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Loss of Epithelial RelA Results in Deregulated Intestinal Proliferative/Apoptotic Homeostasis and Susceptibility to Inflammation

Kris A. Steinbrecher,2*† Eleana Harmel-Laws,* Raquel Sitcheran,‡ and Albert S. Baldwin2‡

NF-κB plays a central, proinflammatory role in chronic intestinal inflammation, yet recent work suggests a predominantly protective function for this transcription factor group in some cell types of the intestine. We herein describe the conditional deletion of the NF-κB RelA gene in murine intestinal epithelia and determine its function in homeostatic control of enterocyte proliferation/apoptosis and susceptibility to colonic inflammation. Mice lacking RelA in ileal and colonic enterocytes were born in expected Mendelian ratios, and RelA-null epithelia differentiated normally. Spontaneous intestinal disease and death occurred with low penetrance in neonates lacking epithelial RelA. IκBα and IκBβ were significantly diminished in RelA-null epithelia, and endotoxin challenge revealed elevated p50 and c-Rel DNA binding activity as compared with controls. Deletion of RelA resulted in diminished expression of antimicrobial (defensin-related cryptdin 4, defensin-related cryptdin 5, RegIIIγ) and antiapoptotic, prorestitutition genes (Bcl-xL, RegIV, IL-11, IL-18), and basal rates of epithelial apoptosis and proliferation were elevated. Mice lacking colonic RelA were sensitive to dextran sodium sulfate-induced colitis. Although experimental colitis enhanced proliferation in cells lacking RelA, sustained epithelial cell apoptosis precluded mucosal healing and decreased animal survival. We conclude that activation of RelA is required for homeostatic regulation of cell death and division in intestinal epithelia, as well as for protection from development of severe, acute inflammation of the intestine. The Journal of Immunology, 2008, 180: 2588–2599.

Control of gene expression by NF-κB is essential for appropriate cellular responses to a wide variety of stimuli and often has seemingly contradictory roles in initiation and resolution of inflammatory disease (1–3). NF-κB functions as a dimeric transcription factor whose subunits are RelA, RelB, c-Rel, p50/NF-κB1, and p52/NF-κB2. Heterodimers of RelA/p50 are primary mediators of NF-κB-target gene expression, although functional redundancy may be possible through contributions of c-Rel or RelB (4, 5). In an unstimulated state, NF-κB is sequestered in the cytoplasm through association with inhibitors of κB (IκBα, IκBβ, IκBe, NF-κB1, and NF-κB2). Upon signal-dependent phosphorylation and proteolysis, IκB proteins release the NF-κB dimer for accumulation in the nucleus and DNA binding. Sufficient steady-state levels and timely resynthesis of IκB is thought to be critical for proper temporal control of NF-κB-mediated gene expression (6, 7). NF-κB activation, such as that induced by TNF-α, is largely dependent on the activity of IκB kinase (IKK) proteins (2). The IKK complex typically consists of IKKα and IKKβ, which have enzymatic activity, and IKKγ/NEMO, which is required for complex formation and stability. These proteins encompass most, but not all, cellular IκB kinase activity (2). The complex relationship between IKK activity and NF-κB regulation is especially relevant to inflammatory disease, in which multiple pathways of NF-κB activation may be induced, both IKK mediated and IKK independent (8).

Accumulating evidence indicates that in genetically susceptible individuals, the initiating events in chronic, recurring intestinal inflammation may be a pathological synergy between defective innate immune responses and uncontrolled lamina propria mononuclear and T cell activation (9). The intestinal epithelial cell (IEC) monolayer is essential to mucosal innate immunity via its role in barrier function, antimicrobial peptide production, and regulation of mucosal immune responses to both commensal and pathogenic microflora (10, 11). Within this context, NF-κB and the signaling pathways that regulate its transcriptional activity are of central importance to both epithelial innate immunity and crosstalk between the mucosal immune system and the epithelial cell layer (12–15). Critical to this are pattern recognition receptors (PRR), such as CARD15/NOD2 and TLR, which are expressed in intestinal epithelium and mucosal immune cells and are activated by bacterial products (16, 17). Studies of PRR-null mice, as well as animals lacking IEC expression of IKK subunits, suggest that

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3Abbreviations used in this paper: IKK, IκB kinase; DAPA, DNA affinity protein-binding assay; Defcr, defensin-related cryptdin; DSS, dextran sulfate sodium; IEC, intestinal epithelial cell; PRR, pattern recognition receptor; Reg, regenerating islet-derived protein; TSLP, thymic stromal lymphopoietin; XIAP, X-linked inhibitor of apoptosis.

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epithelial NF-κB is critical for not only proper responses to luminal microflora, but also for maintaining tissue homeostasis in the intestine (14–19).

It is becoming increasingly clear that the barrier function of the IEC monolayer, enterocyte/immune cell communication, and epithelial cell migration following injury require fully functional NF-κB activity (20, 21). Mice lacking IKKβ or IKKγ/NEMO in IECs are susceptible to chemically induced colitis or develop spontaneous intestinal inflammation, respectively. Indications that NF-κB dimers containing the p50 subunit may be important for the IEC proliferative response required for healing mucosal injury can be seen in studies of p50-null mice that have moderately increased IEC proliferation levels (22). Importantly, mice lacking the p50 NF-κB subunit and one RelA allele (p50−/−/RelA−/−) are susceptible to pathogen-induced intestinal inflammation and are unable to resolve enteric infection (23, 24). Undercrossing the need for tight control of temporal and cell type-specific NF-κB activity, gnotobiotic mice that were associated with commensal microflora before weaning, although at low penetrance. Surviving NF-κB-deficient mice (26). These studies collectively suggest that NF-κB is required for basal ho-

Materials and Methods

Generation of RelAF/F and IEC-RelA−/− mice

Genomic sequences from the mouse RelA gene (MGI:103290), flanked by primer-based restriction sites, were PCR amplified from 129sv/Ea DNA using high-fidelity polymerase (LA Taq; TaKaRa). Initially, a fragment spanning 1185 to +2901 (transcriptional start site designated +1) was cloned into a vector containing one loxP site adjacent to flanked neo-mycin resistance and thymidine kinase expression cassettes. An additional segment of the RelA gene was then amplified with a second loxP site, and this fragment was cloned into the construct such that, upon Cre-mediated recombination, bp +2002 to +4889 was removed. This region contains exons 5–8 of RelA and codes for most of the Rel homology domain. The construct was completed by addition of homologous sequences spanning bp +4889 to +7497. This targeting construct was then sequenced in its entirety to ensure intact RelA coding sequences as well as proper orientation and placement of loxP sites. Electroporation of this construct into 129-derived ES cells, expansion of homologously targeted clones using positive and negative antibiotic selection, and FLPe-mediated removal of the neomycin cassette was performed by the Animal Models Core at the University of North Carolina. Following identification by PCR and Southern blotting of ES cell clones with properly targeted RelA, C57BL/6J blastocysts were injected with selected clones, and mice heterozygous for the loxP-flanked RelA allele (RelAF/F) were bred from chimeric founders. Dele-

letion of RelA from intestinal epithelia was achieved by breeding RelAF/F mice with villin-Cre transgenics (29). All mice used in the present studies were re-genotyped with respect to RelA and Cre following necropsy. All studies were performed with littermate RelAF/F and RelA−/− mice designated as wild-type controls and RelAF/F;villin-Cre animals denoted as IEC-RelA−/−. Animals were housed under specific pathogen-free conditions and given autoclaved chow and filtered air and water. During the course of these studies the standard practice of assessing co-housed sentinel animals containing an unexpected pathogen was not followed. No death or disease was noted in accordance with the Institutional Animal Care and Use Committees of the University of North Carolina and the Cincinnati Children’s Hospital Medical Center.

Isolation of primary intestinal epithelial cells

Terminal ileum (~5 cm proximal to cecum) and colon were removed from control and IEC-RelA−/− mice, rinsed with ice-cold PBS and cut open longitudinally. IEs were isolated using a low temperature protocol modified from Weiser and from Flint et al. (30, 31). Briefly, tissue was placed in chelating solution (5.0 mM Na2HPO4, 96 mM NaCl, 8 mM KH2PO4, 1.5 mM KCl, 0.5 mM DTT, 44 mM sucrose, 6.0 mM EDTA, 5.0 mM EGTA, pH 7.3) for 30 min at 4°C, and then epithelial cells were dissociated by repeated vigorous shaking. Tissue debris was removed and IEs were collected by centrifugation at 150 × g for 10 min at 4°C. Cells were washed in cold PBS and processed for protein in RNA. Viability of primary epithelial cells was confirmed using trypan blue staining and was typically >90%.

Real-time RT-PCR, immunoblotting, and DNA affinity protein-binding assay (DAPA)

Real-time RT-PCR, immunoblotting, and DAPA were performed as previously described except for the following changes (32). SYBR Green I-based detection (QuantitTect SYBR Green Master Mix, Qiagen), and a Stratagene Mx3000P PCR machine were used for gene expression analysis. Primer sequences are: regenerating islet-derived protein (REGIII) forward, 5′-TTCTGTGCTCCCATGAGTAACAAA-3′, REGIII reverse, 5′-CTATACATTGTTGGTTGATA-3′, REGIV forward, 5′-AGAGAAAGTGTTGCTAGGAG-3′, REGIV reverse, 5′-AATGTTGGAAGAGTTTGGCAAGG-3′, TLR4 forward, 5′-GCGTGTTCTATCCTCTTTTGAGG-3′, TLR4 reverse, 5′-CTGATTTCTCGGCATGGGAAG-3′. TLR5 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR5 reverse, 5′-ATGTTTGGTTGGTTGATA-3′, TLR9 forward, 5′-GAGTTTGGTTGGTTGATA-3′, TLR9 reverse, 5′-TCGACCACTTAAATACGCGTT-3′. TLR2 forward, 5′-GCGAGTGTTTTGAGTGGATA-3′, TLR2 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR1 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR1 reverse, 5′-ATGTTTGGTTGGTTGATA-3′, TLR7 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR7 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR8 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR8 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR10 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR10 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR11 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR11 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR12 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR12 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR13 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR13 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR14 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR14 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR15 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR15 reverse, 5′-ATGTTTGGTTGGTTGATA-3′. TLR16 forward, 5′-AGAGAAGGCTGTTTTCTTTGAAAG-3′, TLR16 reverse, 5′-ATGTTTGGTTGGTTGATA-3′.

Immunoblotting was performed using the following Abs: RelA (Santa Cruz Biotechnology) and cleaved caspase-3 (Cell Signaling Technology). In some studies, protein extracts were performed with littermate RelAF/F and RelA−/− mice designated as wild-type controls and RelAF/F;villin-Cre animals denoted as IEC-RelA−/−. Animals were housed under specific pathogen-free conditions and given autoclaved chow and filtered air and water. During the course of these studies the standard practice of assessing co-housed sentinel animals containing an unexpected pathogen was not followed. No death or disease was noted in accordance with the Institutional Animal Care and Use Committees of the University of North Carolina and the Cincinnati Children’s Hospital Medical Center.
were done with the cells in suspension at the time of passaging, effective received nonspecific siRNA. Pilot studies indicated that when transfections per crypt, as well as cells on one crypt side, were counted by an ob-
processed for immunohistochemisry as above. Properly oriented crypts,
EDTA) containing proteinase and phosphatase inhibitors (Sigma-Aldrich) mM NaCl, 10 mM Tris (pH 7.6), 10% glycerol, 1 mM DTT, 0.5 mM EDTA containing proteinase and phosphatase inhibitors (Sigma-Aldrich) as well as 0.1 µg/ml poly(dI-dC) at 4°C overnight. Preincubation of ex-
tects with excess nonbiotinylated dsDNA duplexes confirmed specificity. Duplex–protein complexes were purified using 100-µl Streptavidin Magnes- 
Sphere Paramagnetic beads (Promega) and a magnetic column and washed 
with DNA-binding buffer three times. Isolated proteins were fractionated 
on 4–12% NuPAGE gels (Invitrogen) and immunoblotted for p50, c-Rel, or RelA.

DSS colitis
Intestinal inflammation was induced by providing transgenic or control 
littermate mice with 5% DSS water (m.w. 36,000–50,000; MP Biomedici- 
cals) for either 7 days for acute studies or 5 days followed by 5 days of 
water for healing phase studies. Female mice were used for short-term 
experiments, and male animals were used for resolution studies. As 
has been reported, male mice, independent of genotype, were significantly 
more susceptible to DSS colitis (33). Animal weights and stool scores (0, 
normal; 1, soft; 2, diarrhea/minimal anorectal bleeding; 3, diarrhea/severe 
anorectal bleeding) were recorded daily. Although DSS injury occurs 
throughout the colon and ecum at varying levels, the distal colon is a 
primary site of injury (33). Therefore, mice were sacrificed, the ecum and 
colon were removed and flushed with ice-cold PBS, and the distal portion 
of the colon was cut into three equal segments and fixed in 10% formalin. 
The ecum was frozen for later analysis. For each distal colon sample, 
longitudinal cuts of tissue in which the three distal colon sections had 
clearly visible intestinal lumen were used in histological scoring with the 
observer blinded to genotype. Scoring parameters were modified 
from Cooper et al. and Hogan et al. and included quantitation of the area 
of distal colon involved, edema, erosion/ulceration of the epithelial 
monolayer, crypt loss/damage, and infiltration of immune cells into the 
mucosa (34, 35). Severity was graded on a scale that, for the area 
involved (erosion/ulceration and crypt loss), was defined as 0 (normal), 1 
(0–10%), 2 (10–25%), 3 (25–50%), and 4 (>50%). Edema and immu-
ne cell infiltration were scored as: 0, absent; 1, weak; 2, moderate; and 
3, severe. Total disease score was expressed as the mean of all 
combined scores per genotype.

Immunohistochemistry
Immunohistochemistry was performed as previously described with the 
following alterations (36). Quantitation of cleaved caspase-3 staining in 
epithelial cells of the distal colon was performed in a blinded manner by 
counting positive cells in multiple random microscope fields (100×) 
per tissue section (Fisher Micromaster digital microscope and Micron 
imaging software; Fisher Scientific). BrdU labeling was performed as 
suggested by the manufacturer (BrdU staining kit; Invitrogen). Mice were injected with 50 µg BrdU/g body weight 1.5 h before sacrifice and 
processed for immunohistochemistry as above. Properly oriented cyrpts, 
defined as those with a clear cell column lining both sides of an open 
luminal area, were photographed and the number of BrdU-positive cells per 
crypt, as well as cells on one crypt side, were counted by an ob-
server who was unaware of sample genotype. Microphotographs of his-
tologically stained sections were taken on an Axiosplan 2 microscope 
(Carl Zeiss MicroImaging).

RNA interference
Knockdown of mouse RelA was performed in m-IC142 cells, a nontrans-
formed mouse intestinal cell line (37), m-IC142 cells were grown in DMEM/
Ham’s F12, 60 nM sodium selenate, 5 µg/ml transferrin, 2 mM glutamine, 
50 nM dexamethasone, 1 nM triiodothyronine, 5 µg/ml insulin, 20 nM 
D-glucose, 10 nM epidermal growth factor, 2% FCS, and 20 mM HEPEs 
(pH 7.4). Lipofectamine 2000 (Invitrogen) was used for transfection of 
RelA-specific siGENOME ON-TARGET SMARTpool (L-040776-0, Dhar-
macon) into m-IC142 cells at a concentration of 100 nM. Control cells 
received nonspecific siRNA. Pilot studies indicated that when transfections 
were done with the cells in suspension at the time of passaging, effective knockdown of RelA could be obtained after 72 h. TNF-α treatment (20 
ng/ml, Peprotech) and nuclear extracts were isolated as previously de-
scribed (32). In some studies, an NF-κB luciferase reporter, mutant re-
porter, and IκBα-serine 32/36 to alanine mutant (IκBα-SR) plasmids were cotransfected with siRNA.

Organ culture and PGE2 ELISA
Colonie organ cultures were performed by harvesting distal colon from 
control and IEC-RelA−/− mice given water or DSS for 7 days. Tissue 
was flushed with cold PBS and mesentery was removed. One-centimetre 
trodes of colon were weighed and placed in 24-well plates contain-
ing DMEM, 0.5% FBS, penicillin (100 U/ml), streptomycin (100 
µg/ml), Fungizone (0.25 µg/ml) (Invitrogen), and Primocin (50 µg/ml) 
(InvivoGen). Once all study tissue was collected, media was replaced 
and explants were incubated overnight at 37°C. Supernatants were col-
cleared by centrifugation, and PGE2 levels were measured by 
ELISA (Cayman Chemical).

Statistics
All values are presented as mean ± SE. All comparisons are made between 
control and IEC-RelA−/− mice using the unpaired Student t test. Differ-
ences were considered statistically significant at p < 0.05.

Results
Neonatal mice lacking IEC expression of RelA have normal 
epithelial cell differentiation but are susceptible to spontaneous intestinal failure

Defining the physiological role of RelA in fully developed tissue 
has been difficult due to the TNF-α-dependent hepatocyte apopto-
sis and embryonic lethality that occurs in mice lacking this subunit of NF-κB (27, 28). Therefore, to investigate the role of RelA in 
a tissue-specific manner, we targeted the murine RelA locus with a 
conditional loxP-based allele that would allow removal of a major 
portion of the RelA genomic coding sequence upon in vivo expres-
sion of Cre recombinase. A targeting vector was designed in 
which exons 5–8 were flanked by loxP sequences (Fig. 1A). This 
portion of the RelA gene was selected for deletion because these 
exons code for the majority of the Rel homology domain that is 
esential for DNA binding activity as well as for dimerization of 
RelA with other transcription factors. Additionally, following Cre 
recombinase-mediated removal of exons 5–8, possible splicing 
from the now adjacent exons 4 and 9 resulted in an out-of-frame 
transcript with multiple and immediate stop codons, thereby less-
ening the likelihood of producing a truncated RelA protein with 
potentially hypomorphic or dominant negative properties. Ho-
myzogous mice with this conditional, loxP-flanked, or “floxed” 
RelA gene (RelAflO) are born in expected Mendelian ratios and are 
distinguishable from wild-type mice.

With the goal of investigating the role of RelA in physiological 
maintenance of the intestinal epithelial cell layer as well as its 
response to inflammation, RelAflO mice were bred with transgenic 
animals that express the Cre recombinase under the control 
of villin gene regulatory sequences. The villin-Cre transgene targets 
epithelial cell lineages of the distal small intestine, cecum, and colon, 
and expression occurs in intestinal crypt stem cells begin-
ning before birth (29). IEC-RelA−/− mice were generated and sur-
vived development in expected genetic ratios. PCR analysis re-
vealed, as expected, that rearrangement of the RelA allele did not 
occur in liver, spleen, kidney, lung, and skin but was present in the 
intestine (Fig. 1B and data not shown).

Although some IEC-RelA mice present with gastrointesti-
nal disease before weaning (see results below), most mice lacking RelA in ileal and colonic epithelia survive to adulthood.

We confirmed complete loss of RelA protein in the IEC monol-
layer by Western blotting of whole-cell extracts from isolated colonic epithelial cells of adult mice (Fig. 1C). The occasional trace 
amounts of RelA that remain are likely due to the previously 
characterized low levels of mosaic expression of the villin 
promoter-driven Cre enzyme or to the unavoidable small 
amounts of contaminating nonepithelial cells inherent to IEC 
isoation. In no instance did we note the appearance of smaller
molecular weight species when using several RelA Abs in IECs from IEC-RelA−/− mice, suggesting that Cre-mediated recombination of the conditional allele results in complete loss of RelA protein production (Fig. 1C and unpublished data). As reported by others (22), immunohistochemical analysis of the intestine showed that the RelA protein is expressed in a diffuse pattern in colonic epithelia and is localized to the nucleus of cells at the base and lower third of the crypt (Fig. 1D). Staining for RelA in IEC-RelA−/− mice showed that, as expected, Cre-mediated deletion is specific to the epithelial cell layer.
Because of the well-described role for NF-κB in differentiation of various cell types (38), we initially assessed IEC cell-type specification in control and IEC-RelA\(^{-/-}\) ileum and colon of apparently healthy 10–12-day-old mice. Alkaline phosphatase activity was present in differentiated villus epithelium in both control littermate and IEC-RelA\(^{-/-}\) ileum (Fig. 1E). Immunohistochemistry for lysozyme showed staining in small-bowel crypts and indicated no defects in Paneth cells numbers in wild-type and IEC-RelA\(^{-/-}\) mice (Fig. 1F). Furthermore, chromogranin A and Alcian blue staining also indicated no substantial genotype-based difference in numbers of enterocinocrine cells in the ileum (Fig. 1G) or of goblet cells in either the small or large intestine (Fig. 1, H and I). Hematoxylin-and-eosin staining of ileum and colon also indicated no gross abnormalities in intestinal morphology in neonatal mice (Fig. 1, J and K). Despite the seemingly normal development of the epithelial cell layer in IEC-RelA\(^{-/-}\) pups, we observed that ~10–15% of IEC-RelA\(^{-/-}\) mice displayed abdominal discoloration and diarrhea, which began within 2–3 days after birth. This resulted in poor weight gain (10–12-day-old wild type, 6.6 ± 0.15 g; unaffected IEC-RelA\(^{-/-}\), 6.1 ± 0.09 g; runted IEC-RelA\(^{-/-}\), 3.5 ± 1.1 g; n = 3–6 per genotype). These mice died before day 25. Upon closer examination, severe gastrointestinal pathology was apparent and was typified by thin-walled, pale small intestine and colon that were often distended with air (Fig. 1L) or filled with colon that were often distended with air (Fig. 1L). Hematoxylin-and-eosin staining of ileum and colon also indicated no gross abnormalities in intestinal morphology in neonatal mice (Fig. 1, J and K).

Although complete analysis of this phenotype is ongoing, we initially investigated NF-κB-dependent gene expression levels in isolated ileal IECs from 12–14-day-old control and IEC-RelA\(^{-/-}\) pups. Importantly, IECs were purified when no visible pathology was present in an effort to determine the status of NF-κB-target genes before disease onset, as we reasoned that this would provide an indication of the basis of this sporadic neonatal phenotype. Real-time RT-PCR analysis confirmed low expression of IκBα, an archetypal RelA-dependent gene (Fig. 2). Additionally, several antiapoptotic genes such as RegIV and Bcl-x\(_L\) were decreased. Other antiapoptotic NF-κB-target genes such as survivin and X-linked inhibitor of apoptosis (XIAP) were unaffected (Fig. 2 and data not shown). These results imply that the absence of RelA in the epithelial cell layer may confer susceptibility to stress-induced cell death (39, 40). Genes regulated by the presence of luminal microflora such as Reg3γ, Defcr4, and Defcr5 are also expressed at low levels in ileal epithelia of IEC-RelA\(^{-/-}\) pups as compared with littermate controls (16, 41). Collectively, these data suggest that, although epithelial cell differentiation occurs normally, loss of RelA-mediated gene expression may result in susceptibility to neonatal intestinal failure.

**Deletion of epithelial RelA results in elevated c-Rel and p50 activity**

Previous studies suggest that the total cellular NF-κB transcriptional activity is highly sensitive to loss of individual subunits and that basal activity equilibrates through coordinated alterations in protein levels and DNA binding activity of the remaining IκB/NF-κB complexes (4, 6). Because potentially compensatory NF-κB activity is critical for understanding the effects of loss of RelA in intestinal epithelial cells, we examined IκB proteins as well as the NF-κB subunits p50 and c-Rel in isolated primary cells lacking RelA. As expected (27, 42), RelA deletion in colonic epithelia had no effect on total cellular levels of p50 or c-Rel but did result in dramatically decreased levels of IκBα and IκBβ (Fig. 3A). Next, we isolated nuclear extracts from colonocytes of untreated or LPS-injected control and IEC-RelA\(^{-/-}\) mice and examined nuclear translocation and DNA binding activity of relevant NF-κB subunits. Although the anticipated nuclear accumulation of p50, c-Rel, and RelA was apparent in endotoxin-treated control mice, IECs lacking RelA had elevated translocation of c-Rel and p50 (Fig. 3B). Notably, the DNA binding potential of the c-Rel subunit of NF-κB in intestinal epithelia is thought to be, under normal conditions, low or absent (22, 32). However, as measured by DNA affinity pull-down assays, loss of RelA leads to a shift in the composition of bound NF-κB dimers with substantial c-Rel and p50 binding in epithelia of both ileum (data not shown) and colon (Fig. 3, C and D). This suggests that c-Rel/p50 may control expression of a subset of NF-κB-dependent genes in the RelA-null intestine. To confirm the presence of potentially compensatory NF-κB activity in IECs lacking RelA, we used a more easily controlled in vitro system in which RNA interference was used to greatly diminish RelA protein levels. Knockdown of RelA in the nontransformed mouse intestinal cell line m-IC\(_{cl2}\) resulted in coordinately decreased stability of IκBα and IκBβ protein in unstimulated cells (Fig. 3E). Similarly, the lack of significant levels of nuclear RelA upon TNF-α treatment resulted in elevated c-Rel DNA binding activity (Fig. 3E, lower panel) and suggested that this system was a valid model of in vivo conditions. NF-κB luciferase reporter assays indicated that, although decreased RelA protein significantly blunted luciferase production, both basal and TNF-α-induced NF-κB activity remain (Fig. 3F). Coexpression of a stabilized IκBα mutant (IκBα-SR) resulted in complete loss of NF-κB-mediated luciferase activity and confirms that the remaining reporter gene expression was NF-κB, and likely c-Rel/p50, dependent (Fig. 3F). Having determined that loss of RelA results in a shift in the predominant NF-κB subunit composition and activity in intestinal epithelia, we next determined the impact that this had on cell survival and division of RelA-null IECs.

**RelA is required for proliferative and apoptotic homeostasis of the fully mature intestinal epithelial cell monolayer**

The specific gene expression defects identified in neonatal intestine, along with previous work in p50 and IEC-IKKγ/NEMO-null mice (14, 22), support the possibility that deregulation of basal proliferation and apoptosis may be present in surviving, adult IEC-RelA\(^{-/-}\) mice. Therefore, we determined rates of IEC turnover in adult control and IEC-RelA\(^{-/-}\) intestine. Proliferation was measured by determining the average number of BrdU-labeled cells per well-oriented crypt in the distal colon of IEC-RelA\(^{-/-}\) and wild-type littermate mice. Loss of RelA in the colon results in increased proliferation, with IEC-RelA\(^{-/-}\) mice having nearly twice the number of mitotic cells as compared with control animals.
decreased levels of IκBα reporter assays using plasmids containing an NF-
κB-responsive site (Fig. 4). Large clusters of apoptotic epithelial cells were found in IEC-RelA−/− mice, and most crypts had numerous cells with activated caspase-3 (Fig. 4D).

We next investigated NF-κB-regulated gene expression in IEC-RelA−/− mice using real-time RT-PCR to determine possible causes of altered epithelial proliferation and apoptosis. Antiapoptotic Bcl-xL expression was significantly decreased in isolated RelA-null colonocytes (Fig. 4E). Other NF-κB-target genes such as XIAP, cellular inhibitor of apoptosis 2, Casp8 and FADD-like apoptosis regulator (CFLAR, also called cFLIP), and survivin were not affected. We also found a notable decrease of IL-11 and IL-18 expression in isolated colonocytes of IEC-RelA−/− mice. These interleukins, which target IECs in a paracrine manner, are antiapoptotic and essential for both epithelial restitution and resistance to acute intestinal inflammation (43–45). Because activation of NF-κB by pattern recognition receptors is critically important for epithelial cell interactions with luminal microbiota, we measured expression of several TLR proteins known to be expressed in colonocytes but found no differences between genotypes (Fig. 4E).

IEC-RelA−/− mice that reach maturity show no gross signs of intestinal disease, and yet increased epithelial cell apoptosis may lead to low levels of inflammation. Accordingly, we addressed this possibility by measuring cytokine expression in whole ceca of 12-wk-old littermate control and IEC-RelA−/− mice. No indications of altered levels of proinflammatory genes such as MCP-1/CCL2, IL-6, or RANTES/CCL5 were found (Fig. 4F). Additionally, we found no changes in expression of TSLP or TNF (ligand) superfamilies, member 13 (also called a proliferation-inducing ligand, or APRIL) (data not shown). These cytokines are necessary for IEC-directed dendritic cell maturation and class-switch recombination to IgA in mucosal B cells, respectively (15, 46). Of note, a significant elevation in NOD2 expression was found in ceca of IEC-RelA−/− mice, and TLR4 and TLR9 levels trended upward (p = 0.07). Additional work will be necessary to determine the possibility of aberrant immune cell presence in the mucosa of IEC-RelA−/− mice.

RelA activity protects intestinal epithelia during inflammation

We hypothesized that deregulated cell division and elevated apoptosis in the intestine of IEC-RelA−/− mice would result in increased susceptibility to monolayer injury, exposure of the submucosa to luminal contents, and severe inflammation. To investigate this possibility, we used an established model of chemically induced colitis (administration of DSS in drinking water) that would allow us to closely regulate the initiation of disease. Initially, control and IEC-RelA−/− mice were given 3% DSS for 2 days and then analyzed to determine susceptibility of the IEC monolayer during the initial stages of injury. We did not observe cell death in DSS-treated IEC-RelA−/− mice that was significantly greater than that seen in IECs of untreated IEC-RelA−/− animals (Fig. 4A). Despite this significant increase in dividing cells in RelA-null crypts, only a moderate increase in IECs per crypt was detected (Fig. 4B). This led us to investigate whether the increased proliferation in this cell layer was accompanied by elevated apoptosis. Immunohistochemical staining for cleaved caspase-3 was performed on tissue sections from IEC-RelA−/− and control mice and was quantified by counting positive epithelial cells in random high-power microscope fields. The RelA-null epithelial monolayer had elevated numbers of apoptotic cells that were predominantly crypt epithelia, suggesting a protective role for RelA in intestinal stem or progenitor cells that cannot be compensated for by enhanced c-Rel activity (Fig. 4C). Whole-cell levels of p50 and c-Rel are unchanged.

FIGURE 3. Enhanced NF-κB (c-Rel/p50) activity in intestinal epithelia lacking RelA. A, Immunoblotting of extracts from isolated primary colonocytes of three control and three IEC-RelA−/− mice shows loss of RelA and decreased levels of IκBα and IκBβ. Whole-cell levels of p50 and c-Rel are unchanged. B, Control and IEC-RelA−/− mice were injected with LPS (2.5 μg/g), and IEC nuclear extracts from colon were isolated 90 min later and immunoblotted. C, DNA affinity pull-down assay on nuclear extracts from isolated colon epithelial cells of LPS-injected control and IEC-RelA−/− mice. Competition with excess nonbiotinylated NF-κB site DNA duplexes shows specificity (D). E, Immunoblot of whole-cell extracts from m-ICcl2 cells transfected with RelA siRNA. RelA knockdown (RelA-KD) coincided with decreased IκBα and IκBβ (upper panel). DNA affinity pull-down assay in control and RelA-KD cells showed RelA and c-Rel binding following TNF-α stimulation (20 ng/ml) for 60 min (lower panel). F, NF-κB luciferase reporter assay in control and RelA-deficient cells. Luciferase reporter assays using plasmids containing an NF-κB-responsive (Luc) or NF-κB mutant (Mut) promoter as well as stabilized mutant IκBα (IκB-SR).
Two days on DSS caused enhanced c-Rel DNA-binding activity in isolated RelA-null IECs as compared with controls (data not shown). We next determined the response of IEC-RelA−/−/mice to longer DSS treatment that results in significantly elevated mucosal cytokine production and acute colitis. In these studies, we provided control and IEC-RelA−/−/mice with 3% DSS drinking water for 7 days. All mice showed the expected soft stool and diarrhea but, by days 5–6, gross rectal bleeding was noted in IEC-RelA−/−/mice (Fig. 5A). IEC-RelA−/−/mice lost significant amounts of weight as compared with control animals by study day 7 (Fig. 5B). We then measured the degree of intestinal injury and inflammation in IEC-RelA−/−/mice via histological examination of the distal colon. Integration of several individual scoring parameters indicated that disease severity was elevated in the intestines of mice lacking epithelial RelA (Fig. 5C). IEC-RelA−/−/mice displayed increased mucosal damage and immune cell infiltration as well as substantial, specific injury to the epithelial cell layer, as measured by monolayer ulceration and crypt loss (Fig. 5D). We further assessed inflammation in IEC-RelA−/−/null mice by quantifying expression levels of proinflammatory cytokines and chemokines in the cecum. Although mice lacking RelA in intestinal epithelia had higher levels of IL-6 and MCP-1 following 7 days on DSS as compared with control littermate mice, some cytokines such as CXCL1 and TNF−/−/were found to be similar (Fig. 5E). PGE2 plays an important role in mucosal response to injury, and mice lacking COX-2 are highly susceptible to DSS colitis (47–49). Therefore, we next assessed COX-2 protein levels in the distal colon of control and IEC-RelA−/−/mice given DSS for 7 days. While COX-2 was moderately increased in control mice, we also found enhanced levels in RelA-null animals (Fig. 5F). We next measured prostaglandin production in cell-culture media of colonic explants from water and DSS-treated mice. PGE2 production was elevated in IEC-RelA−/−/tissue relative to samples from colitic control mice (Fig. 5G). As seen in mice lacking enterocyte IKKβ, these data suggest that the epithelia is not the predominant site of NF-kB-mediated COX-2 expression and PGE2 production (40). Elevated levels of PGE2 produced by colitic tissue of IEC-RelA−/−/mice...
may have important implications regarding levels of enterocyte apoptosis and proliferation (49, 50).

Cytokine production that results from DSS treatment may further exacerbate inflammation by eroding the epithelial cell layer in IEC-RelA−/− mice. Therefore, we determined levels of apoptosis in the remaining epithelial cells of IEC-RelA−/− mice. Although the number of cleaved caspase-3-positive cells is more than three times higher in RelA-null epithelia (Fig. 6A), this likely is an underestimation because of the significant loss of intestinal epithelia in these mice. We typically noted fewer epithelia per field in IEC-RelA−/− mice as compared with controls but still observed elevated numbers of apoptotic colonocytes. Continuous regions of cleaved caspase-3-positive epithelial cells were commonly found in IEC-RelA−/− mice, and this was consistent with the severe ulceration and crypt loss typically seen in these animals (Fig. 6B). Based on the elevated levels of apoptosis seen in RelA-null IECs, we next investigated the proliferative response of control and RelA-null epithelial cell layers to DSS-induced inflammation. We found that significantly more BrdU-labeled cells were evident in IEC-RelA−/− mice (Fig. 6C). Quantitation of mitotic cells in well-oriented crypts indicated that IEC-RelA−/− mice have approximately three times as many dividing cells per crypt as compared with control animals on DSS (Fig. 6D). These data collectively imply an essential role for RelA-mediated NF-κB activity in antiapoptotic protection of the intestinal epithelial cell monolayer and coordinated proliferative responses to injury.

RelA is required for epithelial cell monolayer recovery from acute inflammation

NF-κB activity facilitates epithelial cell migration and may mediate effective wound-healing responses (20, 21). Therefore, we speculated that loss of RelA in the intestine would result in progressive disease and tissue damage due to the inability of the epithelial monolayer to reestablish itself and limit the expansion of inflammation. We placed mice on 3% DSS for 5 days and then provided them with water for an additional 5-day recovery period. Histological characterization confirmed that IEC-RelA−/− mice had severe injury (Fig. 7A). Loss of RelA resulted in large ulcerations and widespread crypt loss, whereas the epithelia of littermate control mice showed significant crypt expansion and had

**FIGURE 5.** Mice lacking intestinal RelA are highly susceptible to mucosal injury. IEC-RelA−/− and littermate control mice were given 3% DSS for 7 days and then analyzed. A and B, DSS colitis resulted in more severe stool score and weight loss in mice lacking IEC RelA (n ≥ 10 mice per genotype). C, Disease was quantitatively higher in IEC-RelA−/− mice upon histological scoring. D, Epithelial monolayer ulceration, crypt loss, and extent of immune cell infiltration were higher in IEC-RelA−/− mice (n ≥ 10 mice per genotype). E, Proinflammatory gene expression was elevated in ceca of IEC-RelA−/− mice relative to controls (n ≥ 3 mice per genotype). F, COX-2 protein levels were analyzed by immunoblot in distal colon of wild-type and IEC-RelA−/− mice on water or DSS. G, Distal colon segments from water- or DSS-treated mice were cultured for 24 h, and PGE2 levels in tissue culture media were measured by ELISA (n = 4 water and 6 DSS mice per genotype; two segments measured per mouse).
fewer large ulcerations (Fig. 7B). With the use of immunohistochemistry, we found that the sporadic areas of epithelia regrowth in IEC-RelA−/− mice did not express RelA (data not shown), indicating that it is not absolutely required for epithelial stem cell expansion. The culminating effects of significant and early mucosal damage coupled with poor restitution of the epithelial monolayer following removal of DSS were seen in the enhanced mortality of IEC-RelA−/− mice relative to controls (Fig. 7C). Closer inspection revealed that the epithelia of IEC-RelA−/− mice continued to undergo increased levels of apoptosis (Fig. 7, D and E). Notably, quantitation of disease severity and levels of apoptosis in
IEC-RelA−/− mice as compared with control animals may be significantly underestimated due to the decreased survival of mice lacking IEC RelA. Collectively, these data suggest that RelA is required to protect the epithelial monolayer during the initial stages of mucosal injury as well as during the reestablishment of the epithelial cell layer. However, note that the poor survival of IEC-RelA−/− mice may result from both the more extensive initial DSS-mediated injury as well as the continued loss of epithelial cells to apoptosis, factors that cannot be overcome by elevated IEC proliferation.

Discussion

The NF-κB/IKK signaling system provides an attractive therapeutic target based on its critical role in intestinal inflammatory diseases. However, recent work suggests that blocking IKK activity may have widely differing results in various cell types, with its loss in activated immune cells suppressing inflammation via decreased cytokine production, and epithelial-specific ablation causing apoptosis-induced barrier disruption and initiation of mucosal inflammation (14, 40, 51). Additionally, as the number of IKK substrates grows, it is now clear that the IKK subunits have both pro- and anti-inflammatory functions as well as NF-κB-independent targets (3, 52–55). Furthermore, determining the best approach for therapeutic manipulation of this signaling pathway in intestinal disease has been hampered by the lack of tissue-specific genetic removal of NF-κB subunits in the intestine. Using mice lacking RelA in intestinal epithelial cells, we present data indicating that RelA-mediated NF-κB activity is essential for control of proliferative and apoptotic homeostasis in the intestine. We also show that, despite the presence of c-Rel/p50 activity, the expression of a number of NF-κB-target genes involved in innate immunity is lost in IEC-RelA−/− animals. Evidence for the sensitivity of these animals to intestinal pathology can be seen in the increased levels of perinatal disease and death of IEC-RelA−/− mice as well as sensitivity of adult animals to chemically induced colitis. These data support a protective role of epithelial RelA/NF-κB activity during acute intestinal inflammation but also have implications for its suitability as a drug target due to its functional importance in maintaining the normal physiology of the resting, unchallenged intestine.

An important mechanistic basis for the catastrophic intestinal phenotype seen in some IEC-RelA−/− neonatal mice may be the loss of coordinated IkB/NF-κB feedback regulation. We demonstrate low levels of IkBα proteins in IECs lacking RelA as well as elevated nuclear localization and DNA binding activity of c-Rel/p50. A primary role for IkBα and IkBβ is to block the DNA binding potential of NF-κB, and it is evident that these proteins are required for coordinated gene activation and deactivation (6, 7, 56). For example, loss of IkBα results in sustained NF-κB activity in vitro as well as widespread inflammatory disease in mice (4, 6, 57). Maintaining sufficient levels of IkBα may be especially important in intestinal epithelia where tolerance to PRR ligands through properly controlled NF-κB activity is likely mediated by stabilization of IkB (17, 58, 59). In the developing epithelia of mice and preterm infants, low levels of IkBα/β protein are a suggested driving force in the deregulated NF-κB activity, enhanced cytokine production, and uncontrolled inflammation of necrotizing enterocolitis (12, 60). Although our data are consistent with enhanced c-Rel/p50-mediated proinflammatory gene expression driving intestinal disease in IEC-RelA−/− mice, other possibilities exist. For example, normally innocuous neonatal stress, along with loss of RelA-mediated antimicrobial and antiapoptotic gene expression in the maturing intestine, may result in elevated apoptosis of the epithelial cell layer. The subsequent loss of barrier function could lead to increased exposure of mucosal immune cells to luminal contents and uncontrolled intestinal inflammation similar to that seen in mice lacking IEC expression of IKKγ/NEMO (14). A primary difference between the IEC-RelA−/− mice described here and the recently reported IEC-IKKγ/NEMO−/− animals with respect to the frequency of spontaneous inflammation may be the presence of compensatory c-Rel-mediated antiapoptotic gene expression that, in the absence of a stochastic, disease-initiating event early in life, allows for relatively unaffected IEC-RelA−/− animal growth (22). Additional work will be necessary to define the consequences of RelA deletion in neonatal enterocytes during the initial coordination of mucosal innate and adaptive immune responses. Understanding the function of epithelial RelA, as well as other NF-κB subunits, in the developing intestine may have direct relevance to the pathogenesis of necrotizing enterocolitis.

Our data clearly demonstrate that in adult animals, IEC RelA/NF-κB plays a protective role during intestinal inflammation and this likely occurs through regulation of antiapoptotic gene expression and wound healing. Decreased production of two protective interleukins that are expressed in IECs, IL-18 and IL-11, may have profound effects on antiapoptosis and mucosal restitution in IEC-RelA−/− mice. Animals lacking IL-18 are susceptible to DSS-induced colitis due to poor mucosal repair and are unable to elevate IL-11, an established target gene of IL-18 signaling (44, 61). IL-11, in turn, is highly increased during experimental colitis, and expression of the IL-11 receptor in the intestine is mainly found in IECs. Activation of Akt signaling by IL-11 in colonic epithelia has potent antiapoptotic activity (45, 61). Additionally, loss of Bcl-xL expression in IECs of neonatal and adult IEC-RelA−/− mice is consistent with the elevated basal levels of apoptosis that we observed in these animals. It is possible that c-Rel compensates for loss of RelA in basal expression of some antiapoptotic NF-κB target genes such as cFLIP, XIAP, and survivin. Further work is required to determine whether, under the significant stress of elevated cytokine production and DNA damage associated with experimental murine colitis (62, 63), the ability of c-Rel to provide some measure of antiapoptotic gene expression is surpassed, resulting in widespread epithelial cell death.

IEC-RelA−/− mice given DSS have significant IEC apoptosis, and this results in poorly resolving injury to the intestinal mucosa that often results in animal death. These data are highly similar to those seen in intestine-specific ablation of IKK subunits. Mice lacking IKKβ in intestinal epithelia have more severe inflammation and ulceration in response to DSS challenge that correlated with increased apoptosis and loss of Bcl-xL expression (40). We report herein a large increase in proliferation of the RelA-null epithelial cell layer in response to inflammation. It seems likely that, as shown previously in hepatocyte-null IKKβ mice, the proliferative response in IEC-RelA−/− animals may be secondary to elevated JNK-induced apoptosis (64). Although present at much lower levels, the moderately elevated IEC apoptosis seen in unchallenged IEC-RelA−/− mice resembles that which is thought to initiate colitis in mice with ablated epithelial expression of IKKγ/NEMO. However, a significant difference between mice lacking IKKβ or IKKγ/NEMO in IECs and the IEC-RelA−/− mice described herein is the continued presence of undiminished IKK complex activity, decreased IkB proteins, and altered c-Rel/p50 NF-κB transcriptional potential. This may be especially relevant in light of the well-understood role for c-Rel in proliferation and oncogenesis (65). Accordingly, the elevated proliferative response and uncontrolled inflammation seen in IEC-RelA−/− mice suggest that RelA may have an important role in tumor initiation in the intestine and may be critical in the context of colitis-associated colorectal cancer.
It is increasingly evident that the IKK/NF-κB signaling pathway is an important mediator of mucosal homeostasis. Decreased epithelial IKK activity and, as we have shown, deletion of RelA/NF-κB result in defective antimicrobial and antiapoptotic gene expression, elevated proliferation and cell death of epithelia, and susceptibility to intestinal inflammation. Collectively, it is apparent that IKK/NF-κB activity in the intestinal epithelium is required for suppression of microflora-induced inflammation in early life, as well as for effective mucosal immune responses to infection and injury in adult animals. Although inhibition of NF-κB and its upstream signaling kinases may be a highly effective means of decreasing the severity of active, chronic inflammation, loss of NF-κB-mediated gene expression in epithelial cells may have detrimental effects on the initiation, as well as the eventual resolution, of inflammatory disease.

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References


