

Constitutive intestinal NF- κ B does not trigger destructive inflammation unless accompanied by MAPK activation

Monica Guma,^{1,2,3} Dariusz Stepienak,⁶ Helena Shaked,^{1,2,3}
 Martina E. Spehlmann,⁴ Steve Shenouda,⁵ Hilde Cheroutre,⁶
 Ildelfonso Vicente-Suarez,⁶ Lars Eckmann,⁴ Martin F. Kagnoff,^{4,5}
 and Michael Karin^{1,2,3,5}

¹Laboratory of Gene Regulation and Signal Transduction, Departments of ²Pharmacology, ³Pathology, and ⁴Medicine, and ⁵Laboratory of Mucosal Immunity and the Wm. K. Warren Medical Research Center for Celiac Disease, School of Medicine, University of California, San Diego, La Jolla, CA 92093

⁶La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

Nuclear factor (NF)- κ B, activated by $\text{I}\kappa\text{B}$ kinase (IKK), is a key regulator of inflammation, innate immunity, and tissue integrity. NF- κ B and one of its main activators and transcriptional targets, tumor necrosis factor (TNF), are up-regulated in many inflammatory diseases that are accompanied by tissue destruction. The etiology of many inflammatory diseases is poorly understood, but often depends on genetic factors and environmental triggers that affect NF- κ B and related pathways. It is unknown, however, whether persistent NF- κ B activation is sufficient for driving symptomatic chronic inflammation and tissue damage. To address this question, we generated IKK β (EE)^{IEC} mice, which express a constitutively active form of IKK β in intestinal epithelial cell (IECs). IKK β (EE)^{IEC} mice exhibit NF- κ B activation in IECs and express copious amounts of inflammatory chemokines, but only small amounts of TNF. Although IKK β (EE)^{IEC} mice exhibit inflammatory cell infiltration in the lamina propria (LP) of their small intestine, they do not manifest tissue damage. Yet, upon challenge with relatively mild immune and microbial stimuli, IKK β (EE)^{IEC} mice succumb to destructive acute inflammation accompanied by enterocyte apoptosis, intestinal barrier disruption, and bacterial translocation. Inflammation is driven by massive TNF production, which requires additional activation of p38 and extracellular-signal-regulated kinase mitogen-activated protein kinases (MAPKs).

CORRESPONDENCE

Michael Karin:
 karinoffice@ucsd.edu

Abbreviations used: ChIP, chromatin immunoprecipitation; DSS, dextran sulfate salt; IEC, intestinal epithelial cell; IEL, intestinal intraepithelial lymphocyte; IKK, activated by $\text{I}\kappa\text{B}$ kinase; LP, lamina propria; LPL, LP lymphocyte; MAPK, mitogen-activated protein kinase; MLN, mesenteric LN; Q-RT-PCR, quantitative real-time PCR; TACE, TNF- α -converting enzyme.

NF- κ B is a key regulator of inflammation and innate immunity (Ghosh and Karin, 2002). It has long been considered the prototypical proinflammatory signaling pathway, as it is responsive to proinflammatory cytokines such as IL-1 and TNF, and is in charge of their synthesis (Ghosh and Karin, 2002). In addition, NF- κ B transcription factors activate expression of genes encoding cytokines, chemokines, adhesion molecules, matrix metalloproteinases, cyclooxygenase 2, inducible nitric oxide synthase, and enzymes and molecules with microbicidal activity (Li and Verma, 2002; Lawrence, 2009; Pasparakis, 2009). Through such genes, NF- κ B plays a key role in coordination of inflammation and immune responses. However, by controlling cell proliferation and survival, NF- κ B also maintains tissue integrity and might play

an antiinflammatory role (Karin and Lin, 2002; Pasparakis, 2009). NF- κ B protects cells from death by inducing expression of antiapoptotic proteins, including Bcl-x_L, FLICE-like inhibitory protein, and members of the inhibitor of apoptosis (IAP) family (Karin and Lin, 2002; Pasparakis, 2009).

NF- κ B activity is of particular importance for maintenance of epithelial barriers (Karin and Lin, 2002; Pasparakis, 2009), but it was also proposed that NF- κ B activation in epithelial cells can lead to production of inflammatory chemokines that recruit immune cells to the

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tissue, thereby initiating an inflammatory amplification cascade (Barnes and Karin, 1997). Indeed, persistent activation of NF- κ B signaling pathways is often associated with chronic inflammatory diseases, such as rheumatoid arthritis (Handel et al., 1995; Tak et al., 2001), inflammatory bowel disease (Neurath et al., 1996; Kaser et al., 2010), psoriasis (Lizzul et al., 2005), and asthma (Edwards et al., 2009). NF- κ B activity and nuclear localization have been consistently detected in biopsies from such patients, and are accompanied by enhanced recruitment of inflammatory cells and production of proinflammatory mediators such as IL-1, IL-6, and TNF (Barnes and Karin, 1997; Tak and Firestein, 2001; Pasparakis, 2009). Patients with elevated NF- κ B also suffer a worse outcome in septic shock/multiple organ failure (Böhrer et al., 1997; Arnalich et al., 2000; Liu and Malik, 2006). The etiology of these inflammatory diseases is unknown, but is likely to depend on a combination of genetic susceptibility and environmental triggers, and single nucleotides polymorphisms in NF- κ B or related-pathways have been strongly implicated (Vereecke et al., 2009; Zhang et al., 2011). It is unclear, however, whether the persistent activation of NF- κ B in epithelial cells is sufficient for driving chronic inflammation and tissue damage, given its aforementioned protective role.

NF- κ B is regulated by the activated by I κ B kinase (IKK) complex consisting of the IKK α and IKK β catalytic subunits and IKK γ /NEMO regulatory subunit (DiDonato et al., 1997; Ghosh and Karin, 2002; Rothwarf and Karin, 1999). To determine whether persistent epithelial cell activation of NF- κ B can drive chronic and destructive inflammation, we generated IKK β (EE)^{IEC} mice that selectively express constitutively activated IKK β (Delhase et al., 1999) in intestinal epithelial cells (IECs). It is well established that NF- κ B can stimulate transcription of genes encoding TNF and other proinflammatory cytokines and chemokines in IECs (Eckmann and Kagnoff, 2005). However, the absence of IKK β or IKK γ /NEMO in IECs can result in tissue destruction caused by the extensive apoptosis, and this tissue damage can also result in increased inflammation (Chen et al., 2003; Egan et al., 2004; Greten et al., 2004; Chae et al., 2006; Nenci et al., 2007; Spehlmann and Eckmann, 2009). Hence, in IECs, NF- κ B can have both pro- and antiinflammatory functions, making it difficult to predict which of these opposing functions would prevail in IKK β (EE)^{IEC} mice.

As expected, IKK β (EE)^{IEC} mice expressed copious amounts of inflammatory chemokines and cytokines, but unexpectedly produced only small amounts of TNF. The lamina propria (LP) of IKK β (EE)^{IEC} mice was infiltrated with immune cells, but this inflammation did not progress over time and the intestinal barrier remained intact. When challenged with pathogenic bacteria, LPS, or TCR agonists, IKK β (EE)^{IEC} mice succumbed to damaging acute inflammation accompanied by IEC apoptosis and bacterial translocation, that was driven by massive TNF production, found to require additional activation of p38 and extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPK) on top of the already active NF- κ B. Our results strongly suggest

that persistent NF- κ B activation in epithelial cells is insufficient for driving symptomatic inflammation and tissue damage, because synthesis of key inflammatory mediators, such as TNF, is subject to intricate transcriptional and posttranscriptional controls.

RESULTS

IKK β (EE)^{IEC} mice exhibit persistent NF- κ B activation and target gene expression, but not tissue damage

To persistently activate NF- κ B in enterocytes, we generated transgenic (Tg) IKK β (EE)^{IEC} mice, which express a constitutively active IKK β variant, IKK β (EE) (Delhase et al., 1999), from the villin control region (Madison et al., 2002). Heterozygous IKK β (EE)^{IEC} mice were obtained at the expected Mendelian ratio and their general condition and survival did not differ from WT littermates. IKK β (EE) was expressed throughout the intestine after a decreasing cephalocaudal gradient that paralleled villin expression (Fig. 1 a). Enterocyte I κ B kinase activity was enhanced in IKK β (EE)^{IEC} mice (Fig. 1 b), resulting in nuclear translocation of p65 (RelA), RelB, and p50 (Fig. 1 c) and increased p65 DNA binding (Fig. 1 d).

To investigate NF- κ B–driven gene expression in IECs, we performed microarray analysis and confirmed representative results by quantitative (Q) real time (RT) PCR. Expression of \sim 150 genes was increased >2.5 -fold in IKK β (EE)^{IEC} enterocytes relative to WT counterparts (Fig. 1 e; Fig. S1 a; and Table S1). Many up-regulated genes are known NF- κ B targets, including *Tnfaip3*, *Birc3*, *Nfkbia*, *Ccl20*, *Ccl2*, *Cxcl10*, *Icam1*, *Tnf*, *RelB*, *Csf1* and *Bcl3*. Chromatin immunoprecipitation (ChIP) demonstrated RelA recruitment to the promoters of several of these genes, including *Tnf* (Fig. 1 f). However, IKK β (EE)^{IEC} enterocytes exhibited a very large increase in the secretion of several chemokines and cytokines, including CCL20, CCL2, and M-CSF, whose concentrations reached several nanograms/milliliter; however, the increase in TNF production was very modest and did not exceed 20–25 pg/ml (Fig. 1 g). This small increase in TNF protein secretion was in marked contrast to the 10–20-fold increase in its mRNA amounts (Fig. 1 e). In situ hybridization confirmed that TNF mRNA accumulated in IECs of IKK β (EE)^{IEC} mice (Fig. S1 b). Consistent with increased chemokine production, histological examination revealed marked infiltration with predominantly mononuclear leukocytes in the LP of the villus regions in jejunum and ileum of IKK β (EE)^{IEC} mice. This was paralleled by modest epithelial hyperplasia and crypt deepening without apparent loss of goblet cells or any epithelial ulcerations (Fig. 1 h). Most IKK β (EE)^{IEC} mice displayed no morphological changes in the colon, although a small number of these mice had modest mucosal infiltration with mononuclear cells in the mid-to-distal colon without epithelial ulceration. Submucosa and muscularis layers were not affected in the small intestine or colon of the Tg mice. Infiltration was apparent within 3 wk after birth, but did not progress over time. Even in mice older than 1 yr, small intestinal villi were intact without transmural infiltration or granulomas, and no spontaneous intestinal adenomas or carcinomas were detected.

$\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice did not show any sign of diarrhea, and stool weight and water content did not differ between WT and Tg mice. There was no breakdown of the epithelial barrier as measured by fluorescein-dextran uptake (Fig. S1 c).

Immunofluorescence studies showed an influx of mainly $\text{F}4/80^+$ and $\text{CD}3^+$ cells into the small intestinal LP of $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice (Fig. 2 a), paralleling increased expression of chemokines and cytokines important for macrophage and T cell recruitment and survival, such as $\text{CCL}20$, $\text{CCL}2$, $\text{CXL}10$, and M-CSF (Fig. 2 b and Fig. 1 e). Small intestinal intraepithelial lymphocytes (IELs) and $\text{CD}3^+$ and $\text{F}4/80^+$ cells in the LP were increased by 2- and 3.5-fold, respectively, in $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice (Fig. 2 c). $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ LP $\text{F}4/80^+$ cells showed a mild increase in expression of several inflammatory cytokines (Fig. S2 a). TNF and IL-6 secretion elicited by stimulation with TLR ligands (CpG and LPS) was

modestly increased relative to WT cells (Fig. S2 b). Yet, expression of several immunoregulatory molecules, including IL-10 and PD-L1, which have been suggested to maintain mucosal tolerance (Denning et al., 2007), did not differ between WT and Tg mice (Fig. S2, b and c). We also detected a modest increase in expression of other known regulators of inflammation, such as IL-12 or IL-23, in small intestinal extracts. This might be related to the increased number of $\text{F}4/80^+$ LP cells in $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice (Fig. S2 d). Small intestinal IELs and LP lymphocytes (LPLs) did not show increased cytokine (e.g., $\text{IFN-}\gamma$) staining (Fig. S2 e), or any major differences in their subpopulation distribution between WT and Tg mice (Table I), including $\text{Foxp}3^+$ regulatory T cells, which have also been suggested to play a role in the maintenance of intestinal tolerance (Izcue et al., 2006). Immune responses to oral antigens and oral tolerance were also not altered in $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice

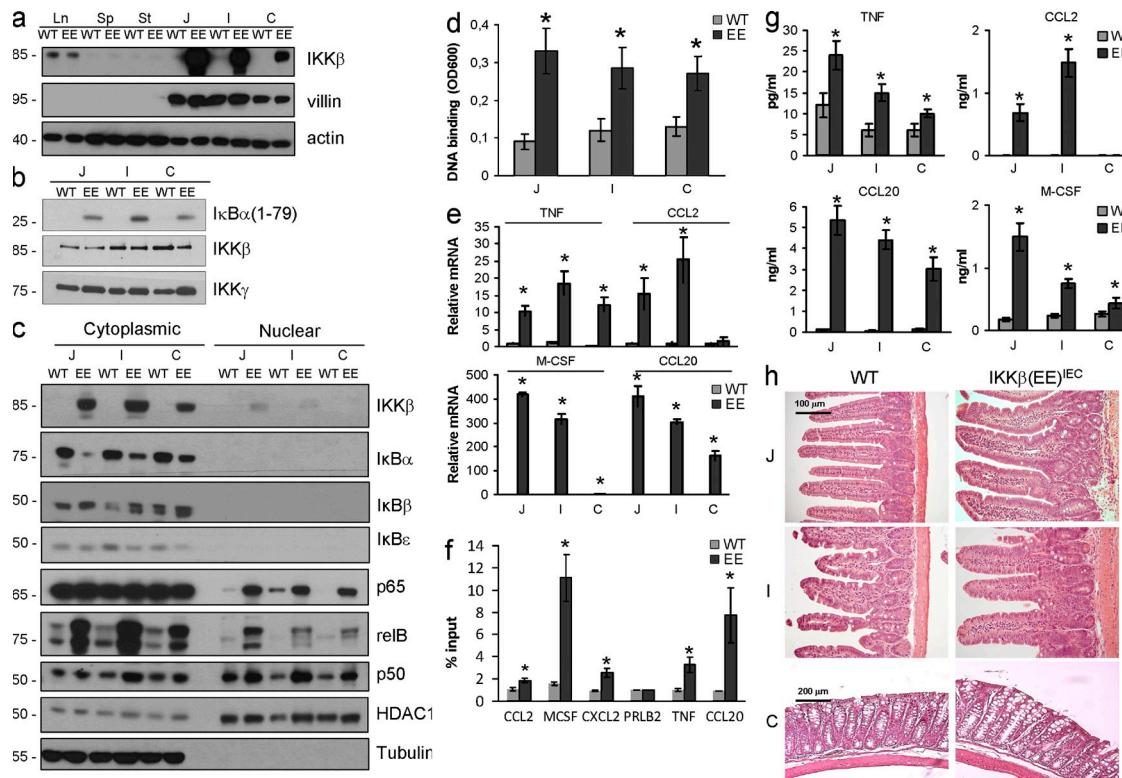


Figure 1. $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice exhibit persistent NF- κB activation, expression of NF- κB target genes, but no tissue damage. (a) Lysates of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ IECs were analyzed for villin and $\text{IKK}\beta$ expression by immunoblotting. Ln, lung; Sp, spleen; St, stomach; J, jejunum; I, ileum; C, colon. Representative of five experiments. (b) IKK activity was measured by immunocomplex kinase assay using GST- $\text{IkB}\alpha(1-79)$ as a substrate in lysates of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ jejunal (J), ileal (I), and colonic (C) enterocytes. Representative of five experiments. (c) Presence of the indicated proteins in cytoplasmic and nuclear extracts of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ enterocytes was examined by immunoblotting. Representative of five experiments. (d) Nuclear extracts of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ enterocytes were analyzed for p65 DNA binding by ELISA. Results (absorbance/micrograms protein) are means \pm SEM ($n = 3$). * $P < 0.05$ versus WT. Data are representative of three independent experiments. (e) WT or $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ enterocyte RNAs were analyzed in triplicates by Q-RT-PCR for expression of the indicated genes. mRNA amounts were normalized to Hprt mRNA. Results are means \pm SEM ($n = 4$). * $P < 0.05$ versus WT. Data are representative of four independent experiments. (f) ChIP was performed with a p65-specific antibody using fixed and sheared chromatin from WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ small bowel enterocytes. Promoter sequences were analyzed by Q-RT-PCR. Results are means \pm SEM ($n = 3$). * $P < 0.05$ versus WT. Data are representative of three independent experiments. (g) WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ enterocyte lysates (75 μg protein) were analyzed by ELISA for the indicated proteins. Results are means \pm SEM ($n = 3$). * $P < 0.05$ versus WT. Data are representative of four independent experiments. (h) Intestinal histology of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice. Representative of 10 experiments.

(Fig. S2, f and g). In summary, persistent NF- κ B activation in IECs results in enhanced localized macrophage and T cell infiltration, but does not cause destructive symptomatic intestinal inflammation, massive TNF production, or altered lymphocyte homeostasis or responsiveness.

Small intestinal apoptosis and enhanced mortality is associated with elevated TNF production in challenged IKK β (EE) IEC mice

We next examined whether constitutive NF- κ B activation protects the epithelium from inflammatory challenge. Unexpectedly, IKK β (EE) IEC mice were extremely sensitive and exhibited considerable mucosal damage upon challenge with all tested stimuli. Administration of a dose of LPS that did not cause mortality in WT mice led to endotoxic shock, with a large and rapid drop in body temperature, and high mortality in IKK β (EE) IEC mice (Fig. 3 a). Even a low LPS dose (0.1 mg/kg) induced severe small intestinal injury characterized by fluid-filled dilations (Fig. 3 b), apoptosis, which was most severe in crypts, and shortening of villi (Fig. 3 b). This occurred despite the enhanced expression of antiapoptotic genes in IKK β (EE) IEC

IECs (Fig. S3 a). Inflammatory cytokines, especially TNF, were substantially elevated in LPS-challenged IKK β (EE) IEC mice (Fig. 3 c). Given the marked increase in TNF, we examined its role in the enhanced susceptibility of IKK β (EE) IEC mice to LPS challenge. Although IKK β (EE) IEC mice lacking TNF (IKK β [EE] IEC x *tnf* $^{-/-}$) or TNFR1 (IKK β [EE] IEC x *tnfrsf1* $^{-/-}$) exhibited similar histology and gene expression profiles to unchallenged IKK β (EE) IEC mice, including immune infiltration of the LP (Fig. 3 d and Fig. S3 b), they were fully protected from LPS-induced tissue damage (Fig. 3 e) and endotoxic shock (Fig. 3 f). Soluble TNFRII (Enbrel) was also protective (Fig. 3, e and f). IL-6 and IL-1 β were also elevated in LPS-challenged IKK β (EE) IEC mice, but neither IL-6 ablation (IKK β [EE] IEC x *il6* $^{-/-}$) nor treatment with an IL-1 receptor antagonist (Anakinra) protected Tg mice from LPS-induced damage (Fig. S3, d and e). Mice were also treated with a cocktail of antibiotics to determine the role of the intestinal microbiota in the exacerbated mucosal responses of IKK β (EE) IEC mice, but the antibiotics treatment did not protect Tg mice from LPS-induced apoptosis.

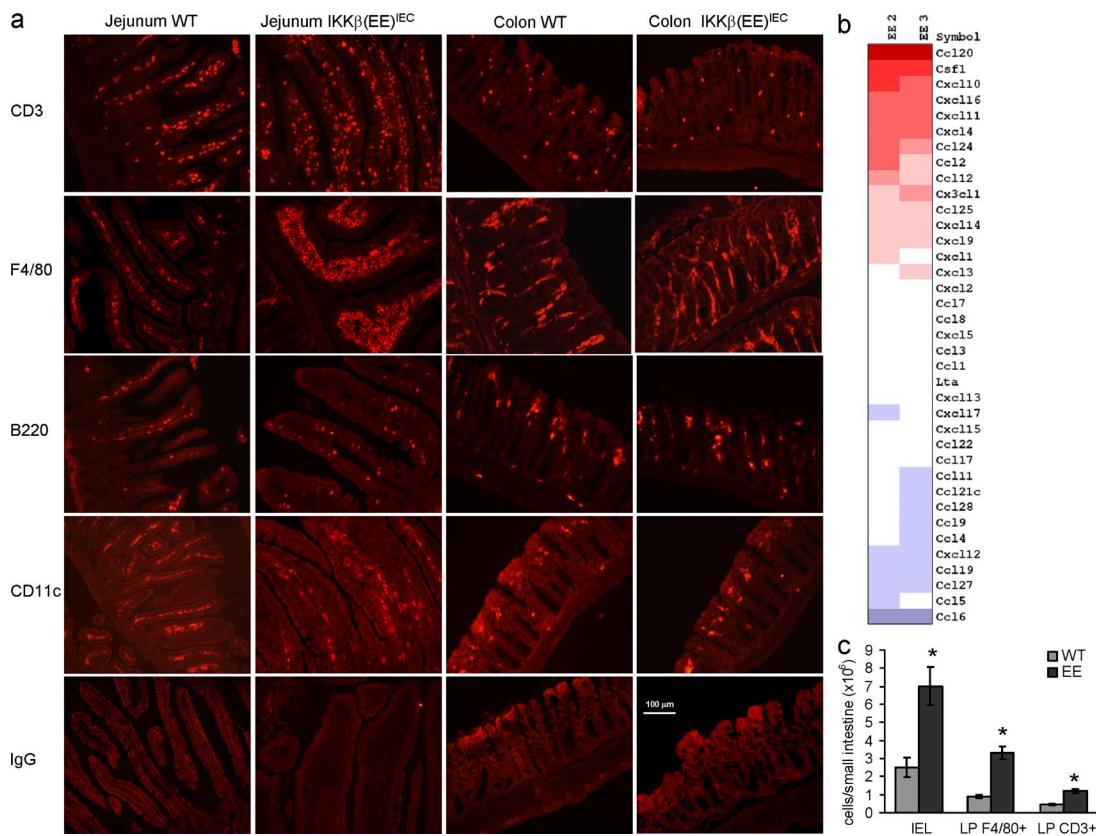


Figure 2. The IKK β (EE) IEC small intestinal LP is infiltrated with F4/80 and CD3 $^{+}$ cells. (a) Frozen sections of WT or IKK β (EE) IEC jejunum and colon were stained for the indicated markers and visualized by immunofluorescence. Representative of five experiments. (b) RNAs from WT or IKK β (EE) IEC enterocytes were subjected to gene expression profiling using whole-genome oligonucleotide arrays (Agilent). Shown are the expression levels of chemokine genes. Positive or negative values represent fold changes between IKK β (EE) IEC mice and littermate controls ($n = 2$). (c) Small intestinal IELs and LPLs were isolated from WT or IKK β (EE) IEC mice and counted. Percentages of F4/80 and CD3 $^{+}$ cells were determined by flow cytometry in cells gated for CD45. Results are means \pm SEM ($n = 6$). *, P < 0.05 versus WT. Data are representative of three independent experiments.

Elevated TNF production and mortality in challenged IKK β (EE)^{IEC} mice depend on MAPK activation

To identify sources of TNF in LPS-challenged IKK β (EE)^{IEC} mice, we isolated LP F4/80⁺ cells before and after LPS challenge, and found that their TNF mRNA expression did not differ between Tg and WT mice, although IL-6 and IL-12 mRNAs were higher in Tg cells (Fig. S4). Yet, the number of F4/80⁺ LP cells was increased in IKK β (EE)^{IEC} mice, suggesting that they may contribute to total circulating TNF. In contrast, membrane-associated and especially soluble TNF were dramatically increased in IECs of LPS-challenged IKK β (EE)^{IEC} mice (Fig. 4, a–c). Despite the threefold increase in TNF mRNA (Fig. 4 d), LPS administration led to only a small further increase of p65 nuclear translocation and DNA binding above the basal level of nonstimulated IKK β (EE)^{IEC} enterocytes (Fig. 4, e and f). A lower LPS dose increased TNF protein production by Tg enterocytes without causing any further increase in its mRNA amounts, but did not elicit TNF production by WT IECs (Fig. 4, g and h).

Table I. Sizes of Peyer's patch, MLN, IEL, and LPL subpopulations

	WT	EE
PP		
CD4 ⁺	11.25 \pm 1.3	12 \pm 1.4
CD8 ⁺	3.75 \pm 0.5	3.4 \pm 0.3
B220 ⁺	86 \pm 5.1	87 \pm 7.2
CD11c ⁺	0.5 \pm 0.1	0.3 \pm 0.2
MLN		
CD4 ⁺	31 \pm 2.5	30 \pm 1.7
CD8 ⁺	21 \pm 1.8	20 \pm 1.2
B220 ⁺	26 \pm 3.5	28 \pm 2.1
CD11c ⁺	4 \pm 0.5	3 \pm 0.7
IELs		
Percentage within CD3 ⁺ cells:		
TCR α β ⁺	45.2 \pm 3.4	45.1 \pm 2.3
TCR γ δ ⁺	54.8 \pm 4.5	54.9 \pm 4.3
Percentage within TCR α β ⁺ cells:		
CD8 α α ⁺ CD8 α β ⁻	41.2 \pm 2.3	37 \pm 3.4
CD8 α β ⁺	30.1 \pm 1.5	30.2 \pm 2.5
CD4 ⁺	24 \pm 1.3	22 \pm 1.5
Percentage within TCR γ δ ⁺ cells:		
CD8 α α ⁺ CD8 α β ⁻	83 \pm 3.2	82 \pm 2.4
CD8 α β ⁺	4.1 \pm 1.1	4.2 \pm 0.9
LPLs		
Percentage within TCR α β ⁺ cells:		
CD8 α β ⁺	15.3 \pm 1.4	12.7 \pm 2.8
CD4 ⁺	78.2 \pm 3.4	78.4 \pm 2.9
CD4 ⁺ Foxp3 ⁺	17.2 \pm 2.3	18.1 \pm 3.2

Peyer's patches (PP), MLN, IEL, and LPL subpopulations. Isolated cells from MLN, small intestinal PP, LPLs and IELs of WT or IKK β (EE)^{IEC} ($n = 4$) were stained with labeled antibodies and analyzed by FACS.

These results suggested that although NF- κ B is activated in Tg enterocytes and is sufficient for increasing basal TNF gene transcription, accumulation of TNF mRNA and especially protein may depend on posttranscriptional events. MAPK signaling pathways, both p38 MAPK and ERK, play important roles in posttranscriptional regulation of TNF production (Kotlyarov et al., 1999; Dumitru et al., 2000; Rousseau et al., 2008; Xu and Deryck, 2010; Gais et al., 2010). LPS administration enhanced p38, MK2, and ERK phosphorylation in IECs of both WT and Tg mice (Fig. 5 a). TNF- α -converting enzyme (TACE) activity was also enhanced after LPS administration, but the extent of elevation was similar in both groups of mice (Fig. S5 a). Oral administration of RWJ 67657, a p38 MAPK inhibitor (Wadsworth et al., 1999), and PD184352, a specific MEK-1/2 inhibitor, decreased phosphorylation of the corresponding MAPKs and their targets (Fig. S5 b), serum TNF (Fig. 5 b), and its release from enterocytes (Fig. 5 c), and protected IKK β (EE)^{IEC} mice from endotoxic shock (Fig. 5 d). These results suggest that additional activation of both MAPK pathways on top of the already active NF- κ B is necessary to induce TNF production and epithelial damage in IKK β (EE)^{IEC} mice. We also examined whether IKK β (EE)^{IEC} IECs were more sensitive to TNF-induced killing than WT IECs. Exogenous administration of TNF induced IEC apoptosis in IKK β (EE)^{IEC} \times *Tnf*^{-/-} mice, although to a lesser extent than in IKK β (EE)^{IEC} mice (Fig. 5 e; 3.6 \pm 0.8 vs. 8.7 \pm 1.2 apoptotic cells per crypt; $P < 0.001$), but did not induce apoptosis in *Tnf*^{-/-} and WT mice.

Citrobacter rodentium infection and CD3 activation cause loss of barrier function in IKK β (EE)^{IEC} mice

To further investigate the effect of chronic NF- κ B activation on maintenance of intestinal barrier function and innate immunity, we challenged IKK β (EE)^{IEC} mice with the Gram-negative bacterium *C. rodentium*, an attaching and effacing (A/E) pathogen that colonizes the mouse colon, activates MAPK signaling in colonic IECs (Fig. 6 a), and typically leads to crypt hyperplasia and mucosal inflammation before its clearance from immunocompetent mice (Borenshtein et al., 2008). Tg and WT mice had similar fecal bacterial counts after *C. rodentium* infection (Fig. 6 b), indicating similar bacterial burden, immune response, and bacterial clearance. However, Tg mice had greater colonic mucosal ulceration and crypt apoptosis (Fig. 6 c) and higher liver bacterial counts than their WT counterparts (Fig. 6 b), suggesting that epithelial barrier disruption enhanced systemic bacterial spread. IKK β (EE)^{IEC} \times *Tnf*^{-/-} mice were protected from the increase in *C. rodentium*-induced colon damage (Fig. 6 d) and subsequent bacterial translocation, as the \log_{10} CFU/gram liver for WT, IKK β (EE)^{IEC}, and IKK β (EE)^{IEC} \times *Tnf*^{-/-} mice at 14 d after infection were 0, 3.2 \pm 0.5, and 0.8 \pm 0.35, respectively. IKK β (EE)^{IEC} mice were also extremely sensitive to oral administration of the irritant dextran sulfate salt (DSS), which causes acute intestinal inflammation, as they developed much more severe colonic ulceration and displayed increased mortality compared with WT mice (Fig. S6, a and b). This effect

was again dependent on TNF, as $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ x $\text{Tnf}^{-/-}$ mice were partially protected against acute DSS-induced colitis, as shown in Fig. S6 c.

Intestinal inflammation is associated in some diseases with exaggerated and poorly controlled T cell–mediated immune responses (Kaser et al., 2010). To mimic antigen stimulation of small intestinal T cells, we injected mice with a low dose of a CD3-activating antibody that triggers only minimal enterocyte

apoptosis and small intestinal damage in normal mice (Miura et al., 2005). In $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice, however, this treatment caused massive enterocyte apoptosis, followed by villous shortening (Fig. 7 a) and a rapid drop in body temperature (Fig. 7 b), as well as increased TNF protein amounts in serum (Fig. S7 a) and release by IECs without a further increase in TNF mRNA (Fig. 7, c and d). As seen with LPS, anti-CD3 administration caused MAPK activation in enterocytes from

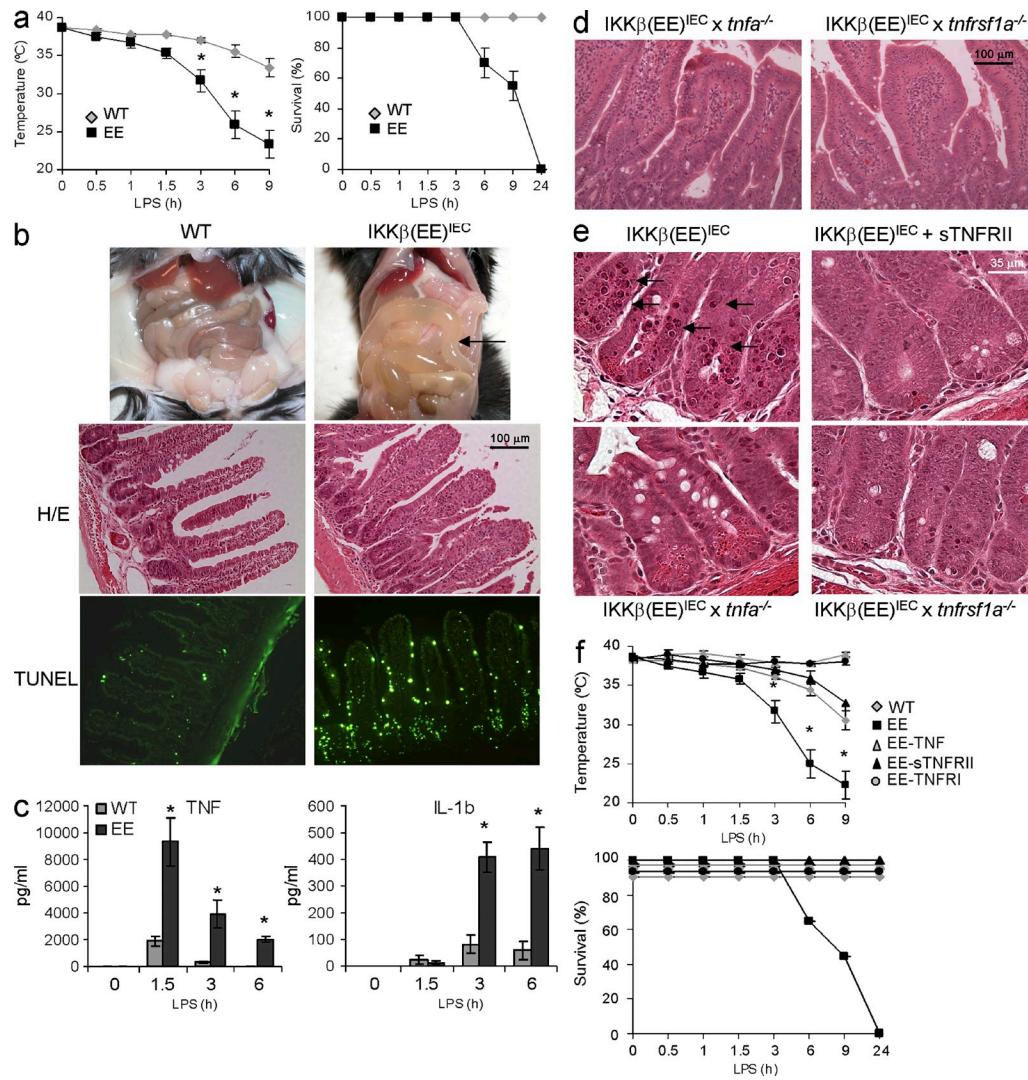


Figure 3. Small intestinal apoptosis and increased endotoxin-induced mortality associated with elevated TNF production in $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice. (a) Rectal temperature and survival of WT (gray diamonds) and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ (black squares) mice after LPS (5 mg/kg *E. coli* O111:B4) injection. Results are means \pm SEM ($n = 8$). *, $P < 0.05$ versus WT. Data are pooled from three independent experiments. (b) Abdomens of WT and $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ mice 4 h after LPS (1 mg/kg) injection. Arrow points at fluid-filled dilated small bowel in Tg mice. Jejunal sections were stained with either H&E (top) or by an in situ TUNEL assay (bottom). Representative of 10 experiments. (c) Circulating TNF and IL-1 β after LPS administration. Results are means \pm SEM ($n = 4$). *, $P < 0.05$ versus WT. Data are representative of three independent experiments. (d) Intestinal histology of $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnfa}^{-/-}$ and $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnfrsf1a}^{-/-}$ mice. Jejunal sections were H&E stained. Representative of five experiments. (e) The indicated mouse strains were analyzed 4 h after LPS injection with or without an accompanying dose of Enbrel (sTNFRII) as indicated. Small intestinal sections were stained with H&E. Representative of three experiments. (f) Rectal temperature and survival of WT (gray diamond), $\text{IKK}\beta(\text{EE})^{\text{IEC}}$ (black square), $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnfrsf1a}^{-/-}$ (black circle), $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnf}^{-/-}$ (gray triangle), $\text{IKK}\beta(\text{EE})^{\text{IEC}} + \text{sTNFRII}$ (black triangle) mice after LPS injection. Results are means \pm SEM ($n = 3$). Survival of WT, $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnfrsf1a}^{-/-}$ (black circle), $\text{IKK}\beta(\text{EE})^{\text{IEC}} \times \text{tnf}^{-/-}$ (gray triangle), and $\text{IKK}\beta(\text{EE})^{\text{IEC}} + \text{sTNFRII}$ (black triangle) is 100%. *, $P < 0.05$ versus $\text{IKK}\beta(\text{EE})^{\text{IEC}}$. Data are representative of three independent experiments.

both WT and Tg mice (Fig. 7 e). No significant differences in intracellular cytokines were found between Tg and WT lymphocytes after anti-CD3 injection (Fig. S7, b and c). Yet, other immune cells that are indirectly activated may also contribute to TNF secretion or IEC apoptosis. Ablation of TNF or TNFR 1 or Enbrel administration protected IKK β (EE)^{IEC} mice from anti-CD3-induced tissue damage and pharmacological inhibition of p38 partially decreased enterocyte TNF production (Fig. 7 f and Fig. S7 d).

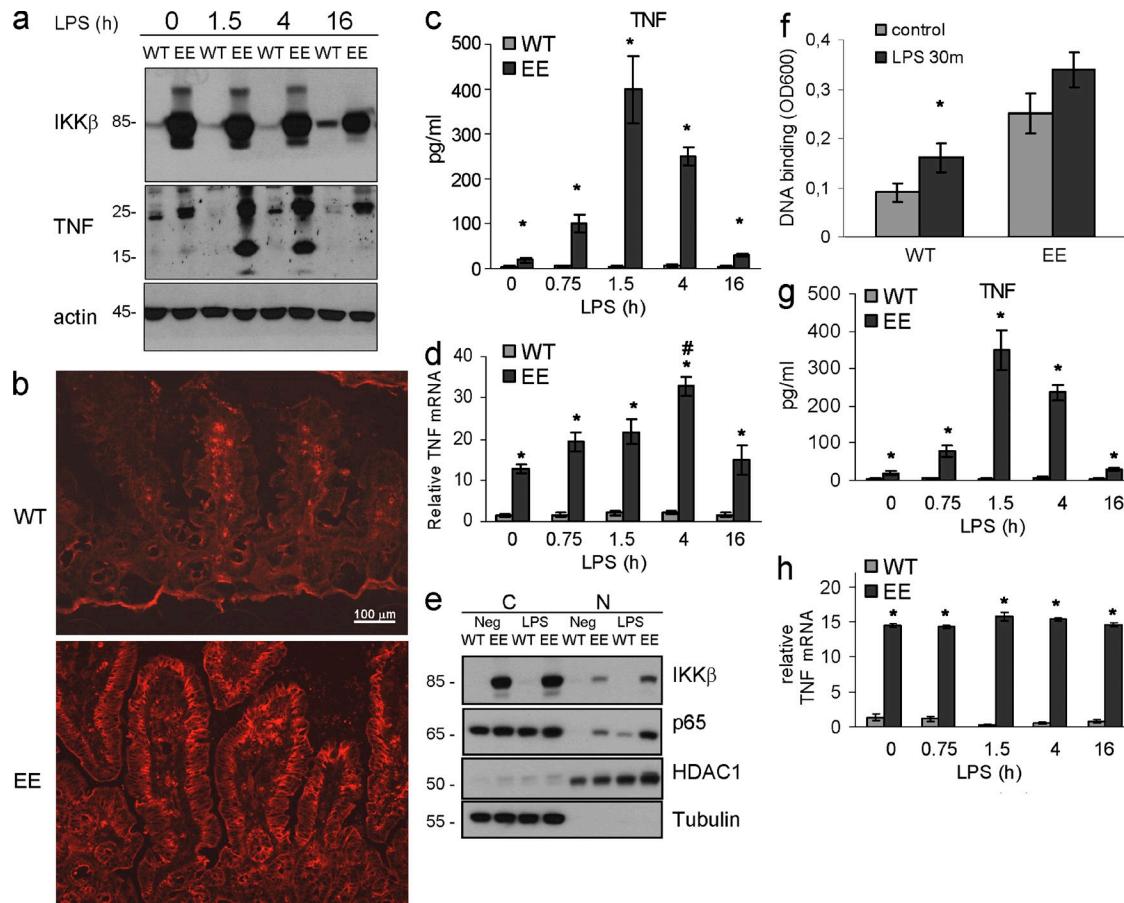


Figure 4. Membrane-associated and especially soluble TNF are dramatically increased in IECs of LPS-challenged IKK β (EE)^{IEC} mice.

(a) Lysates of small intestinal enterocytes were prepared when indicated after LPS injection and analyzed for TNF expression by immunoblotting (trans-membrane, 26 kD; soluble, 17 kD). Representative of three experiments. (b) Frozen sections of WT or IKK β (EE)^{IEC} jejunum prepared 2 h after LPS injection were stained with anti-TNF antibody and visualized by immunofluorescence. Representative of three experiments. (c) Lysates of small intestinal enterocytes (75 μ g protein) of indicated genotypes were prepared when indicated after LPS injection and analyzed for TNF content by ELISA. Results are means \pm SEM ($n = 4$). *, $P < 0.05$ versus WT. Data are representative of four independent experiments. (d) RNAs from small intestinal enterocytes of indicated genotypes were prepared when indicated after LPS injection and analyzed in triplicates by Q-RT-PCR for TNF mRNA and normalized to *Hprt* mRNA. *, $P < 0.05$ versus WT; #, $P < 0.05$ versus other time points in IKK β (EE)^{IEC} mice. Results are means \pm SEM ($n = 4$). Data are representative of four independent experiments. (e and f) LPS administration results in only a mild increase of p65 nuclear translocation and DNA binding in IKK β (EE)^{IEC} enterocytes. (e) WT and IKK β (EE)^{IEC} enterocytes were isolated 30 min after LPS injection and separated into cytosolic (C) and nuclear (N) fractions that were analyzed for their content of the indicated proteins by immunoblotting. Representative of two experiments. (f) Nuclear extracts of WT and IKK β (EE)^{IEC} enterocytes isolated 30 min after LPS injection were analyzed for p65 DNA binding by ELISA. Results are means \pm SEM ($n = 3$). *, $P < 0.05$ versus control. Data are representative of two independent experiments. (g and h) Low LPS dose administration induces TNF protein production by Tg enterocytes without a further increase in its mRNA amounts. (g) Lysates of small intestinal enterocytes (75 μ g protein) of indicated genotypes prepared when indicated after low LPS dose (0.2 mg/kg) injection were analyzed for TNF content by ELISA. *, $P < 0.05$ versus WT. Results are means \pm SEM ($n = 3$). Data are representative of three independent experiments. (h) RNAs from small intestinal IECs prepared when indicated after low LPS dose (0.2 mg/kg) injection were analyzed in triplicates by Q-RT-PCR for TNF mRNA amounts and normalized to *Hprt* mRNA. Results are means \pm SEM ($n = 3$). *, $P < 0.05$ versus WT. Data are representative of three independent experiments.

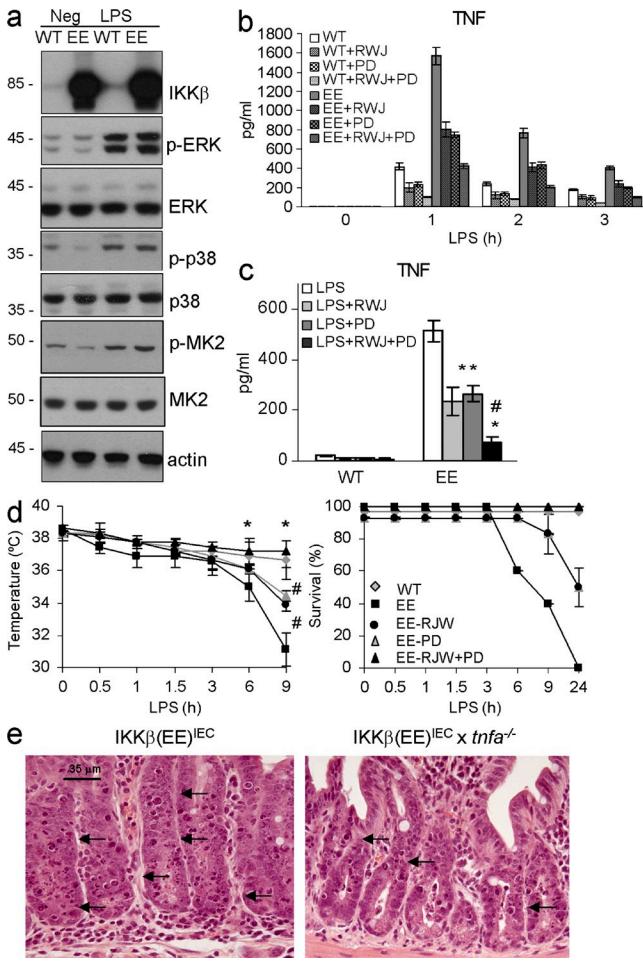


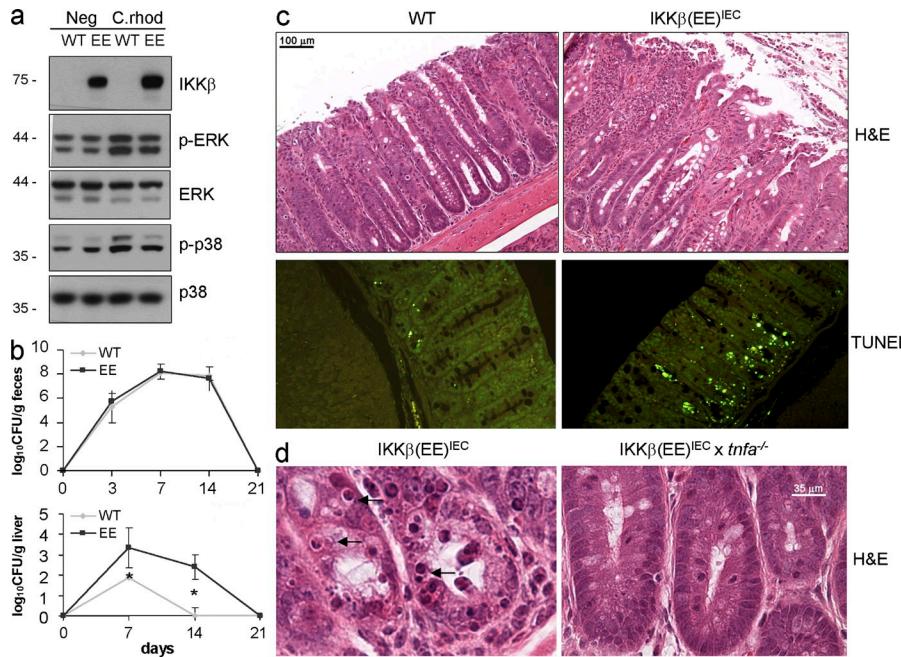
Figure 5. Elevated TNF production in LPS-challenged IKK β (EE)^{IEC} mice and mortality depend on MAPK activation.

(a) Lysates from small intestinal enterocytes prepared 30 min after LPS challenge were analyzed for expression and phosphorylation of the indicated proteins by immunoblotting. Representative of three experiments. (b) Circulating TNF after LPS injection (0.2 mg/kg) with or without RWJ67657 (RWJ) or PD184352 (PD). Results are means \pm SEM ($n = 3$). * $P < 0.05$ versus IKK β (EE)^{IEC}; # $P < 0.05$ versus IKK β (EE)^{IEC} with RWJ or PD. Data are representative of three independent experiments. (c) Small intestinal enterocyte lysates (75 μ g protein) of indicated genotypes were prepared 90 min after LPS injection (0.2 mg/kg) with or without RWJ67657 (RWJ) or PD184352 (PD) and analyzed for TNF content by ELISA. Results are means \pm SEM ($n = 3$). * $P < 0.05$ versus IKK β (EE)^{IEC}; # $P < 0.05$ versus IKK β (EE)^{IEC} with RWJ or PD. Data are representative of three independent experiments. (d) Rectal temperature and survival after LPS injection (0.2 mg/kg) with or without RWJ67657 (RWJ) or PD184352 (PD). Results are means \pm SEM ($n = 6$). WT (gray diamond), IKK β (EE)^{IEC} (black square), IKK β (EE)^{IEC} + RWJ (black circle), IKK β (EE)^{IEC} + PD (gray triangle), and IKK β (EE)^{IEC} + RWJ + PD (black triangle). Survival of WT (gray diamond) and IKK β (EE)^{IEC} + RWJ + PD (black triangle) is 100%, and survival of IKK β (EE)^{IEC} + RWJ (black circle) and IKK β (EE)^{IEC} + PD (gray triangle) is 100% up to 6 h. * $P < 0.05$ for WT and IKK β (EE)^{IEC} + RWJ + PD versus IKK β (EE)^{IEC}; # $P < 0.05$ for IKK β (EE)^{IEC} versus IKK β (EE)^{IEC} + RWJ or PD, and WT and IKK β (EE)^{IEC} + RWJ + PD versus IKK β (EE)^{IEC} + RWJ or PD. Data are pooled from two independent experiments. (e) The indicated mouse strains were analyzed 4 h after TNF administration (mice were injected i.v. with 2 μ g of recombinant murine TNF). Small intestinal sections were stained with H&E. Arrows point at apoptotic cells. Representative of three experiments.

in nonimmune, epithelial, or parenchymal cells triggers the spontaneous development of severe inflammatory conditions (Pasparakis, 2009). In IECs, for instance, NF- κ B mainly plays a protective and antiinflammatory role. IEC-specific ablation of the NEMO/IKK γ regulatory subunit, or of IKK β , resulted in complete or partial loss of barrier function, bacterial translocation, and severe inflammation (Chen et al., 2003; Egan et al., 2004; Greten et al., 2004; Chae et al., 2006; Nenci et al., 2007). The ability of NF- κ B to maintain barrier function and immune homeostasis may depend on suppression of enterocyte death and expression of barrier function genes and defensins. This function may be essential, as the intestinal epithelium has an important function in the maintenance of intestinal immune homeostasis. By forming a mechanical barrier and producing antimicrobial peptides, IECs prevent bacteria from invading the mucosa, where they could encounter immune cells and induce inflammation.

Our results suggest that persistent NF- κ B activation in IECs is sufficient for expression of large amounts of inflammatory chemokines, dramatic mononuclear cell infiltration into the small intestinal LP, and *Tnf* gene transcription, but is insufficient for substantial production of TNF protein and therefore cannot drive symptomatic intestinal inflammation by itself. Consequently, IKK β (EE)^{IEC} mice did not show any sign of diarrhea or intestinal bleeding, and their survival did not differ from WT littermates. The noteworthy absence of epithelial barrier breakdown despite the overall elevated mucosal cytokine levels could support the concept of a protective role of intestinal epithelial NF- κ B. Increased expression of antiapoptotic genes together with the low production of TNF protein by the unchallenged IKK β (EE)^{IEC} IECs could shift the balance toward maintenance of the barrier and intestinal homeostasis.

However, IKK β (EE)^{IEC} mice exposed to relatively mild inflammatory and immune stimuli, at doses that trigger only modest responses in WT mice, showed epithelial apoptosis, intestinal barrier disruption, and subsequent bacterial translocation. These unexpected results are counterintuitive, given the role of NF- κ B in maintenance of barrier function and control of bacterial translocation. These stimuli, which activate additional signaling pathways, especially MAPK, greatly enhanced TNF production in IKK β (EE)^{IEC} mice and led to a widespread epithelial apoptosis and disruption of intestinal integrity. However, neither LPS, which signals via TLR4, which is expressed mainly on macrophages and dendritic cells, nor anti-CD3, which activates TCR, can directly activate MAPKs in IECs. Most likely, they induce production of a cytokine, possibly TNF itself, which acts on IECs to activate MAPKs. Curiously, none of the cytokines produced by IKK β (EE)^{IEC} enterocytes are sufficient for autocrine or paracrine activation of MAPKs in these cells. IKK β (EE)^{IEC} enterocytes were also sensitive to circulating TNF, suggesting that persistent NF- κ B activation up-regulates other proapoptotic pathways that, together with the increase in TNF production, shift the balance toward apoptosis.



We speculate that a similar mechanism may underlie the pathogenesis of chronic inflammatory disorders with relapsing acute flare-ups, such as inflammatory bowel diseases, psoriasis, or rheumatoid arthritis, in which relatively quiescent periods without overt symptomatic inflammation erupt into acute inflammatory flare-ups upon encounter of poorly defined environmental challenges. In these disorders, either direct or indirect chronic NF- κ B activation was observed in nonimmune cells such as IECs, keratinocytes, and synovioocytes (Li and Verma, 2002), single nucleotide polymorphisms in NF- κ B pathway components were found (Vereecke et al., 2009). In addition, these disorders respond to anti-TNF therapy (Sfikakis, 2010). The new findings may also explain microbial translocation in patients and animals experiencing gut insults that increase NF- κ B and cause massive TNF production, such as intestinal ischemia, necrotizing enterocolitis and obstruction (Chen et al., 2003). Our results may also be relevant to the increased translocation of enteric bacteria seen in AIDS patients, who are known to have elevated TNF (Stockmann et al., 2000). Given the increase in IL-1 β -driven inflammation caused by $\text{IKK}\beta$ inhibition (Greten et al., 2007; Hsu et al., 2011), inhibition of MAPKs (ERK and p38) or anti-TNF therapy may be the preferred treatments for such disorders.

MATERIALS AND METHODS

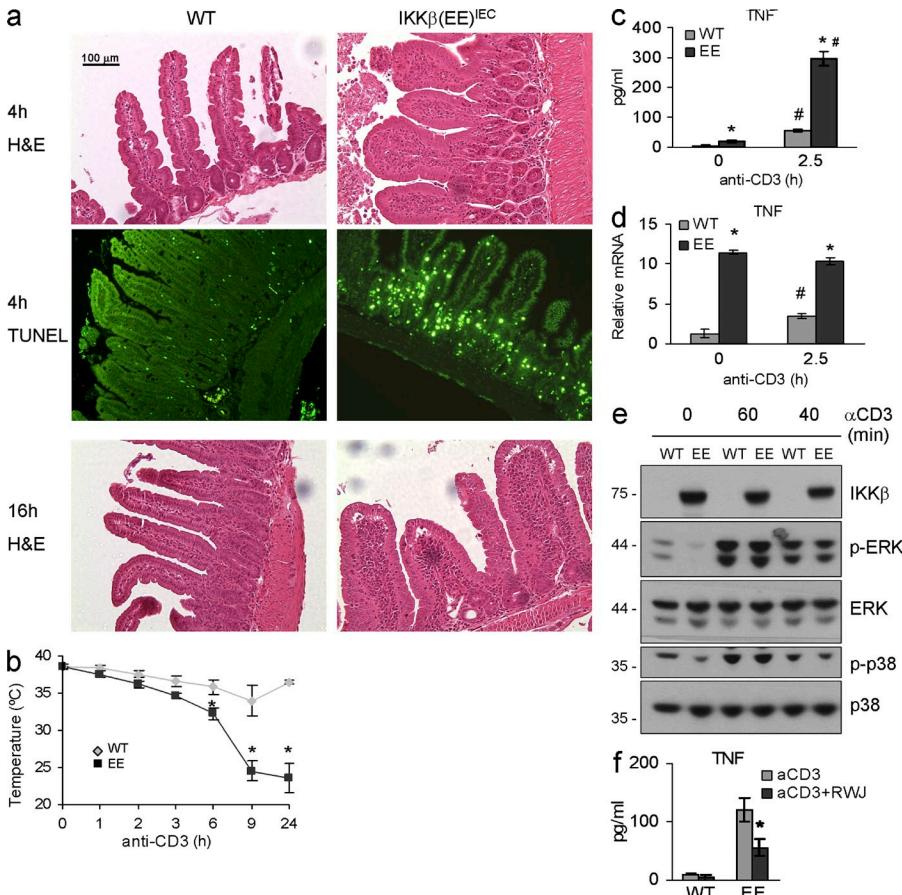
Mice. $\text{IKK}\beta^{\text{EE}}$ cDNA (Delhase et al., 1999) was PCR-amplified and inserted into a plasmid containing the 12.4-kb *Villin* promoter (D. Gumucio, University of Michigan, Ann Arbor, MI; Madison et al., 2002). The 15.7-kb expression cassette was excised by a PmeI digest, purified, and injected into fertilized C57BL/6 oocytes to derive founder mice, one of which transmitted the $\text{Ikk}\beta^{\text{EE}}$ transgene. Tg mice were crossed to $\text{tnf}^{-/-}$, $\text{tnfrsf1}^{-/-}$, and $\text{il6}^{-/-}$ mutants, also in the C57BL/6 background, purchased from The Jackson Laboratory. Mice used in the experiments were 8–12 wk old and were

maintained under standard conditions at a University of California at San Diego animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols were approved by the institutional review board, following National Institutes of Health guidelines.

Reagents. LPS (*Escherichia coli* O111:B4; Sigma-Aldrich) was injected i.p. at a dose of 5 mg/kg unless otherwise indicated. Recombinant mouse TNF from R&D Systems was injected i.v. at dose of 2 μ g/mouse. Antibody against CD3 (clone 145-2C11; BD) was injected i.p. at a dose of 12.5 μ g/mouse, unless otherwise indicated. RWJ 67657 (Wadsworth et al., 1999; p38 MAPK inhibitor) and PD184352 (MEK-1/2 inhibitor) were purchased from Tocris Bioscience and Selleck Chemicals LLC, respectively, diluted in 0.1 N HCl, and administrated by oral gavage (50 mg/kg). Enbrel (sTNFRII; Amgen) was administrated i.v. at 5 mg/kg. IL-1 receptor antagonist (IL-1Ra or Anakinra; Amgen) was administered i.v. at 300 mg/kg. Drugs were administrated 30 min before challenge.

Flow cytometry, intracellular cytokine staining, and cell sorting. For intracellular cytokine staining, surface staining was performed for 25 min with the corresponding cocktail of fluorescently labeled antibodies. After surface staining, cells were resuspended in Fixation/Permeabilization solution (Cytofix/Cytoperm kit; BD), and intracellular cytokine and FoxP3 staining was performed according to the manufacturer's protocol. For cell sorting, a FACS Diva cell sorter (BD) was used. Data were analyzed using FlowJo software (Tree Star).

Histological analysis and TUNEL assay. Intestines were removed, opened longitudinally, cleaned, processed as "Swiss rolls," and fixed in 10% phosphate-buffered formalin for 24 h. Fixed tissues were embedded in paraffin, and 5- μ m sections were prepared and stained with hematoxylin and eosin (H&E). For immunofluorescence studies, tissues were frozen in OCT compound, cut on a cryomicrotome, air-dried, and fixed in acetone. The sections were incubated overnight at 4°C with antibodies against F4/80 (AbD Serotec), CD11c, B220 (BD), CD3, and TNF (Abcam). *In situ* TUNEL assay was performed using ApoAlert DNA fragmentation assay kit (Takara Bio Inc.). Samples were examined under a microscope (Axioplan 2; Carl Zeiss, Inc.).



after anti-CD3 injection (6.25 $\mu\text{g}/\text{mouse}$) in the absence or presence of RWJ 67657 and analyzed for TNF content by ELISA. Results are representative of three independent experiments.

Expression array profiling. Total RNA (isolated by RNeasy kit; QIAGEN) was prepared from enterocytes obtained from small intestines of $\text{IKK}\beta(\text{EE})^{\text{EC}}$ and WT littermates. Purified RNA, 0.5 μg per sample, was labeled using the LRLAK PLUS two-color low RNA input Linear Amplification kit, and then hybridized to a mouse whole-genome microarray 4 \times 44K 60 mer slide according to the manufacturer's instructions (Agilent). Two biological replicates were performed for each experimental condition. GEO submission number is GSE29701.

Isolation of IELs and LPLs. IELs and LPLs were isolated as previously described (Mucida et al., 2007).

Immunoblotting. Whole enterocyte extracts were obtained by lysing cells in 1% SDS and 10 mM Tris-HCl (pH 7.4). Cytoplasmic and nuclear extracts were obtained as previously described (Rius et al., 2008). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes that were incubated with antibodies against $\text{IKK}\beta$ (Millipore); $\text{I}\kappa\text{B}\alpha$, $\text{I}\kappa\text{B}\beta$, $\text{I}\kappa\text{B}\epsilon$, p65, RelB, p50, HDAC1, p38, villin (Santa Cruz Biotechnology, Inc.); TNF (R&D Systems); phospho-MK2, MK2, phospho-p38, phospho-ERK, and ERK1/2 (Cell Signaling Technology); and actin and tubulin (Sigma-Aldrich).

p65 DNA binding. Nuclear extracts were obtained as previously described (Rius et al., 2008) and analyzed for p65 DNA binding by ELISA (Cayman Chemical) according to the manufacturer's instructions.

ChIP. Epithelium from small intestine was scraped into 1% formaldehyde solution and fixed for 10 min at room temperature. Chromatin was precipitated with a p65 antibody (Santa Cruz Biotechnology Inc., San Jose,

CA, sc-372) as described (Lee et al., 2006). Precipitated material was analyzed by Q-RT-PCR. Primer sequences are available upon request. Although not shown, we have confirmed the results of the anti-p65 ChIP with an anti-PolII antibody (Santa Cruz Biotechnology Inc., San Jose, CA, sc-899).

In situ hybridization. Intestinal paraffin sections were hybridized to anti-TNF probe (IMAGE clone #8860739) at 65°C for 72 h as described (Gregoriet et al., 2005).

C. rodentium infection. Mice were infected (5×10^8 bacteria) by oral gavage. Bacterial counts were determined as previously described (Spehlmann et al., 2009).

Intestinal permeability in vivo. In vivo permeability assay to assess barrier function was performed using a FITC-labeled dextran method. Food and water were withdrawn for 4 h, and mice were gavaged with permeability tracer (60 mg/100 g body weight of FITC-labeled dextran; FD-4; Sigma-Aldrich). Serum was collected retroorbitally 4 h after FD-4 gavage, and fluorescence intensity of each sample was measured (excitation, 492 nm; emission, 525 nm).

Kinase assays. Enterocytes were lysed and immunoprecipitated with an anti- $\text{IKK}\gamma$ antibody (BD). The immune complexes were incubated with GST- $\text{I}\kappa\text{B}\alpha(1-79)$ in the presence of $\gamma^{32}\text{P}$ ATP. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane that was exposed to x-ray film and hybridized with antibodies against $\text{IKK}\beta$ (Millipore) and $\text{IKK}\gamma$ (Santa Cruz Biotechnology, Inc.).

Intestinal antigen uptake and processing. Naive CD4⁺ T cells were isolated from spleens and mesenteric LNs (MLNs) of OT-2/CD90.1⁺ mice via negative selection (MACS; Miltenyi Biotec). The purity of CD4⁺ cells exceeded 80%. Cells were labeled with 5 μ M CFSE for 5 min at room temperature, washed twice with PBS, and i.v. injected into either IKK β (EE)^{IEC} or WT recipient mice. Mice were fed with oral OVA: high dose (20% OVA diet; Research Diets Inc.) or low dose (0.1% grade II OVA; Sigma-Aldrich) in nonacidified drinking water. On day 5, MLNs were analyzed for the presence of proliferating CD90.1/CD4⁺ T cells.

OVA oral tolerance. Both WT and IKK β (EE)^{IEC} mice were fed with 20% OVA diet (Research Diets Inc.) for 2 d. On day 7, mice were immunized by a s.c. tail-base injection of 50 μ g of grade V OVA in 100 μ l of complete Freud's adjuvant (Sigma-Aldrich). On day 22, anti-OVA antibody concentrations in serum were determined by ELISA.

TACE activity. Enterocytes were lysed and analyzed for TACE activity by SensoLyte 520 TACE (α -Secretase) Activity Assay kit Fluorometric (Anaspec) according to the manufacturer's instructions.

Statistical analysis. Data are expressed as mean \pm SEM. The analysis used unpaired Student's *t* test for comparing two groups and ANOVA for multiple group comparisons. Bonferroni post-hoc tests were used for multiple pairwise comparisons. All statistical analyses were performed using the SPSS 15.0 (SPSS) statistical package. Results were considered significant at $P < 0.05$.

Online supplemental material. Fig. S1 shows that IKK β (EE)^{IEC} mice exhibit persistent NF- κ B target gene expression, but no tissue damage. Fig. S2 shows that persistent NF- κ B activation in IECs does not cause altered lymphocyte homeostasis and responsiveness. Fig. S3 shows that TNF or TNFR1 deficiencies in IKK β (EE)^{IEC} mice do not alter intestinal histology or gene expression, and IL6 ablation and IL-1 receptor antagonist (Anakinra) treatment does not protect IKK β (EE)^{IEC} mice from intestinal apoptosis after LPS injection. Fig. S4 shows that TNF expression in small intestinal F4/80⁺ LP cells after LPS injection does not differ between WT and IKK β (EE)^{IEC}. Fig. S5 shows that TACE activity does not differ between WT and IKK β (EE)^{IEC}. Fig. S6 shows that DSS exposure causes an increase of colonic damage and high mortality in IKK β (EE)^{IEC} mice. Fig. S7 shows that WT or IKK β (EE)^{IEC} small intestinal T cells do not exhibit significant differences in cytokine production after anti-CD3 injection, and anti-TNF therapy protects IKK β (EE)^{IEC} mice from anti-CD3-induced tissue damage. Table S1 shows the expression profiles of WT and IKK β (EE)^{IEC} IECs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110242/DC1>.

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