Epithelial-intrinsic IKKα expression regulates group 3 innate lymphoid cell responses and antibacterial immunity

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Innate lymphoid cells (ILCs) are critical for maintaining epithelial barrier integrity at mucosal surfaces; however, the tissue-specific factors that regulate ILC responses remain poorly characterized. Using mice with intestinal epithelial cell (IEC)-specific deletions in either inhibitor of κB kinase (IKK)α or IKKβ, two critical regulators of NFκB activation, we demonstrate that IEC-intrinsic IKKα expression selectively regulates group 3 ILC (ILC3)—dependent antibacterial immunity in the intestine. Although IKKβIEC mice efficiently controlled Citrobacter rodentium infection, IKKαIEC mice exhibited severe intestinal inflammation, increased bacterial dissemination to peripheral organs, and increased host mortality. Consistent with weakened innate immunity to C. rodentium, IKKαIEC mice displayed impaired IL-22 production by RORγt+ ILC3s, and therapeutic delivery of rIL-22 or transfer of sort-purified IL-22–competent ILCs from control mice could protect IKKαIEC mice from C. rodentium–induced morbidity. Defective ILC3 responses in IKKαIEC mice were associated with overproduction of thymic stromal lymphopoietin (TSLP) by IECs, which negatively regulated IL-22 production by ILC3s and impaired innate immunity to C. rodentium. IEC–intrinsic IKKα expression was similarly critical for regulation of intestinal inflammation after chemically induced intestinal damage and colitis. Collectively, these data identify a previously unrecognized role for epithelial cell–intrinsic IKKα expression and TSLP in regulating ILC3 responses required to maintain intestinal barrier immunity.

Maintenance of epithelial barrier integrity at mucosal surfaces is essential to limit exposure to commensal and pathogenic microorganisms and to promote intestinal homeostasis (Artis, 2008; Hooper and Macpherson, 2010; Fung et al., 2014; Peterson and Artis, 2014). Defects in epithelial barrier function are associated with multiple infectious and inflammatory diseases, including inflammatory bowel disease (IBD; Pasparakis, 2008; Marchiando et al., 2010; Maloy and Powrie, 2011), and recent studies have highlighted a critical role for innate lymphoid cells (ILCs) in regulating immunity, inflammation, and tissue repair at barrier surfaces such as the intestine (Spits and Cupedo, 2012; Walker et al., 2012).
ILCs represent a heterogeneous family of cells that, based on their expression of the transcription factors T-bet, GATA3, and RORγt, can be categorized into three groups with diverse effector functions. Group 1 ILCs (ILC1s) express IFNγ and T-bet and include NK cells (Spits and Cupedo, 2012), whereas ILC2s express GATA3, RORα, MHCI, IL-5, IL-13, and amphiregulin and regulate inflammation, barrier integrity, and/or tissue homeostasis in the skin, intestine, lung, and adipose tissue (Neill et al., 2010; Monticelli et al., 2011; Molofsky et al., 2013; Roediger et al., 2013; Oliphant et al., 2014; Brestoff et al., 2015; Lee et al., 2015). RORγt+ ILC3s express IL-17A, IFNγ, MHCI, lymphotixin (LT)α1β2, and IL-22 and promote antibacterial immunity, secondary lymphoid structure formation, and the regulation of intestinal inflammation (Buonocore et al., 2010; Kiss et al., 2011; Tumanov et al., 2011; Sonnenberg et al., 2012; Hepworth et al., 2013, 2015; Goto et al., 2014). Although IL-17A and IFNγ production by ILC1s or ILC3s is implicated in the pathogenesis of colitis (Buonocore et al., 2010; Geremia et al., 2011; Spits and Di Santo, 2011), ILC3-derived IL-22 is associated with the promotion of epithelial barrier integrity at multiple tissue sites (Aujla et al., 2008; Satoh-Takayama et al., 2008; Sonnenberg et al., 2012; Goto et al., 2014). Ligation of the IL-22 receptor, expression of which is restricted to nonhematopoietic cell lineages such as epithelial cells, induces expression of host defense genes, mucus, and antimicrobial peptides (AMPs) that are critical for host-protective immunity after exposure to viruses and bacterial infections such as Klebsiella pneumoniae and Citrobacter rodentium (Aujla et al., 2008; Zheng et al., 2008; Kim et al., 2012; Klatt et al., 2012; Ivanov et al., 2013; Goto et al., 2014; Zhang et al., 2014; Muñoz et al., 2015). Although the influence of RORγt+ ILC3s on epithelial barrier function is well characterized, the molecular and cellular pathways that regulate ILC responses in mucosal tissue microenvironments remain poorly understood.

In addition to providing a physical barrier to microorganisms, intestinal epithelial cells (IECs) express cytokines, chemokines, pattern recognition receptors, inflammasomes, and AMPs that permit cross-talk with mucosal immune cells and maintenance of immune homeostasis (Strober, 1998; Pasparakis, 2008; Rescigno, 2011; Welz et al., 2011; Goto and Ivanov, 2013; Dannappel et al., 2014; Kagnoff, 2014; Peterson and Artis, 2014). For example, signals derived from IECs regulate proinflammatory cytokine secretion by DCs (Nenci et al., 2007; Zaph et al., 2007), enhancing their ability to promote regulatory and Th1-type cytokine responses (Rimoldi et al., 2005a,b; Iliev et al., 2009). IECs also secrete cytokines that regulate macrophage function (Smythies et al., 2005) and B cell production of secretory IgA (Xu et al., 2007; Cerutti, 2008). Genetic approaches to interrogate the factors that regulate IEC function have identified a critical role for NFκB-associated genes, including inhibitor of κB kinase (IKK)β or IKKα, which control “canonical” versus “noncanonical” NFκB-dependent gene expression, respectively (Greten et al., 2004; Nenci et al., 2007; Zaph et al., 2007; Eckmann et al., 2008; Vlantis et al., 2011; Bonnegarde-Bernard et al., 2014; Takahashi et al., 2014; Vereecke et al., 2014). Although ILC3s are known to regulate IEC function via IL-17A and IL-22 expression (Aujla et al., 2008; Zheng et al., 2008; Hanash et al., 2012; Muñoz et al., 2015), whether tissue-resident nonhematopoietic cells such as IECs can reciprocally regulate intestinal ILC3 responses remains incompletely defined. In the present study, we demonstrate that mice with IEC-specific deletions in IKKα, but not IKKβ, exhibit impaired innate immunity to C. rodentium infection, identifying a previously unappreciated role for the noncanonical NFκB activation pathway in antibacterial immunity. Critically, mice with IEC-intrinsic IKKα deletions displayed impaired IL-22 production by RORγt+ ILC3s and delivery of recombinant IL-22 or IL-22–competent sort-purified ILCs was sufficient for restoration of intestinal protection against C. rodentium infection. IEC-intrinsic IKKα was also critical for regulation of intestinal inflammation after chemically induced intestinal damage and colitis. Mechanistically, the absence of IKKα expression resulted in elevated thymic stromal lymphopoietin (TSLP) production by colonic epithelial cells, which negatively regulated IL-22 production by ILC3s in vitro and innate immunity to C. rodentium in vivo. Furthermore, neutralization of TSLP in IKKαΔIEC mice could partially restore ILC3 responses and innate immunity to C. rodentium. Collectively, these data highlight a previously unrecognized mechanism by which IECs and ILCs reciprocally regulate intestinal immune homeostasis.

RESULTS

IIEC-intrinsic IKKα, but not IKKβ, expression is critical for immunity to C. rodentium infection

C. rodentium is a natural gram-negative extracellular bacterial pathogen of mice akin to the human pathogen enterohemorrhagic Escherichia coli that causes NFκB activation and colonic lesions after attachment to the epithelial surface (Mundy et al., 2005; Wang et al., 2006; Chandrakesan et al., 2010). Innate immunity to C. rodentium and regulation of intestinal barrier integrity is controlled, in part, by ILC3-dependent IL-22 responses (Satoh-Takayama et al., 2008; Zheng et al., 2008; Kiss et al., 2011; Sonnenberg et al., 2011b; Tumanov et al., 2011). However, the function of IEC-intrinsic NFκB activation and whether it regulates antibacterial immunity and tissue-protective ILC responses is unknown. Using mice with IEC-specific deletions in either IKKβ or IKKα, respectively, we assessed whether IEC-intrinsic canonical versus noncanonical NFκB activation regulates intestinal ILC responses. To do so, IKKβΔIEC or IKKαΔIEC mice in which either the Ilkβ or Ilkα genes are flanked by LoxP sites were crossed with mice expressing Cre recombinase under control of the IEC-specific villin promoter to generate IEC-specific IKKβ (IKKβΔIEC) or IKKα (IKKαΔIEC) knockout mice, as described previously (Nenci et al., 2007). Deletion of IKKβ in IECs from IKKβΔIEC mice and IKKα in IECs from IKKαΔIEC mice was confirmed by Western blotting (Fig. 1 a). To examine the potential influence of IECs on the functions of ILCs under inflammatory conditions, we infected IKKβΔIEC, IKKαΔIEC, and littermate control mice with
C. rodentium. Although IKKβIEC mice exhibited equivalent fecal C. rodentium burdens to IKKβF/F mice at day 5 postinfection (p.i.), IKKαIEC mice displayed higher fecal bacterial titers (Fig. 1b) and enhanced bacterial dissemination to peripheral organs, including the spleen and liver at day 11 p.i. compared with IKKαF/F controls (Fig. 1c and d). Associated with an impaired ability to control C. rodentium infection, IKKαIEC, but not IKKβIEC, mice displayed exacerbated infection-induced weight loss (Fig. 1e), and ~50% of IKKαIEC mice succumbed to infection by day 11 p.i. (Fig. 1f). IKKαIEC mice that
survived beyond day 12 p.i. were able to resolve the infection and regain weight at a similar level to that of control mice (not depicted). Histological analyses demonstrated that deletion of IKKα or IKKβ in IECs was not associated with altered intestinal immune homeostasis in the steady state (Fig. 1 g), consistent with a previous study (Nencic et al., 2007). However, although C. rodentium–infected IKKβ+/F, IKKβΔEC, and IKKα+/F mice exhibited modest intestinal inflammation at day 11 p.i. (Fig. 1 h), infected IKKαΔEC mice exhibited severe inflammation, characterized by disruption of normal epithelial crypt architecture, mucosal hyperplasia, and colonic ulceration (Fig. 1 h), resulting in a significantly higher colonic pathology score relative to control mice (Fig. 1 i). Associated with a loss of intestinal barrier integrity and bacterial dissemination, neutrophil–rich inflammatory foci were observed in the liver of IKKαΔEC mice at day 11 p.i. (Fig. 1 j). Collectively, these data highlight the selective requirement for IEC-intrinsic expression of IKKα for regulation of antibacterial immune responses and intestinal barrier homeostasis.

Figure 2. Colonic IL-22 and AMP expression is reduced in IKKαΔEC mice in the steady state and after C. rodentium infection. (a) IFNγ, IL-17A, IL-6, and IL-22 protein expression in colonic tissue homogenates from naive littermate control IKKαWT and IKKαΔEC mice, as measured by ELISA. (b) IL22, Reg3g, and Reg3b mRNA expression in colonic tissue homogenates from naive mice, as measured by RT-PCR. (c–e) IKKαWT or IKKαΔEC mice were infected with C. rodentium, and IL-22 protein expression (c) and IL22 mRNA (d) were measured in colonic tissue homogenates of naive (N) or infected (INF) mice at day 4 p.i. (e) Reg3g and Reg3b mRNA expression in colonic tissue homogenates from naive or day 4 C. rodentium–infected mice. Gene expression data in b, d, and e were normalized to naive IKKαWT mice. Data for a and b are representative of two independent experiments (IKKαWT, total n = 9; IKKαΔEC, n = 8). Data for c–e are representative of three independent experiments (IKKαWT, total n = 15; IKKαΔEC, n = 14 + 1 naive mouse of each genotype per experiment). Data are expressed as mean ± SEM. *, P < 0.05 compared with IKKαWT control.

Colonic IL-22 and AMP expression are reduced in IKKαΔEC mice in the steady state and after C. rodentium infection

To investigate the mechanisms for dysregulated C. rodentium–induced intestinal inflammation in IKKαΔEC mice, we first examined the expression levels of key cytokines involved in immunity to C. rodentium. Compared with littermate control IKKαWT mice, protein levels of the proinflammatory cytokine IFNγ were significantly elevated in the colons of naïve IKKαΔEC mice, whereas expression levels of IL–17A and IL–6 were not significantly different (Fig. 2 a). In contrast, IL–22 protein (Fig. 2 a) and mRNA (Fig. 2 b) expression was significantly reduced in IKKαΔEC mice in the steady state. This correlated with significant reductions in the mRNA expression levels of the IL–22–dependent AMPs Reg3g and Reg3b in the colon in the absence of IEC-intrinsic IKKα expression (Fig. 2 b). To assess whether the expression of IL–22 and AMPs was compromised after C. rodentium infection, we infected IKKαWT control and mutant IKKαΔEC mice with C. rodentium and analyzed mice at day 4 p.i., an early time point where IL–22 responses reach their peak after C. rodentium infection (Zheng et al., 2008; Ota et al., 2011; Sonnenberg et al., 2011b; Mantà et al., 2013). Importantly, infection-induced IL–22 protein (Fig. 2 c) and mRNA (Fig. 2 d) expression was reduced in IKKαΔEC mice, with concurrent significant reductions in Reg3g and Reg3b expression (Fig. 2 c). Together, these data highlight that IEC-intrinsic IKKα expression regulates both steady state and infection-induced IL–22 responses in the colon, suggesting a potential mechanism by which IECs may regulate barrier integrity in the intestine.

Therapeutic delivery of recombinant IL–22 is sufficient to rescue IKKαΔEC mice from C. rodentium–induced morbidity

IL–22 is critical for innate immunity to infection with C. rodentium (Zheng et al., 2008; Satoh–Takayama et al., 2008; Kiss et al., 2011; Sonnenberg et al., 2011b; Tumanov et al., 2011). In response to C. rodentium infection, IL–22–deficient mice exhibit rapid weight loss, intestinal barrier breakdown, and impaired control of bacterial dissemination resulting in death (Zheng et al., 2008), a phenotype consistent with C. rodentium–infected IKKαΔEC mice (Fig. 1). Epithelial cells possess an
mice are a likely cause of *C. rodentium*–induced morbidity and mortality.

**C. rodentium** infection–induced ILC-dependent IL-22 responses are diminished in IKKαIEC mice

IL-22 can be produced by a variety of immune cells, including T cells, ILCs, neutrophils, and DCs (Zheng et al., 2008; Sonnenberg et al., 2011a; Zindl et al., 2013). However, previous studies have demonstrated that ILC3-dependent IL-22 responses are critical for innate immunity to *C. rodentium* infection (Sonnenberg et al., 2011b; Manta et al., 2013). Consistent with this, analysis of the predominant IL-22–expressing cells in the early stages of *C. rodentium* infection (day 4 p.i.) demonstrated that lineage–negative cells were the predominant source of IL-22 in the mesenteric LNs (mLNs) of control IKKαIEC mice (Fig. 4, a and b). Further characterization revealed that IL-22–expressing CD3–CD5–NK1.1+ cells expressed RORγt, CD25, CD90, and CD4, a phenotype consistent with that of ILC3s (Fig. 4 c). Analysis of RORγt+ ILC responses (lineage+, RORγt+, CD25+ cells) in the mLNs of naïve and day 4 *C. rodentium*–infected IKKαIEC mice revealed that although the frequencies of intestinal RORγt+ ILCs were not significantly different (Fig. 4 d), total numbers of RORγt+ ILCs were significantly reduced in *C. rodentium*–infected IKKαIEC mice compared with IKKαIEC control (Fig. 4 e). Strikingly, when RORγt+ ILCs were assessed for...
their functional capacity to produce IL-22 after PMA and ionomycin stimulation, we observed a significant reduction in infection-induced IL-22 responses in RORγ+ ILCs from IKKαIEC mice compared with IKKαFF littermate controls (Fig. 4 f). Stimulation of cells with rIL-23 ex vivo revealed that RORγ+ ILCs from IKKαIEC mice were functionally capable of producing IL-22 if provided with adequate exogenous stimuli, and although the frequency of IL-22–producing ILC3s tended to be lower in rIL-23–stimulated cultures of IKKαIEC cells than cells from IKKαFF mice (Fig. 4 g), these differences did not reach statistical significance. Importantly, frequencies of CD4+ T cells expressing IL-22 were not significantly altered in
IKKαIEC mice (Fig. 4 h), indicating that IKKα-dependent regulation of IL-22 expression may be selective for ILCs. Collectively, these data indicate that IEC-intrinsic IKKα expression is necessary for optimal C. rodentium infection–induced ILC3-dependent IL-22 responses.

Delivery of sort-purified IL-22–competent ILCs restores immunity to C. rodentium infection in IKKαIEC mice

Although RORγt+ ILCs are the primary source of IL-22 in response to C. rodentium infection (Fig. 4, b and c), cells other than ILCs may contribute to IL-22–mediated immunity to C. rodentium. Therefore, we next assessed whether transfer of ILCs alone could restore immunity to C. rodentium infection in IKKαIEC mice. To do so, ILCs (lineage−CD90+CD25+CD127+) or T cells (CD3+CD90+) were sort purified from naive littermate control IKKαFP mice and pulsed for 1 h ex vivo with rIL-23, rIL-1β, rIL-2, and rIL-7 to promote survival and cytokine production before adoptive transfer into recipient IKKαFP or IKKαIEC mice. Although IKKαIEC mice that received T cells exhibited elevated bacterial titers in the feces at day 12 p.i. (Fig. 5 a) and exacerbated colonic inflammation and histology score compared with IKKαFP control mice (Fig. 5, b and c), IKKαIEC mice that received cytokine-activated ILCs displayed significantly reduced bacterial burdens (Fig. 5 a) and improved intestinal pathology and colonic histology score at day 12 p.i. (Fig. 5, b and c), indicating that ILCs were more potent at restoring antibacterial immunity than T cells. To examine the cell-intrinsic mechanism by which ILCs confer protection against C. rodentium infection, analogous experiments were performed where recipient IKKαFP or IKKαIEC mice were injected with either sort-purified ILCs or T cells from C57BL/6 WT mice or ILCs from C57BL/6 Il22−/− mice. Although IL-22–competent T cells were unable to confer protection against IKKIEC mice (Fig. 4 h), indicating that IKKα-dependent regulation of IL-22 expression may be selective for ILCs. Collectively, these data indicate that IEC-intrinsic IKKα expression is necessary for optimal C. rodentium infection–induced ILC3-dependent IL-22 responses.

Figure 5. Delivery of sort-purified IL-22–competent ILCs restores immunity to C. rodentium infection in IKKαIEC mice. (a–c) IKKαIEC mice were infected with C. rodentium and injected i.v. with either 10⁴ sort-purified CD90+CD25+ T cells or 10⁴ sort-purified lineage−CD90+CD25+CD127+ ILCs from IKKαFP mice on days 0, 2, 4, and 7 p.i. (littermate control IKKαFP mice received T cells only). T cells and ILCs were pulsed with rIL-23, rIL-1β, rIL-2, and rIL-7 for 1 h before injection. (a) C. rodentium CFU in the feces on day 6 p.i. (b) H&E staining of colon tissue sections at day 12 p.i. (c) Pathological score of colon histology. (d–f) Littermate IKKαIEC and IKKαIEC mice were infected with C. rodentium and injected i.v. with either 10⁴ sort-purified T cells or 10⁴ sort-purified ILCs (gating strategies as per a–c) from either C57BL/6 WT or C57BL/6 Il22−/− mice on days 0, 2, 4, and 7 p.i. (d) C. rodentium CFU in the feces on d 6 p.i. (e) H&E staining of colon tissue sections at day 12 p.i. All bars, 50 µm. (f) Pathological score of colon histology. Data for a–c are representative of two independent experiments (IKKαFP + cells, n = 8; IKKαIEC + T cells, n = 8; IKKαIEC + ILCs, n = 6). Data for d–f are representative of two experiments (IKKαFP + WT T cells, n = 7; IKKαIEC + WT T cells, n = 7; IKKαIEC + WT ILCs, n = 6; IKKαIEC + IL-22−/− ILCs, n = 6). Data are expressed as mean ± SEM. *, P < 0.05.
C. rodentium infection in IKKα<sup>ΔIEC</sup> mice, transfer of IL-22–competent ILCs reduced fecal bacterial titers (Fig. 5 d) and intestinal pathology (Fig. 5, e and f) in IKKα<sup>ΔIEC</sup> mice. Critically, ILC2−/− ILCs were unable to confer similar protection (Fig. 5, d–f). Collectively, these data indicate that the provision of IL-22–competent ILCs is sufficient to restore immunity to C. rodentium infection in IKKα<sup>ΔIEC</sup> mice, providing further evidence that defective ILC3–IL-22 responses are a likely cause of C. rodentium–induced immunopathology in IKKα<sup>ΔIEC</sup> mice.

IKKα<sup>ΔIEC</sup> mice exhibit dysregulated TSLP expression by colonic IECs

We next assessed the mechanisms by which IKKα expression within IECs regulates IL-22 production by ILC3. Production of IL-22 by intestinal ILCs is promoted by IL-23, IL-1β, IL-2, LTα1β2, and Ahr ligands (Cella et al., 2009; Hughes et al., 2010; Kiss et al., 2011; Reynders et al., 2011; Kinnebrew et al., 2012; Lee et al., 2012), whereas IEC-derived IL-25 is shown to elevate circulating TSLP levels (Iseki et al., 2011, 2013; Kim et al., 2013; Noti et al., 2014), which has been shown to elevate circulating TSLP levels (Iseki et al., 2012), and examined IL-22 expression. Notably, rIL-23 was able to induce equivalent IL-22 production in ILCs in the presence or absence of rTSLP, suggesting that the ability of TSLP to inhibit IL-22 production is indirect (Fig. 7 b). Next, we examined whether TSLP could regulate ILC–IL-22 responses in vivo. Hydrodynamic tail vein injection with gene-encoding plasmids has been used to induce protein overexpression in several settings (Sebestyén et al., 2006). Here, we treated C57BL/6 WT mice with either a control or TSLP overexpressing cDNA plasmid (as we have previously reported [Siracusa et al., 2011, 2013; Kim et al., 2013; Noti et al., 2014]), which has been shown to elevate circulating TSLP levels (Iseki et al., 2012), and examined IL-22 expression in ILCs 9 d later. Consistent with our in vitro findings using rIL-23 stimulation, TSLP overexpression led to diminished IL-22 production by ILCs isolated from the colonic lamina propria lymphocytes (cLPLs), mLNs, and spleen (Fig. 7 c), corresponding with significantly reduced expression levels of colonic Reg3g and Reg3b (Fig. 7 d). We next performed loss of function experiments to examine whether TSLP regulates innate immunity to C. rodentium. C57BL/6 Rag1<sup>−/−</sup> mice are highly susceptible to C. rodentium infection; however, IL-22 from ILCs does contribute to innate immunity (Zheng et al., 2008; Sonnenberg et al., 2011b). Although C57BL/6 Rag1<sup>−/−</sup> mice exhibited significant weight loss (Fig. 7 e and f) and succumbed to C. rodentium infection by days 20–22 p.i. (Fig. 7 f), C57BL/6 Rag1<sup>−/−</sup> mice deficient in TSLP responsiveness (Reg1<sup>−/−</sup> Tgtp<sup>−/−</sup>) mice) lost less weight, and this correlated with prolonged survival, only succumbing to infection between days 45 and 65 p.i. (Fig. 7, e and f). Collectively, these data suggest that TSLP negatively regulates innate immunity to C. rodentium, potentially via regulation of the ILC3–IL-22 axis.
Neutralization of TSLP partially restores immunity to *C. rodentium* infection in I KKαIEC mice

To directly assess whether overexpression of TSLP in I KKαIEC mice is responsible for impaired immunity to *C. rodentium* infection, we treated I KKαIEC mice every 3 d p.i. with either a neutralizing anti-TSLP mAb or a control rat IgG. Littermate control I KKαIEC mice received rat IgG only. Critically, although I KKαIEC mice treated with rat IgG displayed increased *C. rodentium* CFU in the feces at day 6 p.i. (Fig. 8 a) and liver at day 11 p.i. (Fig. 8 b) compared with I KKαIEC mice, I KKαIEC mice treated with anti-TSLP mAb exhibited substantially reduced bacterial titers (Fig. 8 a and b). TSLP neutralization also partially protected I KKαIEC mice from infection-induced weight loss (Fig. 8 c) and diminished colonic pathology (Fig. 8 d). Importantly, antibody (Ab)-mediated neutralization of TSLP in I KKαIEC mice increased IL-22 production by splenic ILC3 at day 4 p.i. (Fig. 8 e) and restored IL-22 production in the colon to levels observed in I KKαIEC mice (Fig. 8 f). These data demonstrate that dysregulated TSLP expression in the absence of IEC-intrinsic I KKα expression is a potential mechanism by which ILC-dependent innate immunity to *C. rodentium* is impaired in I KKαIEC mice.

IEC-intrinsic I KKα expression regulates inflammation during chemically induced intestinal damage

To assess whether I KKα expression within IECs is protective in other models of intestinal inflammation, we examined the susceptibility of I KKαIEC mice and I KKαIEC mice to dextran sodium sulfate (DSS)–induced colitis. Inclusion of 3% DSS in the drinking water of control I KKαIEC mice caused only...
controls (Fig. 9 d). In line with increased pathology in IKKα\textsuperscript{AEC} DSS-treated mice, levels of the proinflammatory cytokines IFNγ and IL-17A were elevated in IKKα\textsuperscript{AEC} mice at day 4 after DSS treatment (Fig. 9 e). Consistent with what was observed after C. rodentium infection, IL-22 protein levels were significantly reduced in the colon of DSS-treated IKKα\textsuperscript{AEC} mice compared with littermate IKKα\textsuperscript{F/F} controls (Fig. 9 e). In conclusion, IEC-intrinsic IKKα expression can limit inflammation in experimental models of infection-induced colitis and chemical-induced intestinal inflammation.

**DISCUSSION**

RORγt+ ILCs are central for regulation of immunity and barrier function in the intestine by promoting secondary lymphoid structure formation (Kiss et al., 2011), preventing bacterial dissemination (Sonnenberg et al., 2012), controlling immune responses to commensal microbes (Hepworth et al., 2013, 2015), and regulating epithelial cell homeostasis in health and disease (Ota et al., 2011; Sawa et al., 2011; Sonnenberg et al., 2011b; Hanash et al., 2012; Qiu et al., 2012; Kirchberger et al., 2013; Goto et al., 2014). Although several studies have identified some of the transcription factors, microbial factors, and cytokines involved in the development and function of ILC3s (Cording et al., 2014), the cellular and molecular network involved in regulation of ILC3s remains incompletely defined. The present study identifies a previously unappreciated pathway by which IECs selectively regulate ILC3 function via IKKα expression. Ablation of IKKα, but not IKKβ, expression within IECs led to impaired antibacterial immunity and compromised intestinal barrier function that was associated with defective IL-22 production by ILC3s. Immunity to C. rodentium could be restored by therapeutic administration with rIL-22 or with ILCs from IL-22–competent mice that had been pre–primed for IL-22 production. Absence of IKKα expression by IECs led to the overexpression of TSLP, a cytokine that we demonstrate can suppress ILC3–derived IL-22 production in vitro and inhibit innate immunity to C. rodentium infection in vivo. Together, these findings highlight a previously unrecognized mechanism of immune–epithelial cell dialogue that regulates intestinal barrier homeostasis, tissue protection, and antimicrobial ILC3 responses.

The NFκB signaling pathway is fundamental for regulation of IEC function, and although absence of individual IKKα and IKKβ subunits does not result in spontaneous inflammation (Nenci et al., 2007), IEC-intrinsic IKKβ expression is critical for limiting intestinal inflammation in chemically induced models of colitis (Eckmann et al., 2008), ischemia-reperfusion injury (Chen et al., 2003), and helminth infection (Zaph et al., 2007). Furthermore, overexpression of IKKβ within IECs results in uncontrolled epithelial proliferation and intestinal tumorigenesis (Guma et al., 2011; Vlantis et al., 2011). In the present study, we identify a critical role for IKKα-mediated signaling pathways in regulating C. rodentium–induced colitis and demonstrate that IKKβ is dispensable, representing the first demonstration of a differential requirement for IKKβ–versus IKKα–dependent gene expression on IEC function.
Furthermore, our findings are consistent with a previous study demonstrating that hyperactivation of the noncanonical NFκB pathway is associated with elevated immunity to C. rodentium (Hu et al., 2013), suggesting a pivotal role for IKKα-dependent signaling in immunity to bacterial infection.

IKKα-dependent noncanonical NFκB activation is associated with LTβR signaling (Schneider et al., 2004), and LTβR is a critical regulator of intestinal ILC-dependent IL-22 responses (Ota et al., 2011; Tumanov et al., 2011; Macho-Fernandez et al., 2015). Furthermore, mice with IEC-specific deletions in LTβR are highly susceptible to C. rodentium infection (Wang et al., 2010). Together, these data indicate a potential role for impaired LTβR signaling contributing to a reduction in host-protective immunity to C. rodentium in IKKα<sup>AIEC</sup> mice. However, LTβR-mediated regulation of ILCs was primarily mediated through DCs, and the impact of IEC-specific LTβR deletion on ILCs and IL-22 was not assessed (Wang et al., 2010). We did observe significant reductions in expression of the LTβR-dependent genes Cxcl1 and Ccl20 in IECs recovered from IKKα<sup>AIEC</sup> mice; hence it remains plausible that defective LTβR signaling may contribute to impaired IL-22 responses in the absence of IEC-intrinsic IKKα expression. This is consistent with a recent study showing that IEC-intrinsic LTβR expression regulates IL-22-dependent intestinal immune homeostasis during chemically induced colitis (Macho-Fernandez et al., 2015). Notably, despite impaired LTβR-dependent gene expression in IKKα<sup>AIEC</sup> mice, we did not observe reductions in the presence of intestinal lymphoid tissues such as Peyer’s patches (PPs) or isolated lymphoid follicles in IKKα<sup>AIEC</sup> mice (unpublished data), suggesting that in this context, other LTβR-responsive cells may have coordinated lymphoid organogenesis.

IL-22 production by ILC3s can be stimulated by several factors, chiefly IL-23 and IL-1β, but expression of these cytokines was not altered in IKKα<sup>AIEC</sup> mice. In addition to factors that positively regulate ILC3 function, commensal bacteria–dependent expression of IL-25 has been reported to suppress ILC3 responses (Sawa et al., 2011). Although we did not observe significant increases in IL-25 in IKKα<sup>AIEC</sup> mice, expression of the predominately IEC-derived cytokine TSLP was exaggerated in these mice. We demonstrate that exogenous TSLP suppressed IL-22 production by RORγ<sup+t</sup> ILC3s in vitro and in vivo and that Ab-mediated neutralization of TSLP could partially restore innate immunity to C. rodentium infection in IKKα<sup>AIEC</sup> mice. These data indicate that in addition to IL-25, TSLP acts as a negative regulator of ILC3 function in the context of bacterial-driven intestinal inflammation. Further studies are needed to illuminate whether additional IEC-dependent mechanisms, such as growth factors or the intestinal microbiota, may regulate ILC3 location, accumulation, or function.

Previous studies have demonstrated interactions between TSLP and ILC3s (Mjösberg et al., 2012; Kim et al., 2013) and that TSLPR signaling is dispensable for ILC3 development (Vonarbourg et al., 2010). How TSLP regulates IL-22 production by ILC3s remains unclear, but our data suggest that TSLP acts indirectly on ILCs to exert its function, possibly via an accessory TSLP-responsive cell such as a DC. It is becoming increasingly evident that TSLP has diverse cellular targets and biological functions, where TSLP may play both tissue-protective roles in the intestine (e.g., regulation of DC, ILC2, and granulocyte function; Ziegler et al., 2013), as well as nonprotective functions as illustrated in the current study. Thus, it remains possible that the protective effects we observed after TSLP neutralization during C. rodentium infection may be distinct from what would occur in DSS colitis where blocking TSLP–TSLPR interactions has been shown to exacerbate disease (Taylor et al., 2009; Reardon et al., 2011). Furthermore, it is unknown how and why TSLP expression by IECs is dysregulated in the absence of IKKα signaling. Given that TSLP is an IKKβ-dependent gene product (Lee and Ziegler, 2007; Zaph et al., 2007) and that the absence of
IKKα and IKKβ can induce compensatory increases in canonical and noncanonical NFκB activation, respectively (Lawrence et al., 2005; Lam et al., 2008), it is possible that the elevated TSLP production in IKKαΔIEC mice is a result of increased IKK-dependent canonical NFκB activation. Indeed, we did observe increased expression of activated phospho-IKKβ/IKKα (Ser176/177) in colonic IECs from IKKαΔIEC mice compared with IKKαΔIEC (unpublished data), although it remains unclear whether this is sufficient to account for increased TSLP production.

Although deletion of IKKα and IKKβ within IECs does not cause spontaneous colitis (Nenci et al., 2007), the present study highlights multiple steady state defects in intestinal immune responses in IKKαΔIEC mice. Absence of IEC-intrinsic IKKα signaling led to increases in basal IFNγ expression in the intestine, with concurrent reductions in IL-22 and AMP expression. It is also possible that IEC-intrinsic NFκB signaling regulates the composition of the intestinal microbiota, which may have substantial impacts on inflammatory responses, but future studies will be required to investigate the interplay between immune responses and the microbiota in IKKαΔIEC mice. Thus, although IEC-specific deletion of IKKα signaling does not cause evident pathology in the steady state, developmental defects in these animals likely contributed to impaired immunity to infection, as well as increased susceptibility to chemically induced colitis. Further studies comparing the relative roles for IEC-intrinsic IKKα and IKKβ signaling in a variety of other infectious or inflammatory experimental models, potentially making use of inducible Cre-lox technology that could minimize the impact of altered immune development in constitutive ΔIEC mice, will contribute to our understanding of epithelial regulation of host-protective immunity and inflammation. In addition, given the multifaceted functions of IKKα in cell biology (Chariot, 2009), it remains possible that the ability of IKKα to regulate ILC3-dependent mucosal immunity may be independent of its ability to regulate noncanonical NFκB activation.

Collectively, these data identify a previously unrecognized pathway in which tissue-resident IECs selectively regulate the function of ILC3s in an IKKα-dependent fashion, thereby simultaneously promoting antibacterial immunity and maintenance of intestinal immune homeostasis. Elevated NFκB activation is associated with IBDs in humans, and NFκB inhibition therapies have been designed to treat multiple intestinal inflammatory diseases (Atreya et al., 2008). However, NFκB activation can have diverse effects within different cell lineages (Greten et al., 2007; Hsu et al., 2011), and humans carrying mutations in NFκB-related factors such as NOD1 or NOD2 develop intestinal inflammation (Strober et al., 2006). The present study and a previous one (Greten et al., 2007), demonstrate that some factors within the NFκB signaling pathway are critical for limiting intestinal inflammation, suggesting that more specific targeted therapies may be required for the design of optimal therapeutics. This, coupled with studies of dysregulated ILC responses in lesions isolated from IBD patients (Geremia et al., 2011; Bernink et al., 2013; Hepworth et al., 2015), indicates that targeted manipulation of the IEC–ILC axis could be beneficial for the treatment of chronic intestinal inflammatory disorders.

MATERIALS AND METHODS

Animals, cell isolations, and treatments. IKKαΔIEC (Pasparakis et al., 2002) mice used in this study were on a mixed genetic background and crossed with C57BL/6 wild-type mice (Pasparakis et al., 2002) to generate littermate controls and IKKαΔIEC and IKKβΔIEC mouse strains as described previously (Greten et al., 2004; Nenci et al., 2007). Mice were bred at the University of Pennsylvania or Weill Cornell Medical College and maintained in a specific pathogen-free environment. Male or female mice between the ages of 6 and 14 wk were used. Only cohoused littermate controls were used in experiments with F/F and ΔIEC mice. In some other experiments, C57BL/6 wild-type mice were used and obtained from the Jackson Laboratory. C57BL/6 B6-Il22−/−, Rag1−/−, and Tdp−/− mice were bred in-house. Experiments were terminated when mice lost a significant proportion of their original weight (>20%); however, mice that succumbed to infection died naturally. All experiments were performed according to guidelines of the Cornell University or University of Pennsylvania Institutional Animal Care and Use Committee–approved protocols. At necropsy, single cell suspensions of mLN or PPs were prepared by passing through 70-μm nylon mesh filters. Splenocytes were isolated by homogenization followed by red blood cell lysis. IECs were isolated by thoroughly washing colon tissue in PBS and incubating for 10 min at 37°C with 5 ml of 5 mM EDTA in PBS solution, shaking. The epithelial layer was then removed and passed through a 70-μm cell strainer. cPLs were isolated as previously described (Zaph et al., 2007). Reconstituted IL-22 (Pfizer), 50 μg/mouse in PBS, was injected i.p. into mice on days 0, 2, 4, 6, 8, and 10 after C. rodentium infection. Mice were injected i.v. by hydrodynamic tail vein injection with 10 μg control or TSLP encoding cDNA plasmid (Siracusa et al., 2011, 2013; Iseki et al., 2012; Kim et al., 2013; Noti et al., 2014). Previous studies have demonstrated that hydrodynamic injections with similar cDNA constructs have resulted in incorporation and gene expression primarily within hepatocytes (Yang et al., 2001; Sebestyén et al., 2006; Suda and Liu, 2007). Mice were treated with neutralizing mAb against mouse TSLP (Agen) by i.p. injection with 0.5 mg Ab 4 h before infection and every 3 d p.i. Control mice received equivalent amounts of rat IgG.

Adaptive transfer of sort-purified ILCs. ILCs (CD3−CD19−CD11c−NK1.1−CD90+CD127−CD25+) were sort purified from the spleen, PP, and mLNs of naive IKKαΔIEC, C57BL/6 WT, or C57BL/6 IKKβΔIEC (Pasparakis et al., 2002) mice used in this study were on a mixed genetic background. Recombinant IL-22 (Pfizer), 50 μg/mouse in PBS, was injected i.p. into mice on days 0, 2, 4, 6, 8, and 10 after C. rodentium infection. Mice were injected i.v. by hydrodynamic tail vein injection with 10 μg control or TSLP encoding cDNA plasmid (Siracusa et al., 2011, 2013; Iseki et al., 2012; Kim et al., 2013; Noti et al., 2014). Previous studies have demonstrated that hydrodynamic injections with similar cDNA constructs have resulted in incorporation and gene expression primarily within hepatocytes (Yang et al., 2001; Sebestyén et al., 2006; Suda and Liu, 2007). Mice were treated with neutralizing mAb against mouse TSLP (Agen) by i.p. injection with 0.5 mg Ab 4 h before infection and every 3 d p.i. Control mice received equivalent amounts of rat IgG.

C. rodentium infection and assessment of CFU. C. rodentium strain DBS180 (provided by B. Vallance, University of British Columbia, Vancouver, British Columbia, Canada) was prepared by selection of a single colony and culturing in LB broth overnight. C. rodentium DBS100 (provided by B. Vallance, University of British Columbia, Vancouver, British Columbia, Canada) was prepared by selection of a single colony from an overnight culture before i.v. transfer into recipient IKKαΔIEC or IKKβΔIEC mice on days 0, 2, 4, and 7 after C. rodentium infection.

C. rodentium histological analyses and histopathological scoring. Distal colon and liver were fixed in 4% PFA and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin (H&E). For histological scoring, colonic tissue sections were blindly graded on a scale of 0–5 for each of the following parameters: (a) epithelial lesions (crypt elongation, hyperplasia, erosion, and ulceration/necrosis), (b) mural inflammation, and (c) edema for an overall maximal total histology score of 15.
Flow cytometry. Single cell suspensions were stained with anti–mouse fluorochrome-conjugated mAbs against CD3ε (145-2C11), CD4 (RM4-5), CD8 (53-7.3), CD11c (N418), CD19 (1D3), CD25 (PC61.5), CD90.2 (30-H12), CD127 (AB7.34), and NK1.1 (PK136). Intracellular RORγt staining was performed using a commercial kit (clone B2D; eBioscience). For intracellular IL-22 staining, cells were stimulated for 4 h with 50 ng/ml PMA and 750 ng/ml ionomycin in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich), stained with cell surface Abs, fixed and permeabilized using a commercial kit (eBioscience), and stained with fluorochrome-labeled anti–IL-22 (IL22-02; Pfizer). Cells were analyzed by flow cytometry using an LSR II (BD), and further analysis was performed using FlowJo software (Tree Star).

Analysis of IKKα and IKKβ expression. Whole cell extracts from IECs of naive mice were analyzed by SDS-PAGE, followed by immunoblotting with anti–IKKβ Ab (2684; Cell Signaling Technology), anti–IKKα Ab (14A231; EMD Millipore), or control tubulin Ab (T5168; Sigma-Aldrich).

RNA isolations and RT-PCR. Colon tissue was homogenized in TRizol using a TissueLyser (QIAGEN), and RNA was isolated by phenol chloroform extraction and isopropil alcohol precipitation. cDNA was generated per standard protocol with SuperScript II reverse transcription (Invitrogen) and used as input for RT-PCR, using commercially available primer assays (QIAGEN), including B22, Rcg, BgI, IL23a, Il1b, Tgb, Il22, Il25, Ldb, Cxcl20, and Tolf. Data were analyzed using the ΔΔCT method whereby β-actin served as the endogenous gene, and samples were normalized to naive controls. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems).

In vitro cell stimulations. Whole splenocytes or sort-purified lineage– CD90.9CD25.5 ILCs from WT or Rag1–/– C57/6 mice were cultured overnight with 0, 0.1, 1, or 10 ng/ml rTSLP (Amgen) and stimulated with complete media only (RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM Hepes, and 5 × 10−5 M 2-ME) or complete media supplemented with 10 ng/ml recombinant murine IL-23 (eBioscience) for 3 h in the presence of 10 µg/ml brefeldin A, and intracellular IL-22 expression in lineage–CD90.9RORγt– cells was assessed as described in the section Flow cytometry.

ELISA. For tissue homogenates, 1 cm of colonic tissue was mechanically homogenized in 0.5 ml PBS using a TissueLyser (QIAGEN). For organ cultures, 1 cm of colonic tissue was opened longitudinally and cultured overnight in complete media. Cell-free supernatants were analyzed for IL-22 using II22-01 (Pfizer) as a capture Ab and biotin–conjugated II22-03 (Pfizer) as a detection Ab. Cell-free supernatants were analyzed for IFNγ, IL-17A, IL-6 (all from eBioscience), and TSLP (R&D Systems) using standard techniques.

DSS-induced colitis and clinical disease scoring. DSS (MP Biomedicals) was added to drinking water at 3% wt/vol. Mice were weighed regularly, and disease severity was scored as follows: (a) weight loss (no change = 0; <5% = 1; 6–10% = 2; 11–20% = 3; >20% = 4), (b) feces (normal = 0; pasty, semiformalin = 2; liquid, sticky, or unable to defecate after 5 min = 4), (c) blood (no blood = 0; visible blood in rectum = 1; visible blood on fur = 2), and (d) general appearance (normal = 0; plorectal = 1; lethargy and plorectal = 2; moribund = 4).

Statistics. Groups of animals were compared using Mann–Whitney U tests, Student’s t tests, or two-way ANOVA where applicable. P-values <0.05 were considered significant.
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Epithelial regulation of ILC3 responses | Giacomin et al.

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