Manicassamy et al., 2010). However, these authors focused on NLT-DCs in the intestine lamina propria and did not analyze migDCs from the mesenteric LNs. Our analysis shows that WNT-β-catenin target genes are in fact downregulated in migDCs from the CLNs compared to their dermal counterparts (Figure S1), indicating that this pathway is not upregulated during steady-state DC maturation in the skin. This might reflect tissue specificity in pathways regulating NLT-DC maturation, or alternatively, WNT-β-catenin signaling might affect DC function independently of NLT-DC maturation and migration. Another link between WNT-β-catenin signaling and tolerogenic DC maturation was suggested by Jiang et al. (2007), when these authors showed that disruption of homotypic E-cadherin interactions between clusters of bone-marrow-derived DCs (BMDCs) in culture led to β-catenin-mediated maturation, including upregulation of CCR7, CD40, and CD86. This maturation was associated with tolerogenic function rather than T cell priming. We generated IKKβ-deficient BMDCs but found no impact on cluster-disruption-induced maturation, including upregulation of CCR7, CD40, and CD86 (data not shown). Furthermore, a number of the genes induced by BMDCs in this context were in fact downregulated in migDCs from the skin compared to dermal DCs (Figure S1), as were other WNT-β-catenin pathway genes.

In summary, we have uncovered a new role for NF-κB signaling in the accumulation of steady-state migDCs and maintenance of immune tolerance in the skin. Although NF-κB is generally considered as a major pro-inflammatory transcription factor, these data show an important role for NF-κB signaling in immune homeostasis and tolerance that could be significant in the context of autoimmune diseases and cancer—where the molecular mechanisms regulating tolerance to tissue antigens are therapeutic targets. Our analysis of the global gene expression profile of NLT-DCs and their migratory counterparts suggests that a novel NF-κB-regulated gene network is associated with the steady-state maturation of NLT-DCs and their tolerogenic function; however, the homeostatic signals that trigger IKKβ-NF-κB activation in this context are yet to be revealed. Future characterization of the upstream regulators and key nodes in this network might reveal new molecular targets to specifically modulate migDCs and promote immunity or tolerance.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described in the Supplemental Information section.

Mice

K5-mOVA, OT-I, OT-II, *Ccr7*⁻/⁻, and Tg(*Itgax-hBcl2*) mice have been previously described (Azukizawa et al., 2003; Hogquist et al., 1994; Barnden et al., 1998; Förster et al., 1999; Gautier et al., 2009).

Flow Cytometry

Anti-CD11c (HL3), anti-CD8α (53-6.7), anti-MHC II (M5/114), anti-CD45.2 (104), anti-CCR7 (4B12), anti-CD11b (M1/70), anti-CD24 (M1/69), anti-Foxp3 (FJK-16 s), and anti-CD103 (M290) antibodies were all purchased from either BDeBioscience or Biolegend. Anti-Fascin (EP5902) antibody was purchased from Abcam. H-2Kb-OVA257-264 PE tetramers were purchased from Beckman Coulter. Analysis was performed with FACSCanto system (BD) and Flowjo software (Tree Star).

Microarray Analysis

Microarray analyses were performed as reported previously (Yamakita et al., 2011). The genes differentially expressed between steady-state dDCs and migDCs were identified with the Bioconductor Limma statistical package, with cutoff values of 0.05 for the false discovery rate and of 2 for the fold change. A pathway analysis was then performed on the 1,095 genes upregulated in migDCs via the Ingenuity Pathway Analysis facility. Distant regulatory elements analysis was performed with default parameters and a motif search with the Clover software as previously reported (Baranek et al., 2012); p value threshold and score were fixed respectively at 0.05 and 4.

Skin Explants

Ears were split into ventral and dorsal parts and floated split side down on 1 ml medium containing RPMI 10% FCS and 50 μM β-mercaptoethanol.

Isolation of Skin DCs

Epidermis was separated from the dermis and single-cell suspensions were prepared and cultured for 18 hr at 37°C prior to analysis by flow cytometry. For inhibitor experiments, single-cell suspensions of skin DCs were prepared from epidermis and treated with or without various inhibitors for 18 hr at 4°C or 37°C: IKKβ inhibitor BI605906 (Zhang et al., 2014), 10 and 50 μM; JAK3 inhibitor CP690550 (Tocris), 1 μM; anti-mouse TGF-β1 neutralizing Ab (Uyttenhove et al., 2011), 10 μg/ml; anti-mouse CSF2 neutralizing Ab (eBioscience), 5 μg/ml.

Adoptive T Cell Transfer

OT-I and OT-II cells were purified from pooled LNs and spleen by negative selection via a T cell isolation kit (Invitrogen); CD25⁻ OT-II cells were subsequently sorted by flow cytometry using anti-CD4 and anti-CD25 antibodies.

BrdU Labeling In Vivo

Mice were injected i.p. with 1.5 mg of BrdU (Sigma Aldrich) and their drinking water was supplemented with 0.8 mg/ml BrdU and 2% glucose. BrdU staining was performed according to the manufacturer's recommendations (BrdU labeling Flow kit, BD).

FITC Painting

FITC (0.5 mg/ml) was dissolved in acetone and dibutyl phthalate 1:1 and 30 μl was applied on the ear of anesthetized mice.

Topical Immunization

50 μg of H-2Kb-OVA257-264 peptide was mixed in 50 mg of imiquimod cream (Aldara; 3M Pharceuticals) and applied to ears of anesthetized animals.

Histopathology

Hematoxylin and eosin staining was perfomed on paraffin-embedded spleen and kidney tissue. Immunohistochemistry was performed on frozen tissue sections with the indicated antibodies. GC development was evaluated via the following scoring: 1, weak; 2, moderate; 3, marked; 4, high. Serum was analyzed for autoantibody production by a standard autoantibody test with Hep-2 cells fixed on slides (Biomedical Diagnosis).

Statistical Analysis

p values were calculated with either the Mann-Whitney test or the Fisher test when appropriate with Graph pad prism software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.03.003.

AUTHOR CONTRIBUTIONS

M.B. and C.F. performed most of the experiments with technical support from S.D. and C.V. O.D., M.H., and A.F.-L. contributed important data and protocols. J.M., E.P., and M.D. performed bioinformatics analysis. M.B. and T.L. designed the experiments and wrote the manuscript.

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