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IKKβ Links Inflammation and Tumorigenesis in a Mouse Model of Colitis-Associated Cancer

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Summary

A link between inflammation and cancer has long been suspected, but its molecular nature remained ill defined. A key player in inflammation is transcription factor NF-κB whose activity is triggered in response to infectious agents and proinflammatory cytokines via the IκB kinase (IKK) complex. Using a colitis-associated cancer model, we show that although deletion of IKKβ in intestinal epithelial cells does not decrease inflammation, it leads to a dramatic decrease in tumor incidence without affecting tumor size. This is linked to increased epithelial apoptosis during tumor promotion. Deleting IKKβ in myeloid cells, however, results in a significant decrease in tumor size. This deletion diminishes expression of proinflammatory cytokines that may serve as tumor growth factors, without affecting apoptosis. Thus, specific inactivation of the IKK/NF-κB pathway in two different cell types can attenuate formation of inflammation-associated tumors. In addition to suppressing apoptosis in advanced tumors, IKKβ may link inflammation to cancer.

Introduction

For about two millennia, a causal relationship between inflammation and cancer has been suspected. It was Galen who first noted this relationship and later in the 19th century Rudolf Virchow had demonstrated the presence of leukocytes in malignant tissues and claimed that tumors arise from regions of chronic inflammation (Balkwill and Mantovani, 2001). The microenvironment in and around tumors contains cells of the innate immune system that secrete proinflammatory cytokines and chemokines, such as TNFα, IL-1, IL-6, and IL-8, as well as matrix-degrading enzymes, growth factors and reactive oxygen species (ROS), which can promote DNA damage (Coussens and Werb, 2002). This environment enhances cell proliferation, cell survival, cell migration, and angiogenesis, thereby promoting tumor development. Notably, the inflammatory response is similar in many aspects to a wound-healing response and tumors have been considered as wounds that do not heal (Dvorak, 1986). Chronic infection and consecutive inflammation may directly affect the cells that eventually become transformed as well as exert indirect effects on the tumor cell through surrounding cells. One example for direct action of inflammatory stimuli on cells that eventually become transformed is provided by MALT lymphoma, where chronic infection may cause persistent B-cell activation culminating in chromosomal rearrangements that cause cancer (Wotherspoon et al., 1991). Infection of hematopoietic and epithelial cells by viruses or bacteria can directly induce mutations in tumor suppressors and protooncogenes or epigenetically alter signaling pathways that affect cell proliferation and/or survival (Coussens and Werb, 2002). However, it is usually envisioned that inflammation stimulates the formation of epithelial-derived tumors, the most common class of cancers, through an indirect mechanism involving activation of surrounding inflammatory cells (Balkwill and Mantovani, 2001). The interplay between epithelial cells and inflammatory cells is likely to be a crucial, but poorly understood, process during inflammation-associated tumor development.

A key player in inflammatory processes is transcription factor NF-κB (Barnes and Karin, 1997; Chen et al., 2003), which consists of a number of closely related protein dimers that bind a common sequence motif (Ghosh et al., 1998). In resting, nonstimulated cells NF-κB dimers are cytoplasmic but translocate to the nucleus in response to a variety of proinflammatory stimuli. Two major pathways account for nuclear translocation (i.e., activation) of NF-κB. The canonical NF-κB activation pathway, applies to dimers composed of RelA, c-Rel, and p50, which are retained in the cytoplasm by specific inhibitors, the IκB proteins (Ghosh and Karin, 2002). This pathway is normally triggered in response to microbial and viral infections and proinflammatory cytokines all of which activate the IκB kinase (IKK) complex (Ghosh and Karin, 2002). IKK phosphorylates NF-κB bound IκBs and targets them for ubiquitin-dependent degradation and allowing liberated NF-κB dimers to enter the nucleus (Karin and Ben-Neriah, 2000). IκB phosphorylation depends mainly on the IKKβ catalytic subunit of the IKK complex (Li et al., 1999a, 1999b). The second pathway, the alternative pathway, depends on processing of the NF-κB2 precursor protein, which preferentially binds RelB in the cytoplasm, resulting in release of RelB:p52 dimers (Ghosh and Karin, 2002). This pathway depends on the IKKα subunit (Senftleben et al., 2001) and is IKKβ-independent (Dejardin et al., 2002).

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Both pathways ultimately lead to transcription of distinct sets of target genes, mediating different biological functions.

The involvement of the canonical IKKβ/NF-κB activation pathway in acute inflammation and cell survival is well established (Chen et al., 2003; Maeda et al., 2003). In addition to inflammation, persistent NF-κB activation was suggested to contribute to cancer (Karin et al., 2002). Chromosomal rearrangements leading to constitutive NF-κB activation or overexpression of NF-κB subunits were detected in lymphoid malignancies (Gilmore, 1999; Hacker and Karin, 2002). Activated NF-κB was also detected in many solid tumors and its inhibition in tumor cell lines increases their sensitivity to chemotherapeutic drugs and radiation (Amit and Ben-Neriah, 2003). The latter effect is related to the known ability of NF-κB to activate genes whose products inhibit apoptosis (Karin and Lin, 2002), which could be instrumental in tumor development. So far, however, the role of NF-κB in the initiation of solid tumors has not been examined, except for skin tumors, where inhibition of NF-κB was found to promote squamous cell carcinomas (Dajee et al., 2003; Seitz et al., 1998; van Hogerlinden et al., 1999). Yet, the ability of NF-κB to be activated by proinflammatory stimuli and in turn inhibit apoptosis led us to propose that the canonical IKKβ-NF-κB pathway may provide the critical mechanistic link between inflammation and cancer (Karin et al., 2002).

Activation of NF-κB in response to chronic inflammation may be of particular relevance to gastrointestinal (GI) carcinogenesis, especially in gastric cancer and colitis-associated cancer (CAC). The latter arises in patients with inflammatory bowel disease (IBD), particularly ulcerative colitis. CACs comprise up to 5% of all colorectal cancers (Chung, 2000). The cumulative incidence of CAC in patients with ulcerative colitis 25–30 years after diagnosis ranges from 8% to 43% (Ekbom, 1998), accounting for one sixth of all deaths in this group (Munkholm, 2003). Anti-inflammatory therapy with nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of CAC by 75% to 81% (Eaden et al., 2000). NSAIDs were reported to inhibit the IKKβ-dependent NF-κB-signaling pathway (Kopp and Ghosh, 1994; Yamamoto et al., 1999; Yin et al., 1998), in addition to their known ability to inhibit cyclooxygenases (COX) and prostaglandin synthesis (Gupta and Dubois, 2001). Activated NF-κB was detected in lamina propria macrophages and epithelial cells from biopsy specimens or cultured cells of IBD patients (Rogler et al., 1998) as well as in colorectal and cancer but not in adjacent normal tissue (Lind et al., 2001; Yu et al., 2003). Moreover, in human sporadic colonic adenomatous polyps, RelA was detected in nuclei of stromal macrophages that also expressed certain NF-κB target genes, including COX-2 (Hardwick et al., 2001). COX-2 expression in tumor-infiltrating macrophages is an early event in colon carcinogenesis and inhibition of COX-2 activity represents an effective chemopreventive strategy (Janne and Mayer, 2000).

Although the evidence for potential involvement of NF-κB in colitis and colorectal cancer is quite substantial, a direct genetic proof of its role in tumor initiation and promotion was heretofore not available. We examined whether tissue-specific deletion of IKKβ in the two cell types where NF-κB is activated during colitis and CAC, namely enterocytes and macrophages, could prevent tumor initiation and progression in a mouse model of CAC (Okayasu et al., 1996). We show that inactivation of IKKβ in either cell type can indeed reduce the incidence and development of inflammation-associated cancer, but in each cell type IKKβ acts through a different mechanism. While in enterocytes IKKβ contributes to tumor initiation and promotion by suppressing apoptosis, but is not required for inflammation per se, in myeloid cells it is involved in production of inflammatory mediators that promote tumor growth.

Results

Deletion of IKKβ in Enterocytes Greatly Decreases Colitis-Associated Tumor Incidence

Azoxymethane (AOM) is a procarcinogen, which upon metabolic activation causes formation of O^6-methylguanine (Pegg, 1984). AOM induces tumors in the distal colon of rodents and is commonly used to elicit colorectal cancer in experimental animals (Boivin et al., 2003). To investigate the role of IKKβ in CAC, we used a model in which 6–8-week-old mice were injected with a single dose of AOM (12.5 mg/kg) followed by three cycles of dextran sulfate sodium salt (DSS) administration (cycle 1: 2.5%, five days; cycle 2: 2.5%, five days; and cycle 3: 2%, four days) in the drinking water (Figure 1A). Repeated DSS administration causes chronic inflammation, thereby mimicking IBD, which greatly enhances the incidence of AOM-induced tumors (Okayasu et al., 1996).

This protocol was applied to mice that lack IKKβ only in their intestinal epithelial cells (*villin-Cre/Ikkβ*$^{F/\Delta}$ mice) (Chen et al., 2003) and their littermate controls (*Ikkβ*$^{F/\Delta}$ mice). Previous results indicate that deletion of IKKβ in enterocytes does not perturb normal gastrointestinal development or function in nonchallenged mice and has no effect on tissue composition or body weight (Chen et al., 2003; Egan et al., 2004). Regardless of genotype, all mice treated with AOM plus DSS developed tumors, whereas mice receiving DSS alone without AOM did not develop tumors during the observation period (data not shown). We observed a dramatic decrease of 75% in tumor incidence in the IKKβ knockout group (Figure 1B). These tumors were located in the middle to distal colon (Figures 1C and 1D), which is the area where the most severe changes during DSS colitis occur (Okayasu et al., 1990), suggesting that the severity of inflammation correlates with increased tumor incidence. The tumors were mostly broad-based adenomas with high-grade dysplasia and varying degree of inflammatory cell infiltration (Figures 1E–1J). Adenomas did not penetrate the muscularis mucosa in any of the groups. Even though the tumors resemble histologically those that appear in *Apc*$^{Min/+}$ mice, which are also rarely invasive, the latter are mainly localized to the small intestine (Moser et al., 1990). Since AOM leads to induction of mutations throughout the whole intestine, it is likely that the inflammation, which is localized to the large intestine, is crucial for tumorigenesis in the AOM-DSS model. No significant differences in sizes, proliferation index, or apoptotic in-

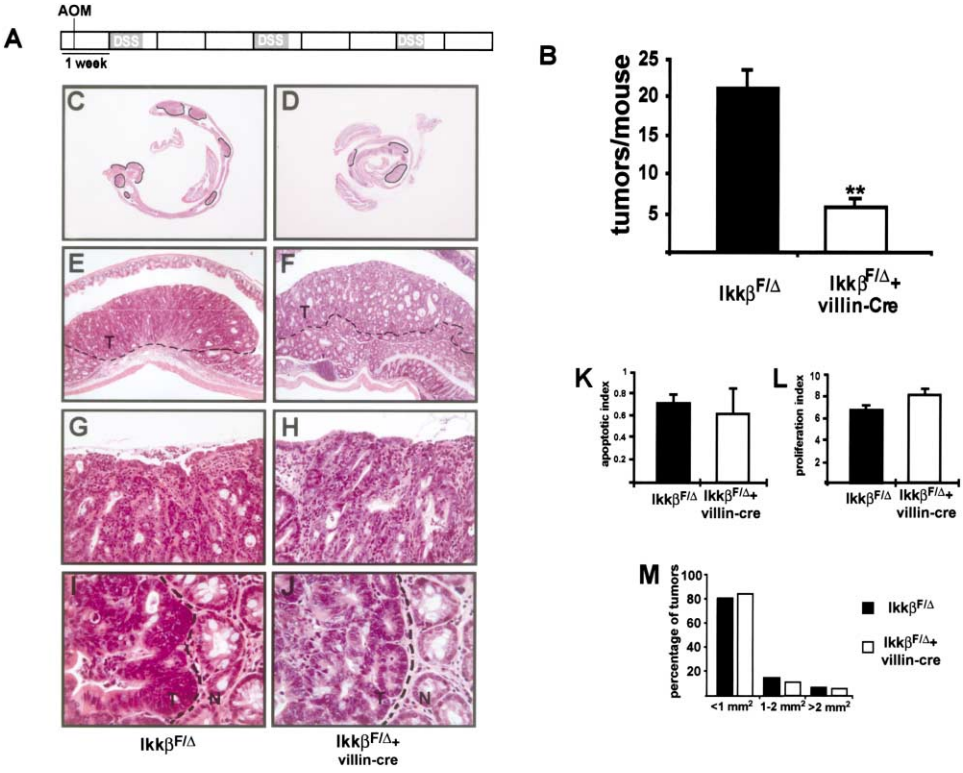

Figure 1. Enterocyte-Specific Deletion of IKKβ Decreases Tumor Incidence

(A) Schematic overview of the CAC model; each rectangle represents one week. After initial AOM injection (12.5 mg/kg), DSS was given in drinking water (gray areas) followed by regular water.

(B) Tumor incidence in *Ikk*$\beta^{F/\Delta}$ and *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice ($n \geq 9$, $p < 0.001$)

(C–J) H&E stainings of tumor morphology.

(C and D) Overview of representative sections of the "Swiss-rolls" that were used for tumor counting. Tumors are marked by black lines.

(E and F) Representative tumors, lines mark borders between adenomas (T) and normal epithelium, 40× magnification.

(G and H) Adenomas show infiltration with inflammatory cells and ulceration on the luminal surface, 100× magnification

(I and J) Nuclei of adenomas (T) show pseudostratification and increased mitotic figures relative to normal tissue (N), 400× magnification.

(K and L) Tumor apoptotic and proliferation indices were determined by TUNEL and BrdU staining, respectively.

(M) Histogram showing size distribution of tumors. Tumor areas were determined by computerized image analysis.

dex between tumors in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice or control *Ikk*$\beta^{F/\Delta}$ mice could be detected at nine weeks after tumor initiation (Figures 1K–1M).

Mutations in the *APC* or β-*catenin* gene that lead to stabilization of β-catenin and transcriptional activation with TCF-4 are very important events in colorectal carcinogenesis (Morin et al., 1997). We therefore used laser capture microdissection to isolate tumor tissue and prepare genomic DNA that was analyzed for mutations in exon 3 of the β-*catenin* gene. Independent of genotype, we found various mutations around the GSK-3β phosphorylation sites of β-catenin (Figure 2A). Activation of the β-catenin/TCF-4 pathway was further confirmed by immunohistochemistry showing a shift of β-catenin from the membrane where it is present in untransformed epithelium toward a cytoplasmic and nuclear pattern in the tumors (Figures 2B and 2C). This was accompanied with nuclear accumulation of two β-catenin target gene products c-Myc and cyclin D1 (Figures 2D–2G). In normal tissue, nuclear c-Myc and cyclin D1 were restricted to a few proliferating cells at the base of colonic crypts. Even though the observed tumors were not invasive, they clearly had a premalignant phenotype. We also analyzed the tumors for the presence of p21, p53, and COX-2 by immunohistochemistry. While in both genotypes strong nuclear staining for p21 was detected within the tumors, only very few infiltrating cells were COX-2 positive (data not shown). None of the tumors was p53 positive, suggesting that they did not harbor mutant *p53* alleles (data not shown).

To examine the possibility that tumors in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice arose from cells that had escaped IKKβ deletion, we analyzed deletion of the floxed allele in DNA from microdissected tumors. At least 8 out of 10 tumors contained the deleted allele (Figure 2H). However, 1 or 2 of the tumors contained higher levels of the nondeleted "floxed" allele, suggesting they arose from cells in which the "floxed" *Ikk*β^{F}-allele was not yet deleted. Thus, the decrease in tumor incidence caused by deletion of IKKβ may be close to 80%. Given that normal enterocytes from *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice do not express any IKKβ (Chen et al., 2003), these results suggest that the few cells within the colonic crypts, which are yet to experience IKKβ deletion, may be preferentially transformed.

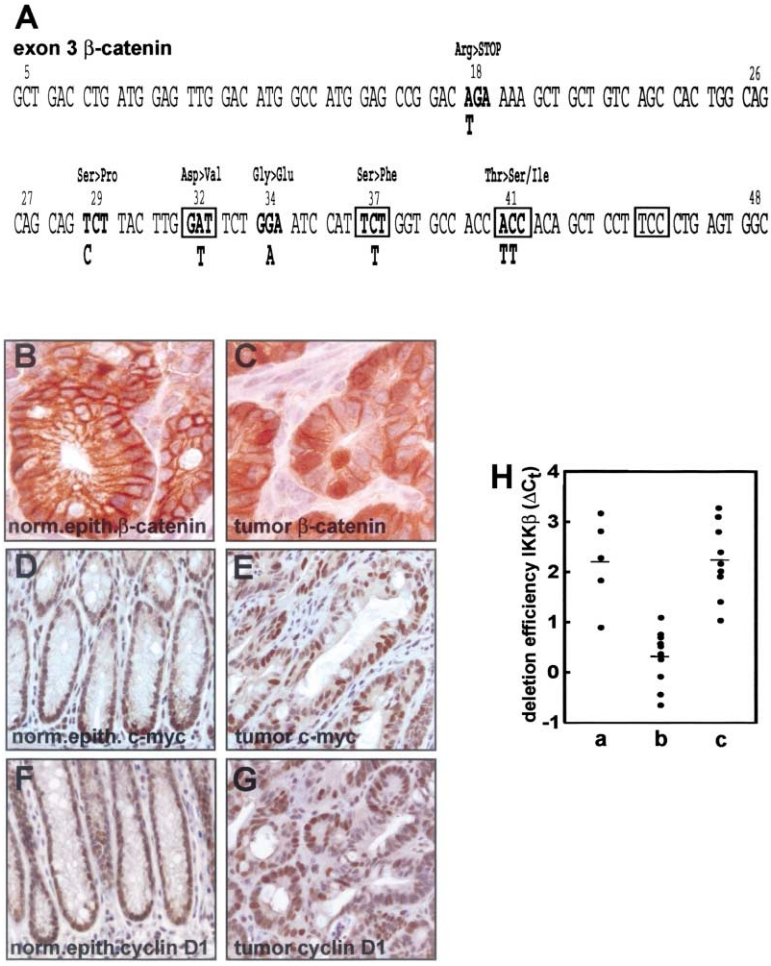

Figure 2. Tumors Harbor β-Catenin Mutations and Exhibit Activation of the β-Catenin Pathway

(A) Mutations within exon 3 of the β-*catenin* gene. DNA was eluted from microdissected tumor cells. Exon 3 that codes for the GSK-3β phosphorylation sites (boxed exons) was amplified and sequenced. Codons containing mutations are in bold.

(B and C) Activation of β-catenin. Immunohistochemical analysis of β-catenin in normal epithelium (B) and in tumors (C).

(D and E) Immunhistochemical analysis of c-Myc in normal epithelium (D) and in tumors (E).

(F and G) Immunohistochemical analysis of Cyclin D1 in normal epithelium (F) and in tumors (G).

(H) Analysis of IKKβ deletion in tumor DNA. DNA from microdissected normal epithelium of *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice (a), normal epithelium and tumors from *Ikk*$\beta^{F/\Delta}$ mice (b) and from tumors of *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice (c) was analyzed by PCR for deletion of *Ikk*β exon 3. ΔC_t values were obtained by subtracting C_t values of two other genes not affected by the deletion from the *Ikk*β C_t values

Given the large reduction in tumor incidence but no apparent effect on tumor size or growth, these results suggest that within enterocytes IKKβ is mostly important during early stages of tumor initiation and/or promotion.

Enterocyte-Specific Deletion of IKKβ Does Not Prevent DSS-Induced Inflammation

Inhibition of NF-κB activation is associated with reduced colonic inflammation and both RelA antisense oligonucleotides and IKKβ inhibitors were found to attenuate the course of colitis (MacMaster et al., 2003; Murano et al., 2000). To test whether reduced colonic inflammation in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice is responsible for the observed differences in tumor incidence, we evaluated the outcome of acute and chronic colitis in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice and their littermate controls. No differences in clinical signs (i.e., weight loss, diarrhea, or rectal bleeding) or colon histology could be detected between the two groups after completion of the nine-week CAC regimen (data not shown). However, at day 15 of the CAC regimen (i.e., five days after termination of the first DSS cycle) *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice exhibited a slightly higher level of histological damage that was not statistically significant (data not shown). We therefore increased the concentration of DSS (from 2.5% to 3.5%) to induce a more acute inflammation. Under these conditions *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice lost significantly more weight than littermate controls (Figure 3A) and showed a significantly higher level of histological damage and greater ulcer numbers (Figures 3B and 3C). Representative histological sections are shown in Figures 3D–3G.

Despite the insignificant increases in histological inflammation, the colons of *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice exposed to 2.5% DSS contained higher levels of mRNAs for proinflammatory proteins, such as TNFα, IL-1β, ICAM, IL-6, as well as the mouse IL-8 homologs MIP-2 and KC (Figure 4A). DSS-treated *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice also exhibited elevated expression of COX-2 and matrix metalloprotease 9 (Figure 4B). These proteins, however, are expressed in myeloid rather than epithelial cells (see below). Collectively, these results indicate that the reduced tumor incidence in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice is not due to reduced inflammation. Since DSS is believed to induce colonic inflammation through physical disruption of the mucosal barrier function thus exposing innate immune cells in the lamina propria to bacteria or bacterial products (Okayasu et al., 1990), the increased inflammation in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice is most likely due to decreased epithelial cell survival (see below). We also examined whether the loss of IKKβ in enterocytes affected the proliferation of the epithelial cells in response to the inflammatory challenge. However, no significant

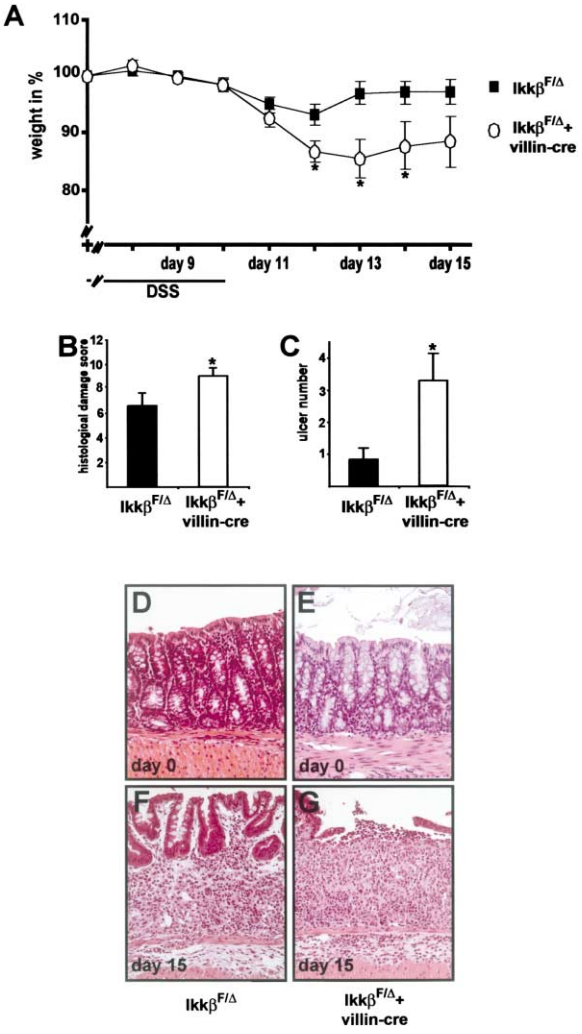

Figure 3. Increased Colonic Inflammation in *villin-Cre/Ikk*$\beta^{F/\Delta}$ Mice Challenged with High Levels of DSS

(A) Weight loss during DSS (3.5%) colitis in *Ikk*$\beta^{F/\Delta}$ and *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice.

(B) Histological damage ($n \geq 7$, $p < 0.05$) and (C) ulcer number ($n \geq 7$, $p < 0.05$) in mice treated with 3.5% DSS.

(D) Histology of untreated *Ikk*$\beta^{F/\Delta}$ and (E) and untreated *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice. Representative histologies of (F) *Ikk*$\beta^{F/\Delta}$ mice and (G) *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice five days after the termination of DSS administration.

difference in BrdU incorporation into colonic epithelial cells of mice given 2.5% DSS could be detected between the two genotypes (Figures 4C and 4D).

Loss of IKKβ in Epithelial Cells Results in Enhanced p53-Independent Apoptosis during Early Tumor Promotion

Alkylating agents, such as AOM, induce rapid p53-dependent apoptosis of epithelial cells in the lower one third of the intestinal crypt (Toft et al., 1999). Previously we found increased radiation-induced apoptosis associated with increased p53 expression in the small intestine of *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice relative to *Ikk*$\beta^{F/\Delta}$ controls (Egan et al., 2004). We therefore examined whether AOM may also induce more apoptosis in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice. However, AOM (without DSS) acted differently from γ-radiation and did not lead to increased apoptosis in the IKKβ-deleted mice (data not shown). In accordance with published results (Toft et al., 1999), the AOM-triggered apoptotic response was almost completely abolished in *p53*$^{-/-}$ mice (data not shown). These results rule out the possibility that *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice had decreased tumor numbers due to differences in the initial response to the carcinogen.

As mentioned above, one of the mechanisms by which DSS leads to development of colitis is induction of epithelial apoptosis. Epithelial apoptosis was also reported in ulcerative colitis patients, leading to inflammation of the colonic mucosa due to loss of barrier function (Iwamoto et al., 1996). We used terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) to examine the DSS-induced apoptotic response in AOM treated or nontreated mice. A high dose of oral DSS (3.5%) induced clusters of TUNEL-positive cells within the surface epithelium of the middle and distal colon, consistent with the greater tissue damage. The areas containing apoptotic cells were significantly larger in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice relative to littermate controls (data not shown). However, exposure to a lower concentration of DSS (2.5%) during the CAC regime resulted in more extensive and uniform epithelial apoptosis than caused by DSS alone. In this case, the difference between *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice and their *Ikk*$\beta^{F/\Delta}$ littermate controls was quite striking (Figures 5A and 5B). Surprisingly, however, at day 7 of the CAC regimen *p53*$^{-/-}$ mice did not show decreased enterocyte apoptosis relative to littermate controls that were exposed to the CAC regimen (data not shown), suggesting the involvement of a p53-independent mechanism.

Biochemical analysis of isolated enterocytes seven days after initiation of the CAC regimen confirmed that exposure to AOM and DSS led to IKK activation (Figure 5C). No IKK activity was detected in *villin-Cre/Ikk*$\beta^{F/\Delta}$ epithelial cells. Immunoblot analysis of anti- and proapoptotic proteins showed marked induction of Bcl-x_L in *Ikk*$\beta^{F/\Delta}$ mice that was absent in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice. By contrast, the levels of the proapoptotic proteins Bak and Bax were slightly higher in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice. This perturbance in expression of Bcl-2 family members could account for the increased apoptosis in the knockout mice. No upregulation of p53, Mdm2, or p21 was detected in enterocytes of either genotype (data not shown). No changes in JNK activity could be detected between the two genotypes (Figure 4C) suggesting that sustained JNK activation (Maeda et al., 2003; Tang et al., 2001) is not responsible for the increase in epithelial apoptosis in AOM+DSS treated *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice.

Deletion of IKKβ in Myeloid Cells Decreases Tumor Growth without an Effect on Apoptosis

To address the role of the IKKβ-dependent NF-κB pathway in myeloid cells, we crossed LysM-Cre mice (Clausen et al., 1999) with *Ikk*$\beta^{F/F}$ mice and generated mice that specifically lack IKKβ in the myeloid lineage. Analysis of DNA of bone-marrow-derived macrophages (BMDM) obtained from *LysM-Cre/Ikk*$\beta^{F/F}$ mice by real-time PCR revealed a deletion efficiency of around 75% in these

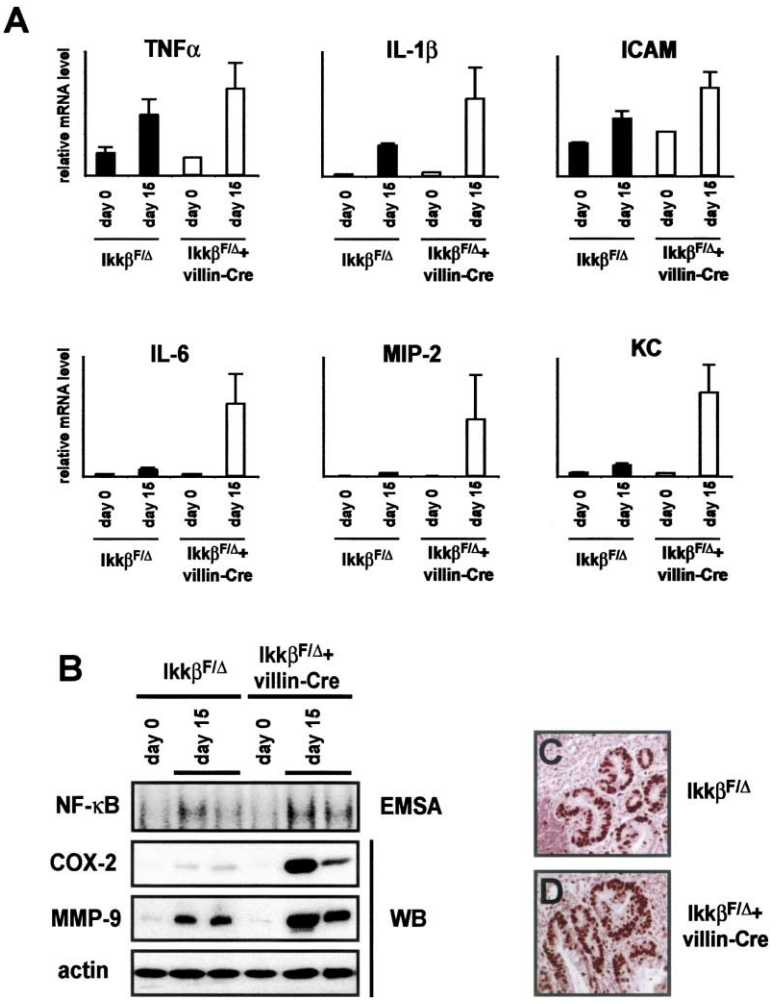

Figure 4. Increased Expression of Proinflammatory Factors during DSS-Colitis in *villin-Cre/Ikk*$\beta^{F/\Delta}$ Mice

(A) Expression of inflammatory genes. Relative mRNA expression levels were examined in whole colon tissues of mice at day 15 of the CAC regimen, 5 days after DSS (2.5%). The levels of the indicated mRNAs were quantitated by real-time PCR and normalized to the level of cyclophilin mRNA ($n \geq 3$).

(B) NF-κB binding activity, COX-2 and MMP-9 expression in whole colonic extracts prepared at days 0 and 15 of the CAC regimen five days after DSS (2.5%) challenge. NF-κB binding activity was determined by EMSA. COX-2 and MMP-9 expression were determined by Western blotting (WB).

(C and D) BrdU incorporation into intestinal epithelial cells at day 15 of the CAC regimen, five days after termination of DSS challenge.

cells. Similar results were obtained by Southern blotting and also for neutrophils (data not shown). Immunoblot analysis confirmed the loss of IKKβ expression (Supplemental Figure S1A available at http://www.cell.com/cgi/content/full/118/3/285/DC1), resulting in diminished NF-κB activation determined by electrophoretic mobility shift assay (Supplemental Figure S1B available on *Cell* website). The effect of the deletion on expression of NF-κB target genes encoding proinflammatory cytokines, chemokines, and other factors, that are known to be involved in colitis and could potentially contribute to tumor progression, was analyzed by real-time PCR. We detected reduced expression of genes encoding IL-1α, IL-1β, IL-6, KC, MIP-2, TNFα, COX-2, and ICAM (Supplemental Figure S1C available on *Cell* website). Thus, inactivation of IKKβ in macrophages reduces expression of many genes that contribute to the inflammatory response.

Deletion of IKKβ in myeloid cells also reduced tumor incidence in the CAC model. *LysM-Cre/Ikk*$\beta^{F/F}$ mice showed an almost 50% reduction in tumor counts (Figure 6A). Although the tumor morphology was similar in both genotypes (Figures 6B and 6C), the *LysM-Cre/Ikk*$\beta^{F/F}$ mice exhibited a greater percentage of smaller tumors than *Ikk*$\beta^{F/F}$ mice (Figure 6D). The apoptotic and proliferation indices determined at nine weeks after initiation of the CAC regimen were similar in both groups (Figures 6E and 6F). To examine whether the IKKβ deletion perturbs recruitment of myeloid cells to the tumors, we stained tumors for F4/80. We did not detect any difference between the two genotypes (Figures 6G and 6H). To understand how IKKβ in myeloid cells affects tumor development, we first compared the apoptotic response to AOM, AOM plus DSS, or DSS alone in *LysM-Cre/Ikk*$\beta^{F/F}$ and *Ikk*$\beta^{F/F}$ mice. No substantial differences in the extent of apoptosis could be detected between the two genotypes after any of the treatments (Figure 7A and data not shown). No changes in Bcl-x_L induction could be observed either (Figure 7B). Thus, the lower tumor incidence in *LysM-Cre/Ikk*$\beta^{F/F}$ mice is not due to accelerated loss of initiated cells, as it is the case for *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice.

We then examined the effect of IKKβ deletion in myeloid cells on production of factors that may act in a paracrine manner to promote tumor growth and development. At five days after termination of the first DSS cycle the *LysM-Cre/Ikk*$\beta^{F/F}$ mice exhibited reduced induction of the mRNAs for IL-1β, ICAM, IL-6, as well as MIP-2 and KC (Figure 7C). Unlike the enterocyte-specific deletion, which resulted in increased COX-2 and MMP-9

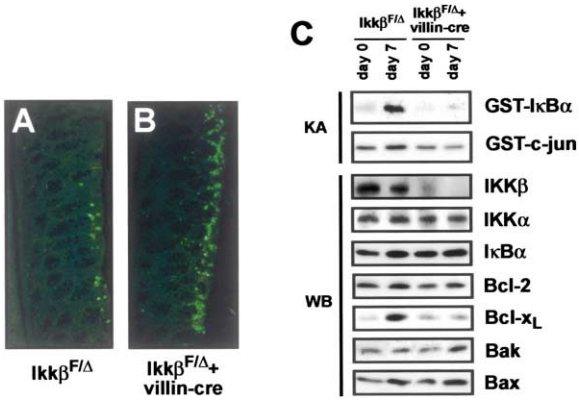

Figure 5. Massive Apoptosis and Defective Bcl-x_L Induction in Colonic Epithelium of *villin-Cre/Ikk*$\beta^{F/\Delta}$ Mice

(A and B) TUNEL stainings of colons of *Ikk*$\beta^{F/\Delta}$ and *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice at day 7 after administration of AOM, two days after beginning of DSS (2.5%) exposure.

(C) Biochemical analysis of enterocytes isolated from mice at day 7 of the CAC regimen. KA: Kinase assay; WB: Western blot analysis.

induction, the myeloid-specific deletion of IKKβ resulted in a substantial decrease in expression of both proteins after DSS treatment (Figure 7D). These results strongly suggest that both of these proteins, as well as most of the other proinflammatory mediators, are expressed by myeloid cells.

To address whether the decreased expression of proinflammatory factors might affect inflammation induced epithelial proliferation we analyzed BrdU incorporation in mice of both genotypes at 15 days after AOM plus DSS treatment. We detected a substantial difference in proliferating epithelial cells, which were present in areas that exhibited extensive histological inflammation in *LysM-Cre/Ikk*$\beta^{F/F}$ mice compared to littermate controls (Figures 7E and 7F). These data support the notion that IKKβ in myeloid cells can promote tumor growth and development through production of tumor-promoting paracrine factors, rather than inhibition of tumor cell apoptosis.

As inhibition of COX-2 is an accepted chemopreventive strategy (Gupta and Dubois, 2001), we tested the effect of COX-2 inhibition on the early apoptosis in our model as well as on the proliferation of enterocytes. Wild-type mice were injected twice daily with the specific COX-2 inhibitor NS-398 starting one day before the AOM administration. However, no differences in the extent of apoptosis on day 7 of the regimen could be detected (Supplemental Figures S2A and S2B available on *Cell* website). Mice treated with NS-398 showed a more severe form of acute colitis characterized by greater weight loss and tissue damage on day 15 (Supplemental Figures S2C and S2D available on *Cell* web-

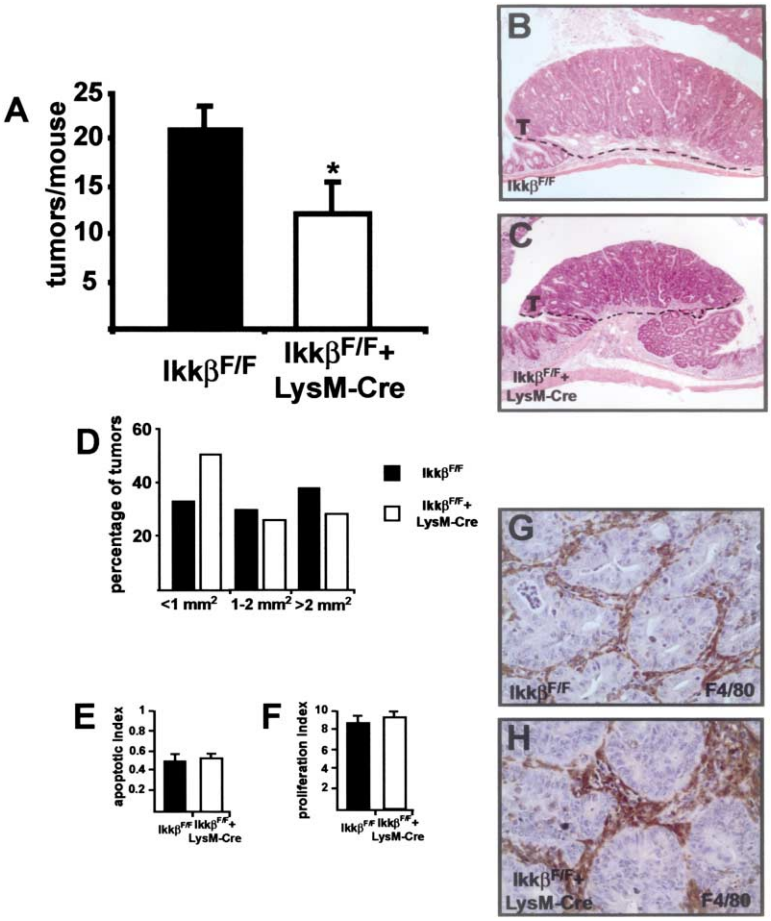

Figure 6. Myeloid-Specific Deletion of IKKβ Decreases Tumor Incidence and Growth

(A) Tumor incidence in *Ikk*$\beta^{F/F}$ and *LysM-Cre/Ikk*$\beta^{F/F}$ mice ($n \geq 7$, $p < 0.03$).

(B–F) Tumor characteristics: (B and C) representative sections stained with H&E of tumors from both genotypes showing decreased tumor size in *LysM-Cre/Ikk*$\beta^{F/F}$ mice; (D) Tumor size distribution determined as for Figure 1M. (E and F) Tumor apoptotic and proliferative indices, determined as described in Figures 1K and 1L.

(G and H) Staining of tumor sections with anti-F4/80 antibody to reveal infiltrating myeloid cells.

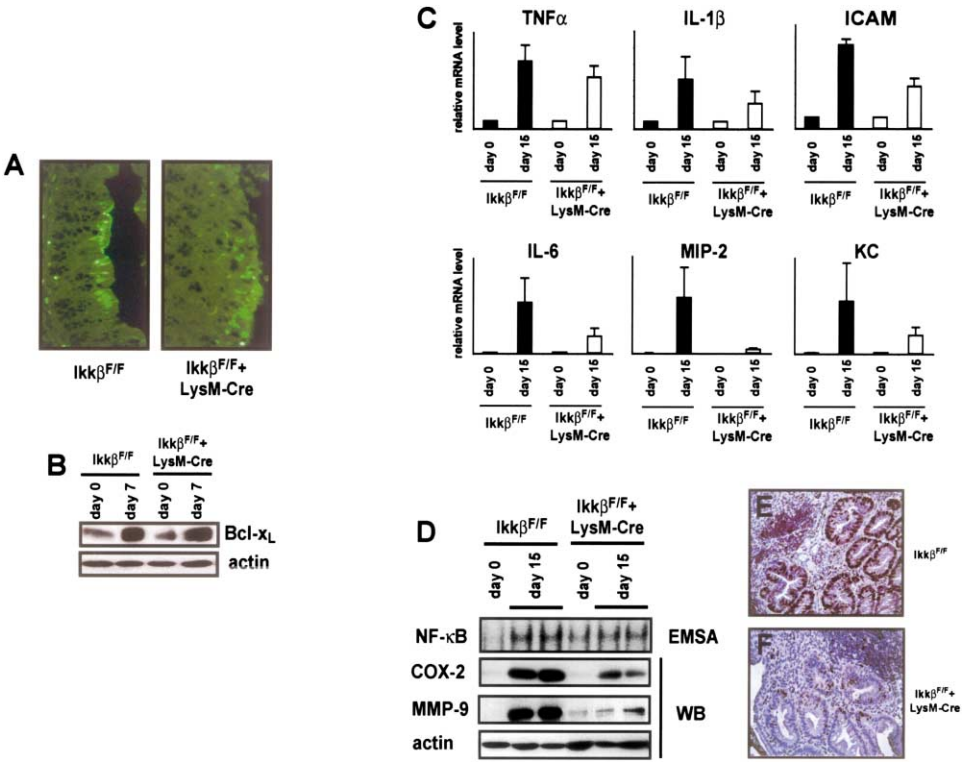

Figure 7. Myeloid-Specific Deletion of IKKβ Does Not Affect Early Apoptosis but Reduces Expression of Potential Tumor-Promoting Factors and Enterocyte Proliferation

(A) TUNEL staining of colons of *Ikk*$\beta^{F/F}$ mice and *LysM-Cre/Ikk*$\beta^{F/F}$ mice at day 7 of the CAC regimen.

(B) Western blot analysis of Bcl-x_L levels in whole colonic extracts of the same mice.

(C) Relative mRNA expression levels in whole colons of mice on day 15 of the CAC regimen. The levels of the indicated mRNAs were quantitated by real-time PCR and normalized to the level of cyclophilin mRNA ($n \geq 3$).

(D) COX-2 and MMP-9 levels in whole colonic extracts of mice on day 15 of the CAC regimen.

(E and F) BrdU incorporation into epithelial cells determined on day 15 of the CAC regimen.

site, and data not shown), but there was no apparent difference in the number of BrdU-labeled cells within areas of inflammation on day 15 (Supplemental Figures S2E and S2F available on *Cell* website). The COX-2 inhibitor, however, was effective in reducing prostaglandin synthesis (Supplemental Figure S2G available on *Cell* website). The failure to observe an ameliorating effect of the COX-2 inhibitor on inflammation in this model is consistent with findings in COX-2 deficient mice (Morteau et al., 2000).

Discussion

A connection between inflammation and cancer has been suspected for a long time. It has become apparent that the interaction between transformed cells and their close surrounding, including innate immune cells, fibroblasts, and endothelial cells, can be instrumental in both tumor promotion and progression of many epithelial tumors, especially in the context of chronic inflammation (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). However, the underlying molecular processes involved in this interaction are still poorly understood. We now show that the IKKβ-dependent NF-κB activation pathway represents a critical molecular link between inflammation and cancer in a mouse model of CAC. While probing the role of IKKβ in linking inflammation and cancer, we also investigated in which cell type it is most likely to act. Our results indicate that IKKβ acts both within enterocytes, which eventually give rise to the transformed component of the tumor, and within myeloid cells, which influence tumor growth. In each cell type IKKβ contributes to tumor promotion through a different mechanism. Our results strongly suggest that in epithelial cells IKKβ contributes to tumor promotion by suppressing apoptosis through the mitochondrial pathway. IKKβ in these cells is required for induction of Bcl-x_L, which is encoded by a typical NF-κB target gene (Chen et al., 2000). The absence of IKKβ in enterocytes prevents Bcl-x_L induction and leads to increased apoptosis of carcinogen-exposed cells a few days after administration of the tumor promoter (DSS). As the results clearly demonstrate, the reduced tumor incidence in *villin-Cre/Ikk*$\beta^{F/\Delta}$ mice is not due to a decreased inflammatory response. Thus, IKKβ in enterocytes plays a critical and direct role in tumor promotion, which is distinct from its role in expression of inflammatory mediators, a function exerted mainly within myeloid cells in this model. A more modest, yet significant, reduction in tumor incidence is obtained by deleting IKKβ in myeloid cells. This deletion has no effect on either the expression of Bcl-x_L or the apoptotic response of carcinogen-

exposed cells. Instead, the deletion of IKKβ in myeloid cells results in a marked decrease in inflammation-induced proliferation of enterocytes and tumor size, indirect effects that were not seen with the enterocyte-specific deletion. Congruently, IKKβ in myeloid cells is required for expression of proinflammatory factors, such as COX-2, MMP-9, KC, and MIP-2, all of which are believed to contribute to tumor growth (Coussens and Werb, 2002).

Activated NF-κB was detected in many different tumor cell lines and primary tumor samples (Barkett and Gilmore, 1999), but until now it was not clear whether NF-κB is instrumental for tumor initiation and promotion. Nonetheless, it is well accepted that inhibition of NF-κB activity in cancer cells can lead to increased sensitivity to chemo- and radiotherapy (Amit and Ben-Neriah, 2003). In the CAC model, which combines chemical carcinogenesis and inflammation, we wanted to determine the role of IKKβ in initiation and early tumor promotion rather than the response of cancer cells to therapy. Given the broad range of genes that NF-κB can regulate, including genes that have immunoregulatory and proinflammatory functions, as well as genes that either inhibit or promote apoptosis (Karin et al., 2002), one can envision that in certain cell types NF-κB activation may promote tumor development while in other cases it may inhibit tumorigenesis. Indeed, it was found that inhibition of NF-κB in keratinocytes promotes development of squamous cell carcinomas in human and mouse models (Dajee et al., 2003; Seitz et al., 1998; van Hogerlinden et al., 1999, 2002). We have also detected a similar increase in tumor incidence in mice lacking IKKβ in hepatocytes (Maeda et al., 2003) using a chemical carcinogenesis model that does not involve inflammation as a tumor promoter (M.K. et al., unpublished data). The present findings, however, show for the first time that in a model that involves inflammation as a tumor promoter NF-κB activation can enhance tumor development and underscore the importance of using conditional knockout mice and different models of cancer to determine the full pathophysiological function of a given signaling pathway. Interestingly, the inhibition of IKK in keratinocytes and enterocytes also results, in diametrically opposed effects on inflammation. While deletion of IKKβ in enterocytes reduced acute inflammation caused by gut ischemia-reperfusion and mediated by TNFα (Chen et al., 2003), its deletion in keratinocytes enhanced TNFα-dependent inflammation (Pasparakis et al., 2002).

The tumors that arise from IKKβ-deficient enterocytes were indistinguishable from those that form in control mice, despite the large decrease in tumor incidence and contained a similar spectrum of β-*catenin* mutations. This points to an important function for IKKβ in enterocytes during very early tumor promotion and suggests it may be dispensable for tumor maintenance and progression once the few tumors that arise in *villin-Cre/Ikkβ*$^{F/\Delta}$ mice have formed. Interestingly, the deletion of IKKβ in myeloid cells had a more pronounced effect on tumor size than on tumor number. This and the effect on the proliferative response to the tumor promoter DSS strongly suggest that IKKβ in myeloid cells is most likely involved in promoting tumor growth through paracrine factors, rather than affecting the very early stages of the tumorigenic process.

This report provides one of the few examples that inactivation of a gene encoding a protein that is not directly involved in the autonomous control of cell proliferation can dramatically reduce tumor development. Ablations of cyclin D1 and c-Jun were shown to reduce the incidence of mammary and hepatocellular carcinomas, respectively (Eferl et al., 2003; Yu et al., 2001). Yet, both cyclin D1 and c-Jun are critical regulators of cell proliferation in the mammary gland and liver, thus, the reductions in tumor incidence caused by their deletion are somewhat expected. Regarding colon cancer, the most striking results were obtained with COX-2 knockout mice (Oshima et al., 1996), results that supported the successful chemoprevention studies in familial adenomatous polyposis (FAP) patients (Steinbach et al., 2000). Like IKKβ, COX-2 is an important mediator of inflammation and does not directly control cell proliferation (Gupta and Dubois, 2001).

Evading apoptosis is one of the hallmarks of cancer (Hanahan and Weinberg, 2000) and induction of apoptosis during tumor promotion represents a critical step for chemoprevention. Upregulation of Bcl-x_L during tumor promotion seems important in both CAC and sporadic colorectal cancer (Yu et al., 2003). One way to avoid upregulation of Bcl-x_L and thereby promote the elimination of initiated cells may entail inhibition of IKKβ. Interestingly, several NSAIDs used in chemoprevention of colorectal cancer, such as sulindac, can act as IKKβ inhibitors (Yamamoto et al., 1999; Yin et al., 1998). The chemopreventive activity of NSAIDs has been explained by three different mechanisms. The most widely accepted explanation is inhibition of COX-2 activity (Gupta and Dubois, 2001). The second proposed mechanism entails inhibition of PPARδ, which lead to induction of apoptosis in colorectal cancer cell lines (He et al., 1999). NSAIDs can also stimulate PPARγ, which was shown to inhibit intestinal tumor development (Girnun et al., 2002). The third putative mechanism entails inhibition of IKKβ, as suggested by our results that genetic ablation of IKKβ can reduce the incidence of CAC. Using a highly specific COX-2 inhibitor, at a dose that does not inhibit IKKβ, we failed to detect increased enterocyte apoptosis in mice subjected to the CAC regimen.

The role of macrophages in tumor development in contrast to tumor progression has not been clearly established, although these cells are important components of the tumor microenvironment (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Elimination of macrophages using a CSF-1 null mutation illustrated that macrophage recruitment is important for progression of mammary gland tumors, since invasive growth and metastasis were significantly attenuated (Lin et al., 2001). Furthermore, it was shown that bone-marrow-derived cells supply MMP-9 during skin carcinogenesis, and that the proliferation of transformed cells was affected by factors derived from surrounding cells (Coussens et al., 2000). Instead of preventing CSF-1 expression, which might also affect neoplastic cells, we chose to inactivate a specific-signaling pathway in myeloid cells, without exerting a direct effect on other cell types. Our findings establish for the first time the role of myeloid cells in inflammation-associated tumor promotion in ad-

dition to their role in tumor progression and invasiveness. Since NF-κB activation accounts for increased transcription of several hundred genes, the observed effect is likely to be due to decreased expression of several genes, whose products enhance tumor development, rather than a single one. Indeed, selective inhibition of COX-2 did not have an effect on either the apoptosis or proliferation of enterocytes in mice exposed to AOM and DSS. Other genes, which probably include KC and MIP-2, have been shown to have growth-stimulating effects on epithelial cells (Driscoll et al., 1995).

In addition to identifying a key molecular mechanism connecting inflammation and cancer, our results suggest that specific pharmacological inhibition of IKKβ may be very effective in prevention of CAC. Nevertheless, given the important role of IKKβ in promoting innate immune responses and suppressing other types of cancer there is a certain risk associated with long-term use of such drugs, a problem that needs to be addressed before realizing the full benefit of IKKβ inhibitors in chemoprevention of CAC or other cancers associated with chronic inflammation.

Experimental Procedures

Tumor Induction and Analysis

6–8-week-old mice (*villin-Cre/Ikkβ*$^{F/\Delta}$ mice and *Ikkβ*$^{F/\Delta}$ littermates on a mixed C57BL/6/129 background; *LysM-Cre/Ikkβ*$^{F/F}$ mice and *Ikkβ*$^{F/F}$ littermates on a mixed C57BL/6/129/FVB background) were injected intraperitoneally (i.p.) with 12.5 mg/kg AOM (NCI). After five days 2.5% DSS (ICN, MW 36 – 50 kDa) was given in the drinking water over five days, followed by 16 days of regular water. This cycle was repeated twice (five days of 2.5% DSS and four days of 2% DSS) and mice were sacrificed ten days after the last cycle. Colons were removed, flushed with PBS, fixed as "Swiss-rolls" in 4% paraformaldehyde at 4°C overnight and paraffin-embedded. Sections (5 μm) were cut stepwise (200 μm) through the complete block and stained with H&E. Tumors counts were performed in a blinded fashion. Tumor sizes were calculated using Scion Image for Windows (Scion Corporation). To determine proliferation rates, mice were injected i.p. with 100 mg/kg BrdU (Amersham) 2.5 hr prior to sacrifice and paraffin sections were stained using the BrdU-in situ detection Kit (BD PharMingen). Apoptosis was determined by TUNEL assay using the ApoAlert DNA fragmentation Assay Kit (BD Clontech). BrdU- and TUNEL-positive cells were counted by an investigator blinded to the genotype. Assessment of colitis disease scores was done as described (L.J. Egan et al., submitted).

Laser Capture Microdissection

Paraffin sections (7.5 μm) were mounted on SL μCut slides (Molecular Machines and Industries) and stained with hematoxylin. Around 4000 cells/tumor as well as normal epithelial cells were laser dissected using a Nikon Laser Microdissection microscope. DNA was eluted overnight at 37°C in 50 μl extraction buffer and 2 μl were used in subsequent PCR reactions. Primers were designed to amplify exon 3 of β-*catenin* and PCR products were subcloned into pBluescript SK+ and sequenced. To quantify deletion of *Ikkβ*, primers within exon 3 of *Ikkβ*, which is flanked by *loxP* sites, were used and real-time PCRs were performed on an ABI 7700 Sequence Detector (Applied Biosystems). Two other chromosomal regions were amplified as internal controls. Ct values of these control genes were averaged and subtracted from Ct values obtained from the *Ikkβ* exon 3 primers. ΔCt values of tumors were compared to ΔCt values of nontransformed knockout epithelial cells and control animals. Primer sequences are available upon request.

Isolation of Bone-Marrow-Derived Macrophages

BMDM from *LysM-Cre/Ikkβ*$^{F/F}$ and *Ikkβ*$^{F/F}$ mice were obtained and cultured as previously described (Park et al., 2002). After differentiation, BMDM were stimulated with 100 ng/ml LPS (Sigma) and DNA, RNA, and proteins were extracted as described (Park et al., 2002). Deletion efficiency of the floxed allele was determined by real-time PCR as described above. For quantification of mRNA levels before and after LPS stimulation, real-time PCR was performed as described (Senftleben et al., 2001). Primer sequences are available upon request.

Isolation of Intestinal Epithelial Cells

Colons were cut open longitudinally and feces was removed by brief washing with PBS. Colons were then cut into 2–3 mm pieces and carefully rocked in Hanks' balanced salt solution (HBSS) containing 30 mM EDTA for 10 min at 37°C. The supernatant was removed and centrifuged. The resulting pellet was washed in ice-cold PBS and pellet was snap-frozen in liquid nitrogen.

IKK Kinase Assay, EMSA, and Immunoblots

IKK immune complex kinase assay and electrophoretic mobility shift assays were performed as previously described (Cao et al., 2001). Protein lysates were separated by SDS-gel electrophoresis and transferred to PVDF membranes. The following antibodies were used for immunoblotting as described (Senftleben et al., 2001): anti-IKKα and anti-IKKβ (Imgenex), anti-IκBα, anti-c-Myc, anti-cyclin D1, anti-p21, anti-p53, anti-Bak (Santa Cruz), anti-β-catenin (Upstate), anti-Bcl-2, anti-Bcl-x_L, anti-Bax (BD PharMingen), anti-COX-2 (Cayman), and anti-MMP-9 (Chemicon).

Statistical Analysis

Data are expressed as mean ± SEM. Differences were analyzed by Student's t test. Values ≤ 0.05 were considered significant.

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