

# Epithelial-intrinsic IKK $\alpha$ 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  $\kappa$ B kinase (IKK) $\alpha$  or IKK $\beta$ , two critical regulators of NF $\kappa$ B activation, we demonstrate that IEC-intrinsic IKK $\alpha$  expression selectively regulates group 3 ILC (ILC3)-dependent antibacterial immunity in the intestine. Although IKK $\beta$ <sup>ΔIEC</sup> mice efficiently controlled *Citrobacter rodentium* infection, IKK $\alpha$ <sup>ΔIEC</sup> mice exhibited severe intestinal inflammation, increased bacterial dissemination to peripheral organs, and increased host mortality. Consistent with weakened innate immunity to *C. rodentium*, IKK $\alpha$ <sup>ΔIEC</sup> mice displayed impaired IL-22 production by ROR $\gamma$ T<sup>+</sup> ILC3s, and therapeutic delivery of rIL-22 or transfer of sort-purified IL-22-competent ILCs from control mice could protect IKK $\alpha$ <sup>ΔIEC</sup> mice from *C. rodentium*-induced morbidity. Defective ILC3 responses in IKK $\alpha$ <sup>ΔIEC</sup> 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 IKK $\alpha$  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 IKK $\alpha$  expression and TSLP in regulating ILC3 responses required to maintain intestinal barrier immunity.**

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Abbreviations used: Ab, antibody; AMP, antimicrobial peptide; cLPL, colonic lamina propria lymphocyte; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IKK, inhibitor of  $\kappa$ B kinase; ILC, innate lymphoid cell; LT, lymphotoxin; mLN, mesenteric LN; p.i., postinfection; PP, Peyer's patch; TSLP, thymic stromal lymphopoietin.

Maintenance of epithelial barrier integrity at mucosal surfaces is essential to limit exposure to commensal and pathogenic microorganisms

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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.,

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2013; McKenzie et al., 2014; Sonnenberg, 2014; Artis and Spits, 2015). ILCs represent a heterogeneous family of cells that, based on their expression of the transcription factors T-bet, GATA3, and ROR $\gamma$ t, can be categorized into three groups with diverse effector functions. Group 1 ILCs (ILC1s) express IFN $\gamma$  and T-bet and include NK cells (Spits and Cupedo, 2012), whereas ILC2s express GATA3, ROR $\alpha$ , MHCII, 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). ROR $\gamma$ t<sup>+</sup> ILC3s express IL-17A, IFN $\gamma$ , MHCII, lymphotoxin (LT) $\alpha$ 1 $\beta$ 2, 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 IFN $\gamma$  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, mucins, 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 ROR $\gamma$ t<sup>+</sup> ILC3s on epithelial barrier function is well characterized, the molecular and cellular pathways that regulate ILC responses in mucosal tissue microenvironments remain poorly understood.

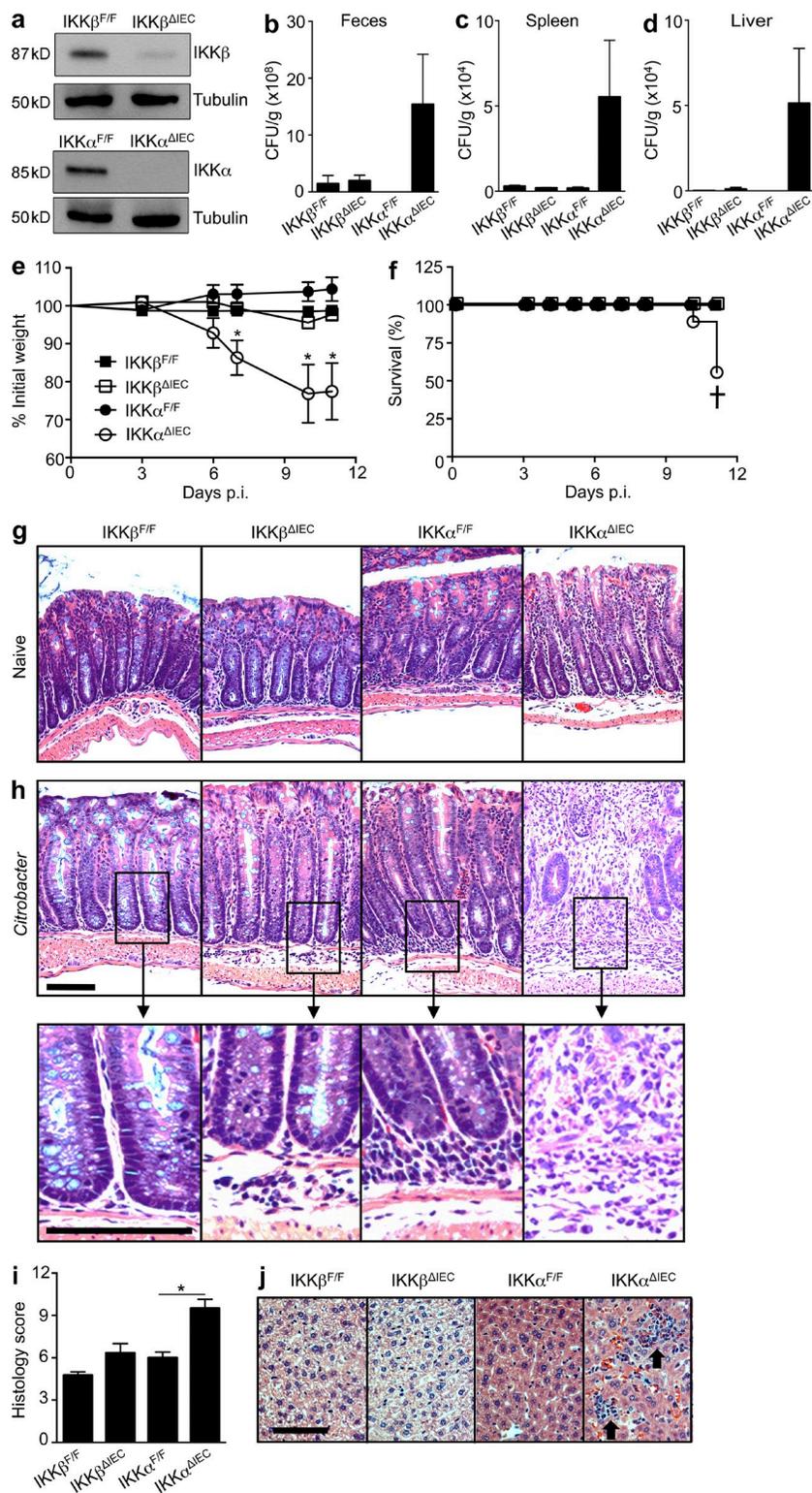
In addition to providing a physical barrier to microorganisms, intestinal epithelial cells (IECs) express cytokines, chemokines, pattern recognition receptors, inflammasomes, and AMPs that permit cross-talk with mucosal immune cells and maintenance of immune homeostasis (Strober, 1998; Pasparakis, 2008; 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 T<sub>H</sub>2-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 NF $\kappa$ B-associated genes, including inhibitor of  $\kappa$ B kinase (IKK) $\beta$  or IKK $\alpha$ , which control “canonical” versus “noncanonical” NF $\kappa$ B-dependent gene expression, respectively (Greten et al., 2004; Nenci et al., 2007; Zaph et al., 2007; Eckmann et al., 2008; Vlantis et al.,

2011; Bonnégarde-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 IKK $\alpha$ , but not IKK $\beta$ , exhibit impaired innate immunity to *C. rodentium* infection, identifying a previously unappreciated role for the noncanonical NF $\kappa$ B activation pathway in antibacterial immunity. Critically, mice with IEC-intrinsic IKK $\alpha$  deletions displayed impaired IL-22 production by ROR $\gamma$ t<sup>+</sup> ILC3s and delivery of recombinant IL-22 or IL-22-competent sort-purified ILCs was sufficient for restoration of protection against *C. rodentium* infection. IEC-intrinsic IKK $\alpha$  was also critical for regulation of intestinal inflammation after chemically induced intestinal damage and colitis. Mechanistically, the absence of IKK $\alpha$  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 IKK $\alpha$ <sup>ΔIEC</sup> mice could partially restore ILC3 responses and innate immunity to *C. rodentium*. Collectively, these data highlight a previously unrecognized mechanism by which IECs and ILC3s reciprocally regulate intestinal immune homeostasis.

## RESULTS

### IEC-intrinsic IKK $\alpha$ , but not IKK $\beta$ , expression is critical for immunity to *C. rodentium* infection

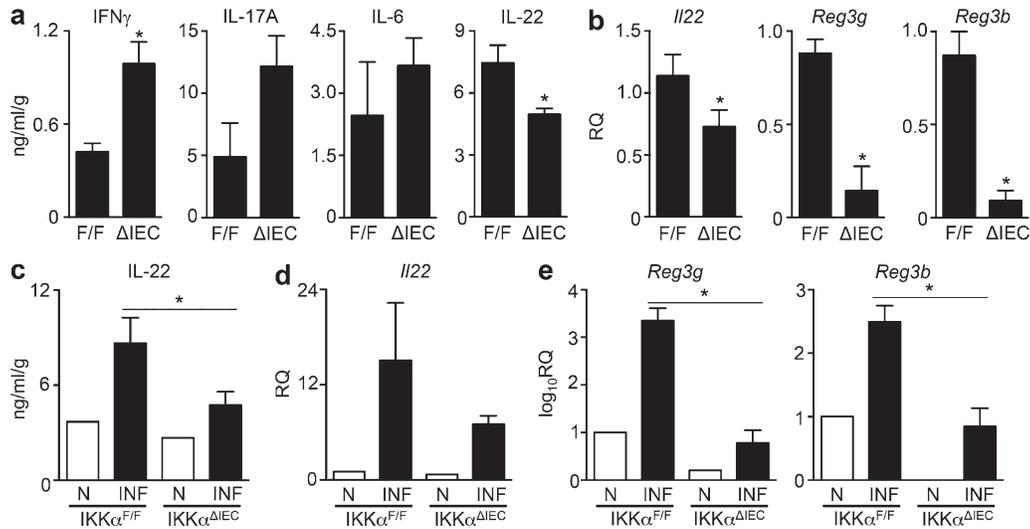
*C. rodentium* is a natural gram-negative extracellular bacterial pathogen of mice akin to the human pathogen enterohemorrhagic *Escherichia coli* that causes NF $\kappa$ B activation and colonic lesions after attachment to the epithelial surface (Mundy et al., 2005; Wang et al., 2006; Chandrakesan et 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 NF $\kappa$ B activation and whether it regulates antibacterial immunity and tissue-protective ILC responses is unknown. Using mice with IEC-specific deletions in either IKK $\beta$  or IKK $\alpha$ , respectively, we assessed whether IEC-intrinsic canonical versus noncanonical NF $\kappa$ B activation regulates intestinal ILC responses. To do so, IKK $\beta$ <sup>F/F</sup> or IKK $\alpha$ <sup>F/F</sup> mice in which either the *Ikk $\beta$*  or *Ikk $\alpha$*  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 IKK $\beta$  (IKK $\beta$ <sup>ΔIEC</sup>) or IKK $\alpha$  (IKK $\alpha$ <sup>ΔIEC</sup>) knockout mice, as described previously (Nenci et al., 2007). Deletion of IKK $\beta$  in IECs from IKK $\beta$ <sup>ΔIEC</sup> mice and IKK $\alpha$  in IECs from IKK $\alpha$ <sup>ΔIEC</sup> 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 IKK $\beta$ <sup>ΔIEC</sup>, IKK $\alpha$ <sup>ΔIEC</sup>, and littermate control mice with



**Figure 1. IEC-intrinsic IKK $\alpha$ , but not IKK $\beta$ , expression is critical for immunity to *C. rodentium* infection.** (a) IKK $\beta$  and IKK $\alpha$  expression in IECs from naive IKK $\beta$ <sup>F/F</sup>, IKK $\alpha$ <sup>F/F</sup>, IKK $\beta$ <sup>ΔIEC</sup>, and IKK $\alpha$ <sup>ΔIEC</sup> mice as detected by Western blot. (b–j) Littermate control IKK $\beta$ <sup>F/F</sup> and IKK $\alpha$ <sup>F/F</sup> mice and mutant IKK $\beta$ <sup>ΔIEC</sup> and IKK $\alpha$ <sup>ΔIEC</sup> mice were infected with *C. rodentium*. (b–d) *C. rodentium* CFU in the feces on day 5 p.i. (b), spleen on day 11 p.i. (c), and liver on day 11 p.i. (d). (e and f) Percentage of initial body weight (e) and percent survival (f) at the indicated time points p.i. (g) H&E staining of colon tissue sections from naive mice. (h) H&E staining of colon tissue sections from *C. rodentium*-infected mice at day 11 p.i., including high-magnification insets (bottom). (i) Pathological score of colon histology. (j) H&E staining of liver tissue sections of day 11 infected mice. Arrows indicate neutrophil-rich inflammatory foci. All bars, 50  $\mu$ m. Data for a are representative of two independent experiments using pooled IECs from three mice. Data for b–j are representative of three to four independent experiments (IKK $\beta$ <sup>F/F</sup>, total  $n = 13$ ; IKK $\alpha$ <sup>F/F</sup>,  $n = 18$ ; IKK $\beta$ <sup>ΔIEC</sup>,  $n = 12$ ; and IKK $\alpha$ <sup>ΔIEC</sup> mice,  $n = 16$ ). Data for f are pooled from three independent experiments. Data are shown as mean  $\pm$  SEM. † indicates infection-induced mortality. \*,  $P < 0.05$  compared with IKK $\alpha$ <sup>F/F</sup>.

*C. rodentium*. Although IKK $\beta$ <sup>ΔIEC</sup> mice exhibited equivalent fecal *C. rodentium* burdens to IKK $\beta$ <sup>F/F</sup> mice at day 5 postinfection (p.i.), IKK $\alpha$ <sup>ΔIEC</sup> mice displayed higher fecal bacterial titers (Fig. 1 b) and enhanced bacterial dissemination to peripheral organs, including the spleen and liver at day 11 p.i. compared

with IKK $\alpha$ <sup>F/F</sup> controls (Fig. 1, c and d). Associated with an impaired ability to control *C. rodentium* infection, IKK $\alpha$ <sup>ΔIEC</sup>, but not IKK $\beta$ <sup>ΔIEC</sup>, mice displayed exacerbated infection-induced weight loss (Fig. 1 e), and ~50% of IKK $\alpha$ <sup>ΔIEC</sup> mice succumbed to infection by day 11 p.i. (Fig. 1 f). IKK $\alpha$ <sup>ΔIEC</sup> mice that



**Figure 2. Colonic IL-22 and AMP expression is reduced in IKKα<sup>ΔIEC</sup> 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 IKKα<sup>F/F</sup> and IKKα<sup>ΔIEC</sup> 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) IKKα<sup>F/F</sup> or IKKα<sup>ΔIEC</sup> 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 IKKα<sup>F/F</sup> mice. Data for a and b are representative of two independent experiments (IKKα<sup>F/F</sup>, total *n* = 9; IKKα<sup>ΔIEC</sup>, *n* = 8). Data for c–e are representative of three independent experiments (IKKα<sup>F/F</sup>, total *n* = 15; IKKα<sup>ΔIEC</sup>, *n* = 14 + 1 naive mouse of each genotype per experiment). Data are expressed as mean ± SEM. \*, *P* < 0.05 compared with IKKα<sup>F/F</sup> control.

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 (Nenci et al., 2007). However, although *C. rodentium*-infected IKKβ<sup>F/F</sup>, IKKβ<sup>ΔIEC</sup>, and IKKα<sup>F/F</sup> mice exhibited modest intestinal inflammation at day 11 p.i. (Fig. 1 h), infected IKKα<sup>ΔIEC</sup> 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 IKKα<sup>ΔIEC</sup> 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.

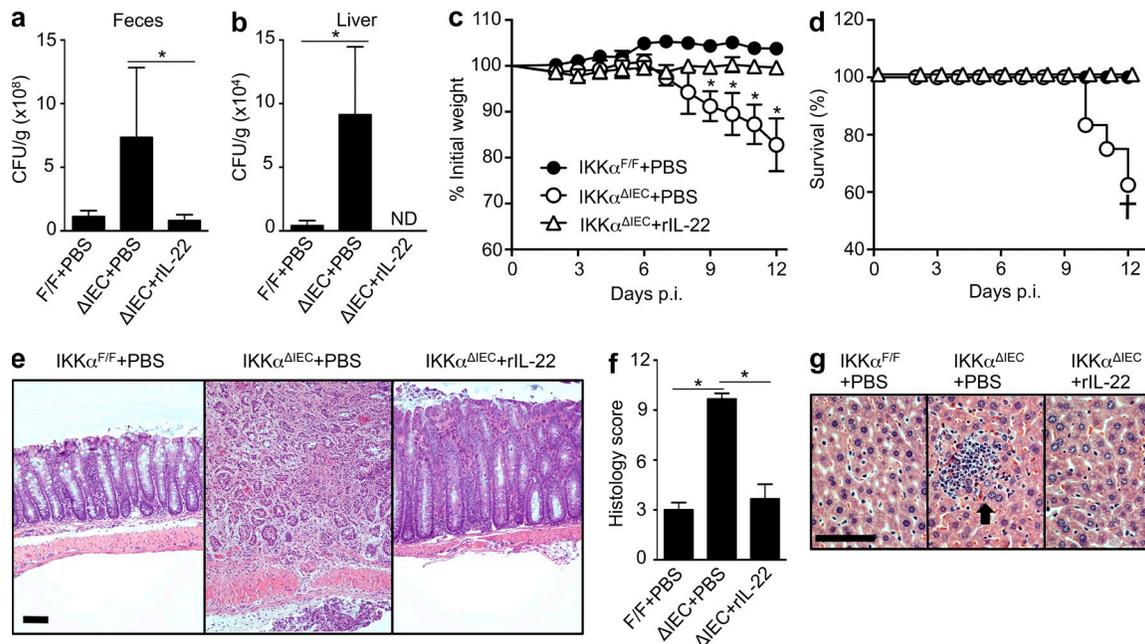
#### Colonic IL-22 and AMP expression are reduced in IKKα<sup>ΔIEC</sup> mice in the steady state and after *C. rodentium* infection

To investigate the mechanisms for dysregulated *C. rodentium*-induced intestinal inflammation in IKKα<sup>ΔIEC</sup> mice, we first examined the expression levels of key cytokines involved in immunity to *C. rodentium*. Compared with littermate control IKKα<sup>F/F</sup> mice, protein levels of the proinflammatory cytokine IFNγ were significantly elevated in the colons of naive IKKα<sup>ΔIEC</sup> 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 IKKα<sup>ΔIEC</sup> 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 IKKα<sup>F/F</sup> control and mutant IKKα<sup>ΔIEC</sup> 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; Manta et al., 2013). Importantly, infection-induced IL-22 protein (Fig. 2 c) and mRNA (Fig. 2 d) expression was reduced in IKKα<sup>ΔIEC</sup> mice, with concurrent significant reductions in *Reg3g* and *Reg3b* expression (Fig. 2 e). 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 IKKα<sup>ΔIEC</sup> 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 IKKα<sup>ΔIEC</sup> mice (Fig. 1). Epithelial cells possess an



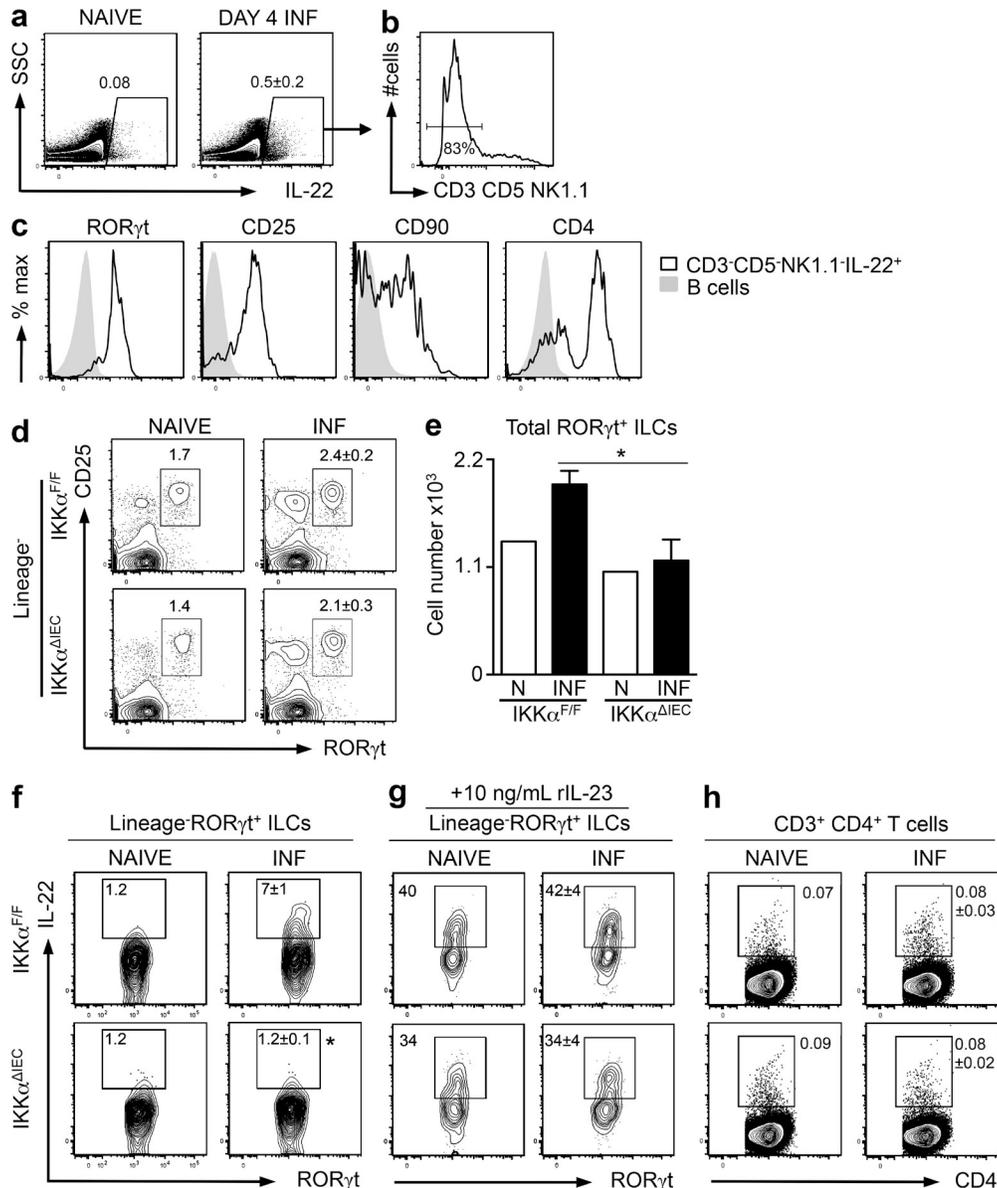
**Figure 3. Therapeutic delivery of recombinant IL-22 is sufficient to rescue IKK $\alpha^{\Delta IE C}$  mice from *C. rodentium*-induced morbidity.** IKK $\alpha^{\Delta IE C}$  mice were infected with *C. rodentium* and injected i.p. with either PBS or 50  $\mu$ g rIL-22 every 2 d (littermate control IKK $\alpha^{F/F}$  mice received PBS only). (a and b) *C. rodentium* CFU in the feces on day 5 p.i. (a) and liver on day 12 p.i. (b). (c and d) Percentage of initial body weight (c) and percent survival (d) at the indicated time points p.i. Data are pooled from three identical experiments. (e) H&E staining of colon tissue sections of *C. rodentium*-infected mice at day 12 p.i. (f) Pathological score of colon histology. (g) H&E staining in liver tissue sections of infected mice at day 12 p.i. Arrows indicate neutrophil-rich inflammatory foci. All bars, 50  $\mu$ m. Data are representative of three independent experiments (IKK $\alpha^{F/F}$  + PBS total  $n = 14$ ; IKK $\alpha^{\Delta IE C}$  + PBS,  $n = 14$ ; IKK $\alpha^{\Delta IE C}$  + rIL-22,  $n = 12$ ) and expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ . ND, not detected. † indicates infection-induced mortality.

array of mechanisms by which they can control mucosal immunity, and epithelial IKK $\alpha$  expression may have diverse effects on intestinal immune homeostasis beyond regulating IL-22 responses. To determine whether delivery of exogenous IL-22 was sufficient to restore protective immunity in IKK $\alpha^{\Delta IE C}$  mice, IKK $\alpha^{\Delta IE C}$  mice were infected with *C. rodentium* and treated with either PBS or rIL-22 every other day. Although IKK $\alpha^{\Delta IE C}$  mice treated with PBS exhibited elevated bacterial titers in the feces at day 6 p.i. (Fig. 3 a) and enhanced bacterial dissemination to the liver at day 12 p.i. (Fig. 3 b) compared with control IKK $\alpha^{F/F}$  mice, rIL-22-treated IKK $\alpha^{\Delta IE C}$  mice displayed significant reductions in bacterial burdens (Fig. 3, a and b), indicating restoration of host-protective immunity. Consistent with this, although PBS-treated IKK $\alpha^{\Delta IE C}$  mice underwent significant weight loss and infection-induced mortality, therapeutic administration of rIL-22 to IKK $\alpha^{\Delta IE C}$  mice rescued mice from infection-induced weight loss and fatal *C. rodentium* infection (Fig. 3, c and d). Restoration of host-protective immunity correlated with reductions in colonic histopathology at day 12 p.i. (Fig. 3 e), lower colonic pathology score (Fig. 3 f), and reduced neutrophilic infiltrates in the liver at day 12 p.i. in rIL-22-treated IKK $\alpha^{\Delta IE C}$  mice (Fig. 3 g). These data indicate that delivery of exogenous IL-22 is sufficient to restore antibacterial immunity in mice with IEC-specific deletion of IKK $\alpha$ , suggesting that defective IL-22 responses in IKK $\alpha^{\Delta IE C}$

mice are a likely cause of *C. rodentium*-induced morbidity and mortality.

### *C. rodentium* infection-induced ILC-dependent IL-22 responses are diminished in IKK $\alpha^{\Delta IE C}$ mice

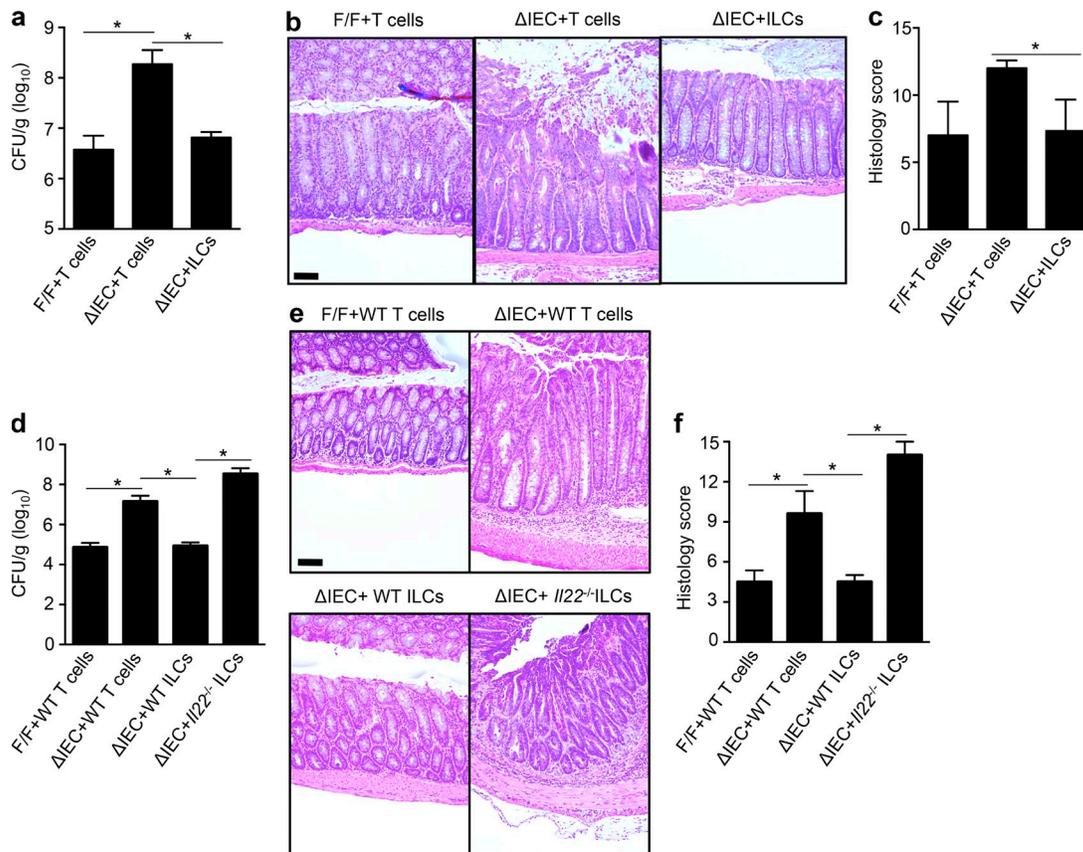
IL-22 can be produced by a variety of immune cells, including T cells, ILCs, neutrophils, and DCs (Zheng et al., 2008; Sonnenberg et al., 2011a; Zindl et al., 2013). However, previous studies have demonstrated that ILC3-dependent IL-22 responses are critical for innate immunity to *C. rodentium* infection (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 IKK $\alpha^{F/F}$  mice (Fig. 4, a and b). Further characterization revealed that IL-22-expressing CD3<sup>-</sup>CD5<sup>-</sup>NK1.1<sup>-</sup> cells expressed ROR $\gamma$ t, CD25, CD90, and CD4, a phenotype consistent with that of ILC3s (Fig. 4 c). Analysis of ROR $\gamma$ t<sup>+</sup> ILC responses (lineage<sup>-</sup>, ROR $\gamma$ t<sup>+</sup>, CD25<sup>+</sup> cells) in the mLNs of naive and day 4 *C. rodentium*-infected IKK $\alpha^{F/F}$  and IKK $\alpha^{\Delta IE C}$  mice revealed that although the frequencies of intestinal ROR $\gamma$ t<sup>+</sup> ILCs were not significantly different (Fig. 4 d), total numbers of ROR $\gamma$ t<sup>+</sup> ILCs were significantly reduced in *C. rodentium*-infected IKK $\alpha^{\Delta IE C}$  mice compared with IKK $\alpha^{F/F}$  control (Fig. 4 e). Strikingly, when ROR $\gamma$ t<sup>+</sup> ILCs were assessed for



**Figure 4.** *C. rodentium* infection–induced ILC-dependent IL-22 responses are diminished in  $IKK\alpha^{\Delta IE C}$  mice.  $IKK\alpha^{F/F}$  mice were infected (INF) with *C. rodentium* and sacrificed at day 4 p.i. (a) Representative plots displaying frequencies of IL-22<sup>+</sup> cells in the mLNs. (b) Expression of T cell (CD3 and CD5) and NK cell (NK1.1) markers among IL-22<sup>+</sup> cells. (c) Expression of ILC surface markers in gated CD3<sup>−</sup>CD5<sup>−</sup>NK1.1<sup>−</sup>IL-22<sup>+</sup> cells (black, open histograms) compared with CD19<sup>+</sup> B cells (gray, closed histograms). (d) Representative plots displaying frequencies of CD3<sup>−</sup>CD5<sup>−</sup>CD19<sup>−</sup>CD11c<sup>−</sup>NK1.1<sup>−</sup>, RORγt<sup>+</sup>CD25<sup>+</sup> ILCs in the mLNs of naive and *C. rodentium*–infected littermate control  $IKK\alpha^{F/F}$  and  $IKK\alpha^{\Delta IE C}$  mice. (e) Total mLN ILCs. (f) Frequencies of IL-22–expressing RORγt<sup>+</sup> ILCs in the mLN after ex vivo PMA and ionomycin stimulation. (g) Frequencies of IL-22–expressing RORγt<sup>+</sup> ILCs in the mLNs after stimulation with rIL-23, PMA, and ionomycin. (h) Frequencies of CD3<sup>+</sup>CD4<sup>+</sup> T cells in the mLNs expressing IL-22 after ex vivo PMA and ionomycin stimulation. Data for a–f and h are representative of five experiments ( $IKK\alpha^{F/F}$ , total  $n = 21$ ;  $IKK\alpha^{\Delta IE C}$ ,  $n = 17 + 1$  naive mouse of each genotype per experiment), and data for g are representative of three experiments ( $IKK\alpha^{F/F}$ , total  $n = 13$ ;  $IKK\alpha^{\Delta IE C}$ ,  $n = 11 + 1$  naive mouse of each genotype per experiment). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$  compared with  $IKK\alpha^{F/F}$  INF.

their functional capacity to produce IL-22 after PMA and ionomycin stimulation, we observed a significant reduction in infection–induced IL-22 responses in RORγt<sup>+</sup> ILCs from  $IKK\alpha^{\Delta IE C}$  mice compared with  $IKK\alpha^{F/F}$  littermate controls (Fig. 4 f). Stimulation of cells with rIL-23 ex vivo revealed that RORγt<sup>+</sup> ILCs from  $IKK\alpha^{\Delta IE C}$  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  $IKK\alpha^{\Delta IE C}$  cells than cells from  $IKK\alpha^{F/F}$  mice (Fig. 4 g), these differences did not reach statistical significance. Importantly, frequencies of CD4<sup>+</sup> T cells expressing IL-22 were not significantly altered in



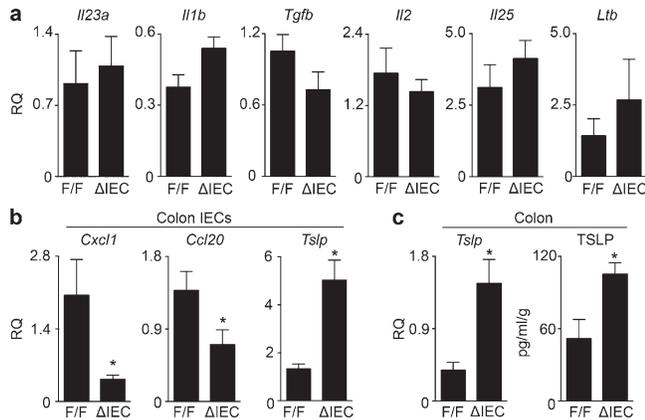
**Figure 5. Delivery of sort-purified IL-22-competent ILCs restores immunity to *C. rodentium* infection in  $IKK\alpha^{\Delta IE C}$  mice.** (a–c)  $IKK\alpha^{\Delta IE C}$  mice were infected with *C. rodentium* and injected i.v. with either  $10^4$  sort-purified  $CD90^+CD3^+$  T cells or  $10^4$  sort-purified lineage $^-CD90^+CD25^+CD127^+$  ILCs from  $IKK\alpha^{F/F}$  mice on days 0, 2, 4, and 7 p.i. (littermate control  $IKK\alpha^{F/F}$  mice received T cells only). T cells and ILCs were pulsed with rIL-23, rIL-1 $\beta$ , 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  $IKK\alpha^{F/F}$  and  $IKK\alpha^{\Delta IE C}$  mice were infected with *C. rodentium* and injected i.v. with either  $10^4$  sort-purified T cells or  $10^4$  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  $\mu$ m. (f) Pathological score of colon histology. Data for a–c are representative of two independent experiments ( $IKK\alpha^{F/F}$  + T cells,  $n = 8$ ;  $IKK\alpha^{\Delta IE C}$  + T cells,  $n = 8$ ;  $IKK\alpha^{\Delta IE C}$  + ILCs,  $n = 6$ ). Data for d–f are representative of two experiments ( $IKK\alpha^{F/F}$  + WT T cells,  $n = 7$ ;  $IKK\alpha^{\Delta IE C}$  + WT T cells,  $n = 7$ ;  $IKK\alpha^{\Delta IE C}$  + WT ILCs,  $n = 6$ ;  $IKK\alpha^{\Delta IE C}$  + *IL-22* $^{-/-}$  ILCs,  $n = 6$ ). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ .

$IKK\alpha^{\Delta IE C}$  mice (Fig. 4 h), indicating that  $IKK\alpha$ -dependent regulation of IL-22 expression may be selective for ILCs. Collectively, these data indicate that IEC-intrinsic  $IKK\alpha$  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 $IKK\alpha^{\Delta IE C}$ mice

Although  $ROR\gamma^t$  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  $IKK\alpha^{\Delta IE C}$  mice. To do so, ILCs (lineage $^-CD90^+CD25^+CD127^+$ ) or T cells ( $CD3^+CD90^+$ ) were sort purified from naive littermate control  $IKK\alpha^{F/F}$  mice and pulsed for 1 h ex vivo with rIL-23, rIL-1 $\beta$ , rIL-2, and rIL-7 to promote survival

and cytokine production before adoptive transfer into recipient  $IKK\alpha^{F/F}$  or  $IKK\alpha^{\Delta IE C}$  mice. Although  $IKK\alpha^{\Delta IE C}$  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  $IKK\alpha^{F/F}$  control mice (Fig. 5, b and c),  $IKK\alpha^{\Delta IE C}$  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  $IKK\alpha^{F/F}$  or  $IKK\alpha^{\Delta IE C}$  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



**Figure 6. IKK $\alpha^{\Delta IEC}$  mice exhibit dysregulated TSLP expression by colonic IECs.** (a–c) Littermate IKK $\alpha^{F/F}$  and IKK $\alpha^{\Delta IEC}$  mice were infected with *C. rodentium* and sacrificed at day 4 p.i. (a) Relative mRNA expression of *Il23a*, *Il1b*, *Tgfb*, *Il2*, *Il25*, and *Ltb* in colon tissue homogenates. (b) Relative *Cxcl1*, *Ccl20*, and *Tslp* mRNA expression levels in colonic IECs. (c) *Tslp* mRNA and TSLP protein levels in colonic tissue homogenates. Gene expression data were normalized to naive IKK $\alpha^{F/F}$  mice. Data for a and c are representative of three independent experiments (IKK $\alpha^{F/F}$ , total  $n = 15$ ; IKK $\alpha^{\Delta IEC}$ ,  $n = 14$ ). Data for b is representative of two experiments (IKK $\alpha^{F/F}$ , total  $n = 10$ ; IKK $\alpha^{\Delta IEC}$ ,  $n = 8$ ). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ .

*C. rodentium* infection in IKK $\alpha^{\Delta IEC}$  mice, transfer of IL-22-competent ILCs reduced fecal bacterial titers (Fig. 5 d) and intestinal pathology (Fig. 5, e and f) in IKK $\alpha^{\Delta IEC}$  mice. Critically, *Il22*<sup>-/-</sup> 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 IKK $\alpha^{\Delta IEC}$  mice, providing further evidence that defective ILC3–IL-22 responses are a likely cause of *C. rodentium*-induced immunopathology in IKK $\alpha^{\Delta IEC}$  mice.

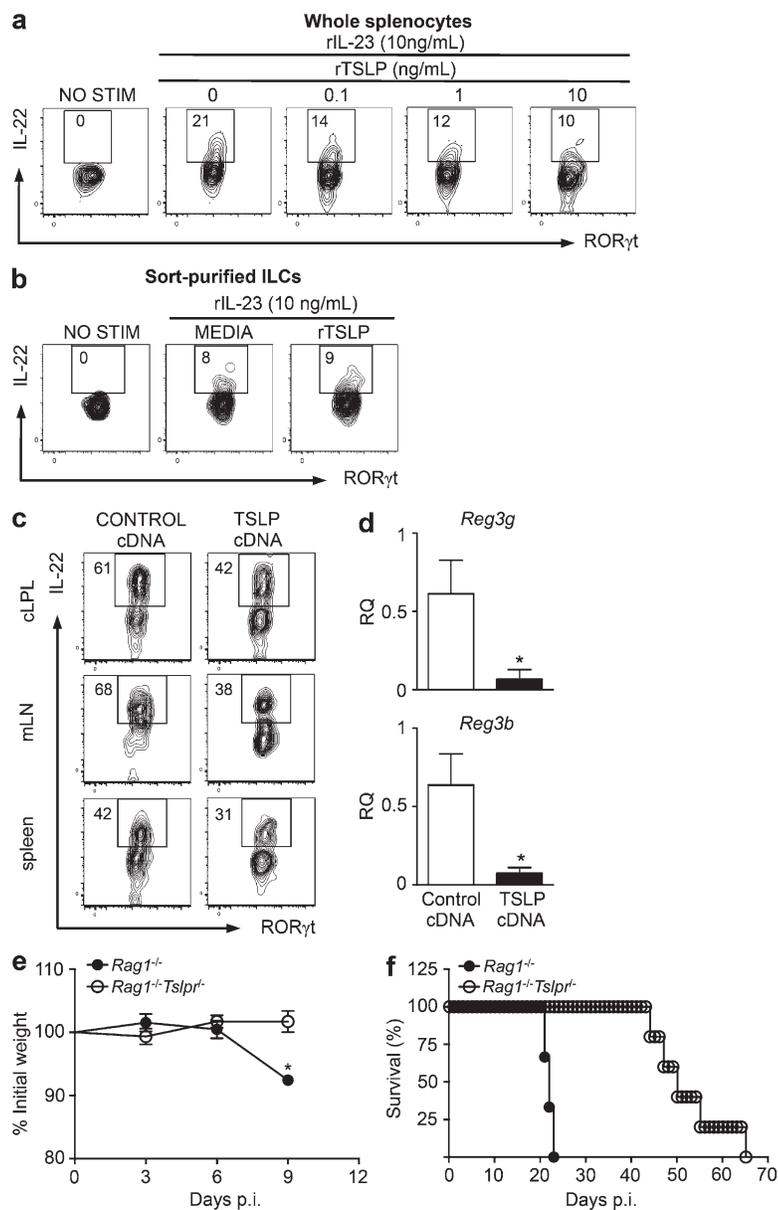
#### IKK $\alpha^{\Delta IEC}$ mice exhibit dysregulated TSLP expression by colonic IECs

We next assessed the mechanisms by which IKK $\alpha$  expression within IECs regulates IL-22 production by ILC3. Production of IL-22 by intestinal ILCs is promoted by IL-23, IL-1 $\beta$ , IL-2, LT $\alpha$ 1 $\beta$ 2, 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 a negative regulator of ILC responses in the intestine (Sawa et al., 2011). Analysis of cytokine expression in whole colonic tissue isolated from day 4 *C. rodentium*-infected IKK $\alpha^{\Delta IEC}$  versus littermate control IKK $\alpha^{F/F}$  mice revealed no significant differences in expression of *Il23a*, *Il1b*, *Tgfb*, *Il2*, *Il25*, or *Ltb* mRNA (Fig. 6 a). Colonic IECs were isolated, and as expected, gene expression of the LT $\beta$ R-dependent chemokines *Cxcl1* and *Ccl20* were significantly diminished in IECs from IKK $\alpha^{\Delta IEC}$  mice (Fig. 6 b), given that LT $\beta$ R signals through the non-canonical NF $\kappa$ B pathway (Matsushima et al., 2001; Dejardin

et al., 2002). Notably, analysis of other IEC-intrinsic factors that may regulate ILC function revealed elevated expression of TSLP mRNA (Fig. 6 b) that correlated with significantly increased *Tslp* mRNA and TSLP protein levels in whole colonic tissue homogenates (Fig. 6 c).

#### TSLP acts as a negative regulator of ILC-derived IL-22 production

Although TSLP has been shown to regulate the function of ILC2s (Mjösberg et al., 2012; Kim et al., 2013), a role for TSLP in influencing other ILC subsets has not been described. To assess whether TSLP regulates ILC3 function, we cultured splenocytes from WT C57BL/6 mice overnight with various concentrations of rTSLP, followed by 3-h stimulation with rIL-23, and examined IL-22 expression. Critically, although rIL-23 induced potent IL-22 expression in ROR $\gamma$ <sup>+</sup> ILCs in the absence of TSLP, addition of rTSLP to splenocyte cultures limited IL-22 expression by ILCs in a dose-dependent manner (Fig. 7 a), suggesting that TSLP is a negative regulator of IL-22 production by ILCs. To assess whether TSLP acts directly on ILCs to regulate IL-22 production, we sort purified splenic lineage<sup>-</sup>CD90<sup>+</sup>CD127<sup>+</sup> cells from C57BL/6 *Rag1*<sup>-/-</sup> mice and cultured cells overnight with various concentrations of rTSLP, followed by 3-h stimulation with rIL-23, 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 ROR $\gamma$ <sup>+</sup> 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 succumbed to *C. rodentium* infection by days 20–22 p.i. (Fig. 7 f), C57BL/6 *Rag1*<sup>-/-</sup> mice deficient in TSLP responsiveness (*Rag1*<sup>-/-</sup>*Tslpr*<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.



**Figure 7. TSLP acts as a negative regulator of ILC-derived IL-22 production.** (a) C57BL/6 WT mouse splenocytes were cultured overnight in the presence of increasing doses of rTSLP and stimulated for 3 h with rIL-23 or were left unstimulated (NO STIM), and intracellular IL-22 expression was assessed in lineage<sup>-</sup>ROR $\gamma$ <sup>+</sup>CD90<sup>+</sup> ILC populations. (b) ILCs were sort purified from C57BL/6 *Rag1*<sup>-/-</sup> mice and cultured overnight in the presence of rTSLP, followed by 3-h stimulation with rIL-23 or medium (NO STIM), and intracellular IL-22 expression was assessed. (c and d) WT C57BL/6 mice were injected with either a control cDNA plasmid or a TSLP-overexpressing cDNA plasmid for 9 d, and (c) IL-22 production by ROR $\gamma$ <sup>+</sup> ILCs in the cPLPs, mLNs, and spleen was assessed after 3-h stimulation with rIL-23 in the presence of brefeldin A. (d) *Reg3g* and *Reg3b* mRNA expression in colonic tissue homogenates. (e and f) C57BL/6 *Rag1*<sup>-/-</sup> or double mutant *Rag1*<sup>-/-</sup>*Tslpr*<sup>-/-</sup> mice were infected with *C. rodentium*. Percentage of initial body weight (e) and percent survival (f) at the indicated time points p.i. Data for a and b are representative of two independent experiments (total *n* = 6/treatment), data for c and d are representative of two experiments (total *n* = 6/group), and data for e and f are representative of two experiments (*Rag1*<sup>-/-</sup>, *n* = 10; *Rag1*<sup>-/-</sup>*Tslpr*<sup>-/-</sup>, *n* = 8). Data are expressed as mean  $\pm$  SEM. \*, *P* < 0.05.

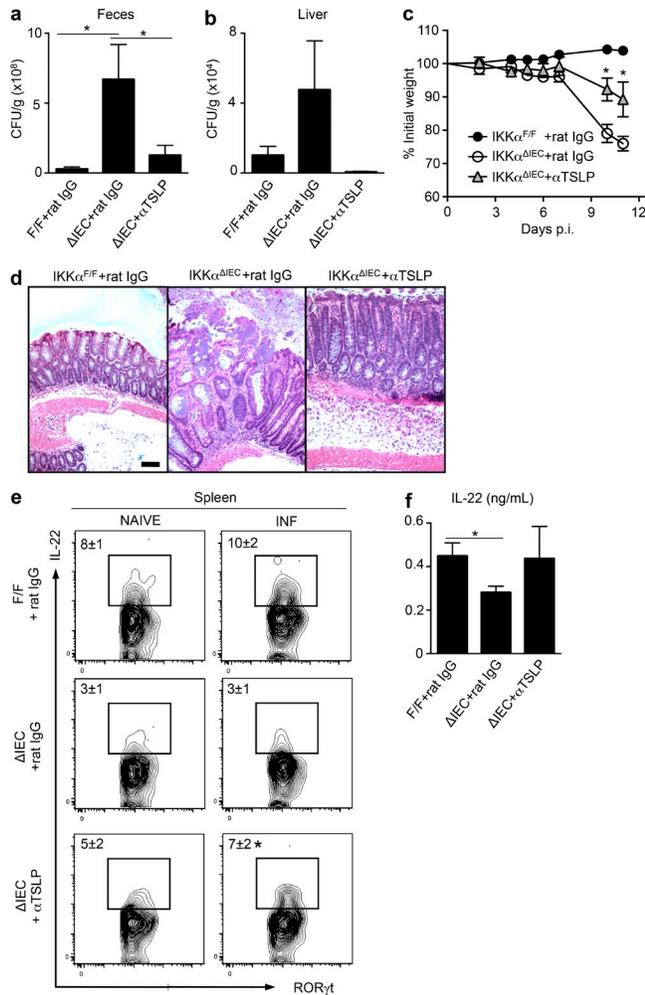
### Neutralization of TSLP partially restores immunity to *C. rodentium* infection in IKK $\alpha$ <sup>ΔIEC</sup> mice

To directly assess whether overexpression of TSLP in IKK $\alpha$ <sup>ΔIEC</sup> mice is responsible for impaired immunity to *C. rodentium* infection, we treated IKK $\alpha$ <sup>ΔIEC</sup> mice every 3 d p.i. with either a neutralizing anti-TSLP mAb or a control rat IgG. Littermate control IKK $\alpha$ <sup>F/F</sup> mice received rat IgG only. Critically, although IKK $\alpha$ <sup>ΔIEC</sup> 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 IKK $\alpha$ <sup>F/F</sup> mice, IKK $\alpha$ <sup>ΔIEC</sup> mice treated with anti-TSLP mAb exhibited substantially reduced bacterial titers (Fig. 8, a and b). TSLP neutralization also partially protected IKK $\alpha$ <sup>ΔIEC</sup> mice from infection-induced weight loss (Fig. 8 c) and diminished colonic pathology (Fig. 8 d). Importantly, antibody (Ab)-mediated

neutralization of TSLP in IKK $\alpha$ <sup>ΔIEC</sup> 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 IKK $\alpha$ <sup>F/F</sup> mice (Fig. 8 f). These data demonstrate that dysregulated TSLP expression in the absence of IEC-intrinsic IKK $\alpha$  expression is a potential mechanism by which ILC-dependent innate immunity to *C. rodentium* is impaired in IKK $\alpha$ <sup>ΔIEC</sup> mice.

### IEC-intrinsic IKK $\alpha$ expression regulates inflammation during chemically induced intestinal damage

To assess whether IKK $\alpha$  expression within IECs is protective in other models of intestinal inflammation, we examined the susceptibility of IKK $\alpha$ <sup>F/F</sup> mice and IKK $\alpha$ <sup>ΔIEC</sup> mice to dextran sodium sulfate (DSS)-induced colitis. Inclusion of 3% DSS in the drinking water of control IKK $\alpha$ <sup>F/F</sup> mice caused only



**Figure 8. Neutralization of TSLP partially restores immunity to *C. rodentium* infection in  $IKK\alpha^{\Delta IE C}$  mice.**  $IKK\alpha^{\Delta IE C}$  mice were infected with *C. rodentium* and injected i.p. with either 0.5 mg rat IgG control or anti-TSLP mAb every 3 d (littermate control  $IKK\alpha^{F/F}$  mice received rat IgG only). (a and c) *C. rodentium* CFU in the feces on day 6 p.i. (a) and liver on day 11 p.i. (b). (c) Percentage of initial body weight. (d) H&E staining of colon tissue sections of day 11 *C. rodentium*-infected mice. Bar, 50  $\mu$ m. (e) IL-22 expression by splenic ILC3s at day 4 p.i., after ex vivo PMA and ionomycin stimulation. (f) IL-22 protein expression within colon homogenate tissue from day 4 infected mice. Data for a–d are representative of three experiments ( $IKK\alpha^{F/F}$  + rat IgG,  $n = 13$ ;  $IKK\alpha^{\Delta IE C}$  + rat IgG,  $n = 10$ ;  $IKK\alpha^{\Delta IE C}$  + anti-TSLP,  $n = 9$ ), and data for e and f are representative of two experiments ( $IKK\alpha^{F/F}$  + rat IgG,  $n = 8$ ;  $IKK\alpha^{\Delta IE C}$  + rat IgG,  $n = 6$ ;  $IKK\alpha^{\Delta IE C}$  + anti-TSLP,  $n = 6$ ). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$  compared with  $IKK\alpha^{\Delta IE C}$  + rat IgG.

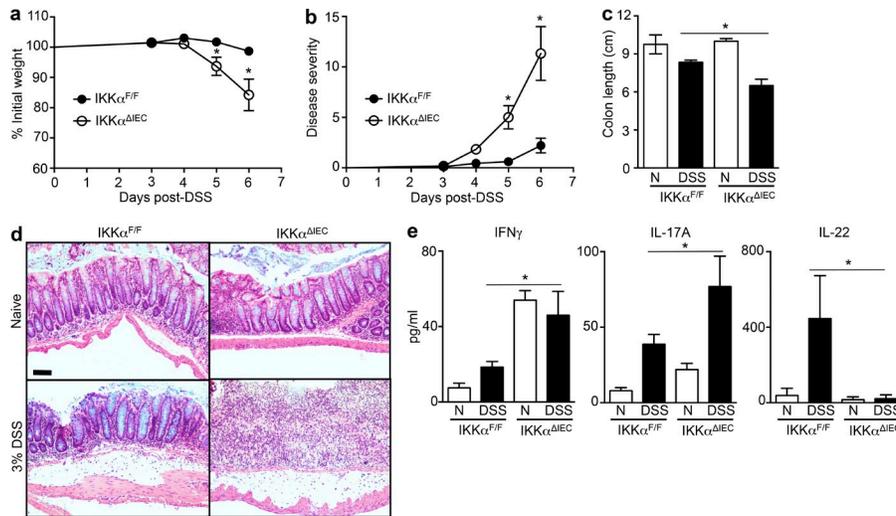
modest weight loss, increased disease severity score, and colon shortening (Fig. 9, a–c) compared with mice that received normal drinking water (naive). However,  $IKK\alpha^{\Delta IE C}$  mice experienced rapid weight loss from day 5 after DSS feeding (Fig. 9 a) and had to be sacrificed by day 6 because of escalating disease severity (Fig. 9 b).  $IKK\alpha^{\Delta IE C}$  mice displayed exacerbated colonic shortening (Fig. 9 c) and markedly increased colonic immunopathology compared with  $IKK\alpha^{F/F}$  littermate

controls (Fig. 9 d). In line with increased pathology in  $IKK\alpha^{\Delta IE C}$  DSS-treated mice, levels of the proinflammatory cytokines IFN $\gamma$  and IL-17A were elevated in  $IKK\alpha^{\Delta IE C}$  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  $IKK\alpha^{\Delta IE C}$  mice compared with littermate  $IKK\alpha^{F/F}$  controls (Fig. 9 e). In conclusion, IEC-intrinsic  $IKK\alpha$  expression can limit inflammation in experimental models of infection-induced colitis and chemical-induced intestinal inflammation.

## DISCUSSION

ROR $\gamma$ <sup>+</sup> 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  $IKK\alpha$  expression. Ablation of  $IKK\alpha$ , but not  $IKK\beta$ , 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  $IKK\alpha$  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 NF $\kappa$ B signaling pathway is fundamental for regulation of IEC function, and although absence of individual  $IKK\alpha$  and  $IKK\beta$  subunits does not result in spontaneous inflammation (Nenci et al., 2007), IEC-intrinsic  $IKK\beta$  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  $IKK\beta$  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  $IKK\alpha$ -mediated signaling pathways in regulating *C. rodentium*-induced colitis and demonstrate that  $IKK\beta$  is dispensable, representing the first demonstration of a differential requirement for  $IKK\beta$ - versus  $IKK\alpha$ -dependent gene expression on IEC function.



**Figure 9. IEC-intrinsic IKK $\alpha$  expression regulates inflammation and pathology during chemically induced colitis.** Litter-mate IKK $\alpha^{F/F}$  and IKK $\alpha^{\Delta IEC}$  mice were treated with either 3% DSS in the drinking water or maintained on normal drinking water (naive, N). (a) Percentage of original body weight. (b) Gross disease score. (c) Colon length at day 6 after DSS. (d) H&E staining of colon tissue sections of naive and DSS-treated mice at day 6 after DSS. Bar, 50  $\mu$ m. (e) IFN $\gamma$ , IL-17A, and IL-22 protein expression in overnight colonic organ culture supernatants at day 4 after DSS. Data for a–d are representative of three independent experiments (IKK $\alpha^{F/F}$ ,  $n = 17$ ; IKK $\alpha^{\Delta IEC}$ ,  $n = 14 + 2$  naive of each genotype per experiment). Data for e are representative of two independent experiments (IKK $\alpha^{F/F}$ ,  $n = 8$ ; IKK $\alpha^{\Delta IEC}$ ,  $n = 8 + 2$  naive of each genotype per experiment). Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$  compared with IKK $\alpha^{F/F}$  + DSS.

Furthermore, our findings are consistent with a previous study demonstrating that hyperactivation of the noncanonical NF $\kappa$ B pathway is associated with elevated immunity to *C. rodentium* (Hu et al., 2013), suggesting a pivotal role for IKK $\alpha$ -dependent signaling in immunity to bacterial infection.

IKK $\alpha$ -dependent noncanonical NF $\kappa$ B activation is associated with LT $\beta$ R signaling (Schneider et al., 2004), and LT $\beta$ R 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 LT $\beta$ R are highly susceptible to *C. rodentium* infection (Wang et al., 2010). Together, these data indicate a potential role for impaired LT $\beta$ R signaling contributing to a reduction in host-protective immunity to *C. rodentium* in IKK $\alpha^{\Delta IEC}$  mice. However, LT-mediated regulation of ILCs was primarily mediated through DCs, and the impact of IEC-specific LT $\beta$ R deletion on ILCs and IL-22 was not assessed (Wang et al., 2010). We did observe significant reductions in expression of the LT $\beta$ R-dependent genes *Cxcl1* and *Ccl20* in IECs recovered from IKK $\alpha^{\Delta IEC}$  mice; hence it remains plausible that defective LT $\beta$ R signaling may contribute to impaired IL-22 responses in the absence of IEC-intrinsic IKK $\alpha$  expression. This is consistent with a recent study showing that IEC-intrinsic LT $\beta$ R expression regulates IL-22-dependent intestinal immune homeostasis during chemically induced colitis (Macho-Fernandez et al., 2015). Notably, despite impaired LT $\beta$ R-dependent gene expression in IKK $\alpha^{\Delta IEC}$  mice, we did not observe reductions in the presence of intestinal lymphoid tissues such as Peyer's patches (PPs) or isolated lymphoid follicles in IKK $\alpha^{\Delta IEC}$  mice (unpublished data), suggesting that in this context, other LT $\beta$ -responsive cells may have coordinated lymphoid organogenesis.

IL-22 production by ILC3s can be stimulated by several factors, chiefly IL-23 and IL-1 $\beta$ , but expression of these cytokines was not altered in IKK $\alpha^{\Delta IEC}$  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 IKK $\alpha^{\Delta IEC}$  mice, expression of the predominantly IEC-derived cytokine TSLP was exaggerated in these mice. We demonstrate that exogenous TSLP suppressed IL-22 production by ROR $\gamma$ T<sup>+</sup> ILC3s in vitro and in vivo and that Ab-mediated neutralization of TSLP could partially restore innate immunity to *C. rodentium* infection in IKK $\alpha^{\Delta IEC}$  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 ILC2s (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 IKK $\alpha$  signaling. Given that TSLP is an IKK $\beta$ -dependent gene product (Lee and Ziegler, 2007; Zaph et al., 2007) and that the absence of

IKK $\alpha$  and IKK $\beta$  can induce compensatory increases in canonical and noncanonical NF $\kappa$ B activation, respectively (Lawrence et al., 2005; Lam et al., 2008), it is possible that the elevated TSLP production in IKK $\alpha^{\Delta\text{IEC}}$  mice is a result of increased IKK $\beta$ -dependent canonical NF $\kappa$ B activation. Indeed, we did observe increased expression of activated phospho-IKK $\beta$ /IKK $\alpha$  (Ser176/177) in colonic IECs from IKK $\alpha^{\Delta\text{IEC}}$  mice compared with IKK $\alpha^{\text{F/F}}$  mice (unpublished data), although it remains unclear whether this is sufficient to account for increased TSLP production.

Although deletion of IKK $\alpha$  and IKK $\beta$  within IECs does not cause spontaneous colitis (Nenci et al., 2007), the present study highlights multiple steady state defects in intestinal immune responses in IKK $\alpha^{\Delta\text{IEC}}$  mice. Absence of IEC-intrinsic IKK $\alpha$  signaling led to increases in basal IFN $\gamma$  expression in the intestine, with concurrent reductions in IL-22 and AMP expression. It is also possible that IEC-intrinsic NF $\kappa$ B 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 IKK $\alpha^{\Delta\text{IEC}}$  mice. Thus, although IEC-specific deletion of IKK $\alpha$  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 IKK $\alpha$  and IKK $\beta$  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  $\Delta\text{IEC}$  mice, will contribute to our understanding of epithelial regulation of host-protective immunity and inflammation. In addition, given the multifaceted functions of IKK $\alpha$  in cell biology (Chariot, 2009), it remains possible that the ability of IKK $\alpha$  to regulate ILC3-dependent mucosal immunity may be independent of its ability to regulate noncanonical NF $\kappa$ B activation.

Collectively, these data identify a previously unrecognized pathway in which tissue-resident IECs selectively regulate the function of ILC3s in an IKK $\alpha$ -dependent fashion, thereby simultaneously promoting antibacterial immunity and maintenance of intestinal immune homeostasis. Elevated NF $\kappa$ B activation is associated with IBDs in humans, and NF $\kappa$ B inhibition therapies have been designed to treat multiple intestinal inflammatory diseases (Atreya et al., 2008). However, NF $\kappa$ B activation can have diverse effects within different cell lineages (Greten et al., 2007; Hsu et al., 2011), and humans carrying mutations in NF $\kappa$ B-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 NF $\kappa$ B 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.** IKK $\alpha^{\text{F/F}}$  (Liu et al., 2008) and IKK $\beta^{\text{F/F}}$  (Pasparakis et al., 2002) mice used in this study were on a mixed genetic background and crossed with C57BL/6 *villin*-Cre mice (Pasparakis et al., 2002) to generate littermate controls and IKK $\alpha^{\Delta\text{IEC}}$  and IKK $\beta^{\Delta\text{IEC}}$  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  $\Delta\text{IEC}$  mice. In some other experiments, C57BL/6 wild-type mice were used and obtained from the Jackson Laboratory. C57BL/6 *Il22*<sup>-/-</sup>, *Rag1*<sup>-/-</sup>, and *Tslpr*<sup>-/-</sup> 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 mLNs or PPs were prepared by passing through 70- $\mu$ m 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 a 5 mM EDTA in PBS solution, shaking. The epithelial layer was then removed and passed through a 70- $\mu$ m cell strainer. cLPLs were isolated as previously described (Zaph et al., 2007). Recombinant IL-22 (Pfizer), 50  $\mu$ g/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  $\mu$ g 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 (Amgen) 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.

**Adoptive transfer of sort-purified ILCs.** ILCs (CD3<sup>-</sup>CD19<sup>-</sup>CD11c<sup>-</sup>NK1.1<sup>-</sup>CD90<sup>+</sup>CD127<sup>+</sup>CD25<sup>+</sup>) were sort purified from the spleen, PPs, and mLNs of naive IKK $\alpha^{\text{F/F}}$ , C57BL/6 WT, or C57BL/6 *Il22*<sup>-/-</sup> mice using a FACSAria III sorter (BD). T cells (CD3<sup>+</sup>CD90<sup>+</sup>) were simultaneously sorted as a control cell population. Sorted ILCs and T cells were incubated for 1 h at 37°C with 10 ng/ml rIL-7, 10 ng/ml rIL-2, 10 ng/ml rIL-23, and 10 ng/ml rIL-1 $\beta$  (R&D Systems) to support cell viability and optimal IL-22 production before i.v. transfer into recipient IKK $\alpha^{\Delta\text{IEC}}$  or IKK $\alpha^{\text{F/F}}$  mice on days 0, 2, 4, and 7 after *C. rodentium* infection.

***C. rodentium* infection and assessment of CFU.** *C. rodentium* strain DBS100 (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. Mice were inoculated with  $\sim 10^{10}$  CFU in 200  $\mu$ l by oral gavage. Analysis of CFU from overnight cultures or mechanically homogenized fecal pellets, livers, and spleens was determined via serial dilutions on MacConkey agar.

***C. rodentium* histological analyses and histopathological scoring.** Distal colon and liver were fixed in 4% PFA and embedded in paraffin, and 5- $\mu$ m 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 CD3e (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD11c (N418), CD19 (1D3), CD25 (PC61.5), CD90.2 (30-H12), CD127 (A7R34), and NK1.1 (PK136). Intracellular ROR $\gamma$ t 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  $\mu$ g/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 LSRII (BD), and further analysis was performed using FlowJo software (Tree Star).

**Analysis of IKK $\alpha$  and IKK $\beta$  expression.** Whole cell extracts from IECs of naive mice were analyzed by SDS-PAGE, followed by immunoblotting with anti-IKK $\beta$  Ab (2684; Cell Signaling Technology), anti-IKK $\alpha$  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 isopropanol 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 *Il22*, *Reg3g*, *Reg3b*, *Il23a*, *Il1b*, *Tgfb*, *Il2*, *Il25*, *Ltb*, *Cxcl1*, *Cd20*, and *Tslp*. Data were analyzed using the  $\Delta\Delta$ CT method whereby  $\beta$ -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<sup>-</sup>CD90<sup>+</sup>CD25<sup>+</sup> ILCs from WT or *Rag1*<sup>-/-</sup> C57BL/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  $\mu$ g/ml streptomycin, 2 mg/ml L-glutamine, 25 mM Hepes, and 5  $\times$  10<sup>-5</sup> M 2-ME) or complete media supplemented with 10 ng/ml recombinant murine IL-23 (eBioscience) for 3 h in the presence of 10  $\mu$ g/ml brefeldin A, and intracellular IL-22 expression in lineage<sup>-</sup>CD90<sup>+</sup>ROR $\gamma$ t<sup>+</sup> 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 IL22-01 (Pfizer) as a capture Ab and biotin-conjugated IL22-03 (Pfizer) as a detection Ab. Cell-free supernatants were analyzed for IFN $\gamma$ , 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, semiformed = 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; piloerection = 1; lethargy and piloerection = 2; motionless = 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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