

IKK α Promotes Intestinal Tumorigenesis by Limiting Recruitment of M1-like Polarized Myeloid Cells

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SUMMARY

The recruitment of immune cells into solid tumors is an essential prerequisite of tumor development. Depending on the prevailing polarization profile of these infiltrating leucocytes, tumorigenesis is either promoted or blocked. Here, we identify I κ B kinase α (IKK α) as a central regulator of a tumoricidal microenvironment during intestinal carcinogenesis. Mice deficient in IKK α kinase activity are largely protected from intestinal tumor development that is dependent on the enhanced recruitment of interferon γ (IFN γ)-expressing M1-like myeloid cells. In IKK α mutant mice, M1-like polarization is not controlled in a cell-autonomous manner but, rather, depends on the interplay of both IKK α mutant tumor epithelia and immune cells. Because therapies aiming at the tumor microenvironment rather than directly at the mutated cancer cell may circumvent resistance development, we suggest IKK α as a promising target for colorectal cancer (CRC) therapy.

INTRODUCTION

An inflammatory microenvironment is an essential component of epithelial tumors that develop on the basis of chronic inflammatory conditions as well as of those malignancies that emerge in an inflammation-independent manner (Quante et al., 2013). In

both instances, recruitment of various types of adaptive and innate immune cells can be observed. Depending on the dominating cell type and polarization profile of the infiltrating cells, tumorigenesis is promoted or suppressed (Grivennikov et al., 2010). Recently, an immune score relying on the intratumoral localization of cytotoxic and memory T cells was established in colorectal cancer (CRC) (Fridman et al., 2012). This immune score has a powerful prognostic value and exemplifies the importance of immune cells for this tumor entity. Moreover, a large number of functional *in vivo* studies have provided substantial evidence demonstrating a key role of myeloid cells in colorectal cancer as well as other tumor entities (Grivennikov et al., 2010). In analogy to the Th1/Th2 classification of T cells, macrophages have been suggested to be grouped into classically activated M1 (in response to interferon γ [IFN γ] or microbial products) or alternatively activated M2 macrophages (in response to interleukin 4 [IL-4]; Gordon and Taylor, 2005). In the context of tumor-associated macrophages, M1 macrophages are considered to behave in a tumoricidal manner whereas M2 macrophages promote tumorigenesis (Mantovani et al., 2002). However, the exact molecular and cellular basis underlying the tumor-promoting lymphocyte and myeloid cell polarization within the tumor microenvironment is still poorly defined.

Nuclear factor (NF)- κ B activation leads to the establishment of a protumorigenic inflammatory microenvironment of various malignancies (Bollrath and Greten, 2009). NF- κ B is tightly controlled by the I κ B-kinase (IKK) complex, which consists of two catalytic subunits, namely the IKK α and IKK β proteins, as well as the regulatory subunit IKK γ (Chariot, 2009). The classical NF- κ B

activation controls key functions for tumor initiation, promotion, and progression both in tumor as well as in infiltrating myeloid cells (Karin and Greten, 2005). In contrast to classical IKK γ /IKK β -dependent NF- κ B signaling, alternative NF- κ B activation depends solely on IKK α (Vallabhapurapu and Karin, 2009). Moreover, IKK α comprises a nuclear localization signal and can therefore also confer important nuclear functions (Chariot, 2009). Whereas, in most malignancies, IKK β -dependent NF- κ B signaling clearly promotes tumorigenesis, the role of IKK α in this context is more complex. Inhibition of IKK α prolongs survival and suppresses occurrence of metastatic diseases in models of mammary and prostate cancer (Cao et al., 2007; Luo et al., 2007; Tan et al., 2011; Zhang et al., 2013). In contrast, loss of IKK α enhances susceptibility to carcinogen-induced squamous cell carcinomas (SCC) in the skin and leads to development of spontaneous lung SCC (Liu et al., 2008; Xiao et al., 2013). Interestingly, the latter depends in part on the development of an excessive inflammatory environment triggered by IKK α mutant macrophages (Xiao et al., 2013).

Globally sporadic CRC comprises the second most common cause of cancer in women and the third most common cause in men (Jemal et al., 2009). In over 80% of the cases, it is initiated by APC and CTNNB mutations that cause persistent activation of the Wnt pathway (Fearon, 2011). We could recently demonstrate that proinflammatory IKK β -dependent NF- κ B signaling enhances β -catenin promoter binding, causing dedifferentiation of postmitotic epithelia and tumor stem cell expansion during Wnt-dependent tumor initiation (Schwitalla et al., 2013a). Moreover, canonical NF- κ B activation controls development of epithelial-mesenchymal transition (EMT) and myeloid cell recruitment in Tp53-deficient invasive carcinomas (Schwitalla et al., 2013b). In contrast, IKK α directly phosphorylates β -catenin, thus increasing its abundance to promote cyclin D1 expression (Albanese et al., 2003), and in colorectal cancer cells, an active IKK α isoform was described (Margalef et al., 2012). However, functional genetic evidence supporting a cell autonomous or nonautonomous role of IKK α and/or the alternative NF- κ B activation pathway in colorectal carcinogenesis is lacking.

RESULTS

Impaired IKK α Activation Suppresses Intestinal Tumorigenesis

To functionally examine the role of IKK α during early intestinal tumorigenesis, we employed *Ikk α ^{AA/AA}* knockin mice, which contain alanines instead of serines in the activation loop of IKK α and express therefore a nonactivatable form of this kinase (Cao et al., 2001). Whereas *Ikk α ^{AA/AA}* mice are characterized by impaired development of Peyer's patches (Sentfleben et al., 2001), intestinal epithelial cell (IEC) differentiation was indistinguishable from littermate controls. *Ikk α ^{AA/AA}* mice displayed regular numbers and distribution of goblet cells, Paneth cells, as well as enteroendocrine cells (data not shown). Similarly, proliferation and apoptosis rates of unchallenged intestinal epithelial cells in small and large intestine were unaltered (data not shown). To induce intestinal tumorigenesis, *Ikk α ^{AA/AA}* mice and littermate controls were repetitively challenged with the procarcinogen azoxymethane (AOM), which is commonly used to induce ade-

noma growth in the distal colon of rodents. Expression of mutant IKK α markedly reduced number of adenomas (>75%) when animals were analyzed 20 weeks after the first carcinogen exposure (Figure 1A). *Ikk α ^{AA/AA}* mice developed only few relatively small tumors that displayed slower proliferation rates (Figures 1B and 1C). Instead, in *Ikk α ^{AA/AA}* mice multifocal low-grade intraepithelial neoplasia was frequently observed. To confirm the *Ikk α ^{AA/AA}*-dependent antitumorigenic effect in a genetic model of adenomatous polyposis, we crossed *Ikk α ^{AA/AA}* mice to *Apc^{Min/+}* mice and monitored their survival. Similarly, loss of IKK α function conferred a protective effect and prolonged survival of *Apc^{Min/+}* mice significantly (median survival of 236.5 days in homozygous *Ikk α ^{AA/AA}* mutants versus 184.5 days in heterozygous *Ikk α ^{AA/WT}* mutants and 166 days in *Ikk α ^{WT/WT}* wild-type *Apc^{Min/+}* mice; $p < 0.0001$; Figure 1D). Accordingly, when we analyzed 4-month-old *Apc^{Min/+}* animals, tumor incidence and size as well as proliferation of tumor epithelia was significantly decreased in *Ikk α ^{AA/AA}* mutant mice (Figures 1E–1H; data not shown). Furthermore, consistent with lower tumor burden, anemia—usually developing in *Apc^{Min/+}* mice as tumorigenesis progresses—was normalized in *Apc^{Min/+}/Ikk α ^{AA/AA}* compound mutants (Figures 1I and 1J). Collectively, these results suggested that *Ikk α ^{AA/AA}* mediated antiproliferative effects during early tumor stages, which led to marked tumor suppression in both models of intestinal tumorigenesis.

Ikk α ^{AA/AA} Mice Block Tumor Cell Proliferation Independently of Alternative NF- κ B Activation

Tamoxifen-inducible β -cat^{c.a.} mice comprise an excellent model to study Wnt-dependent tumor initiation. These mice are characterized by IEC-restricted stabilization of β -catenin causing rapid expansion of intestinal crypts and loss of differentiated IEC, and within 4 weeks, β -cat^{c.a.} mice succumb to this marked crypt hyperproliferation (Schwitalla et al., 2013a). Similarly to the results obtained in AOM-induced and *Apc*-dependent tumor models, mutant IKK α blocked proliferation and expansion of c-myc-expressing β -catenin mutant crypts within 2 weeks after tamoxifen induction (Figures 2A–2C). This was associated with decreased CDK1 and CDK2 activity when mice were analyzed 15 days after the first tamoxifen administration (Figures 2D and 2E). Accordingly, impaired IKK α activation prolonged survival of β -cat^{c.a.} mutant animals (Figure 2F). Interestingly, loss of NF- κ B2/p100 did not affect survival, indicating that IKK α acted independently of the alternative NF- κ B activation pathway (Hayden and Ghosh, 2004). In line with this notion, we also did not observe any differences in p100 processing in β -cat^{c.a.}/*Ikk α ^{AA/AA}* IEC (data not shown).

Prolonged Survival of IKK α Mutant β -cat^{c.a.} Mice Depends on IFN γ

To further explore the underlying IKK α -controlled proliferative mechanism, we performed a microarray analysis comparing RNA isolated from wild-type, *Ikk α ^{AA/AA}*, β -cat^{c.a.}, or β -cat^{c.a.}/*Ikk α ^{AA/AA}* IEC 15 days after the first tamoxifen administration. A total of 732 genes were significantly differentially expressed. In IEC from β -cat^{c.a.}/*Ikk α ^{AA/AA}* compared to β -cat^{c.a.} mice, a general downregulation of Wnt-dependent transcripts rather than control of particular gene subsets was observed. These different

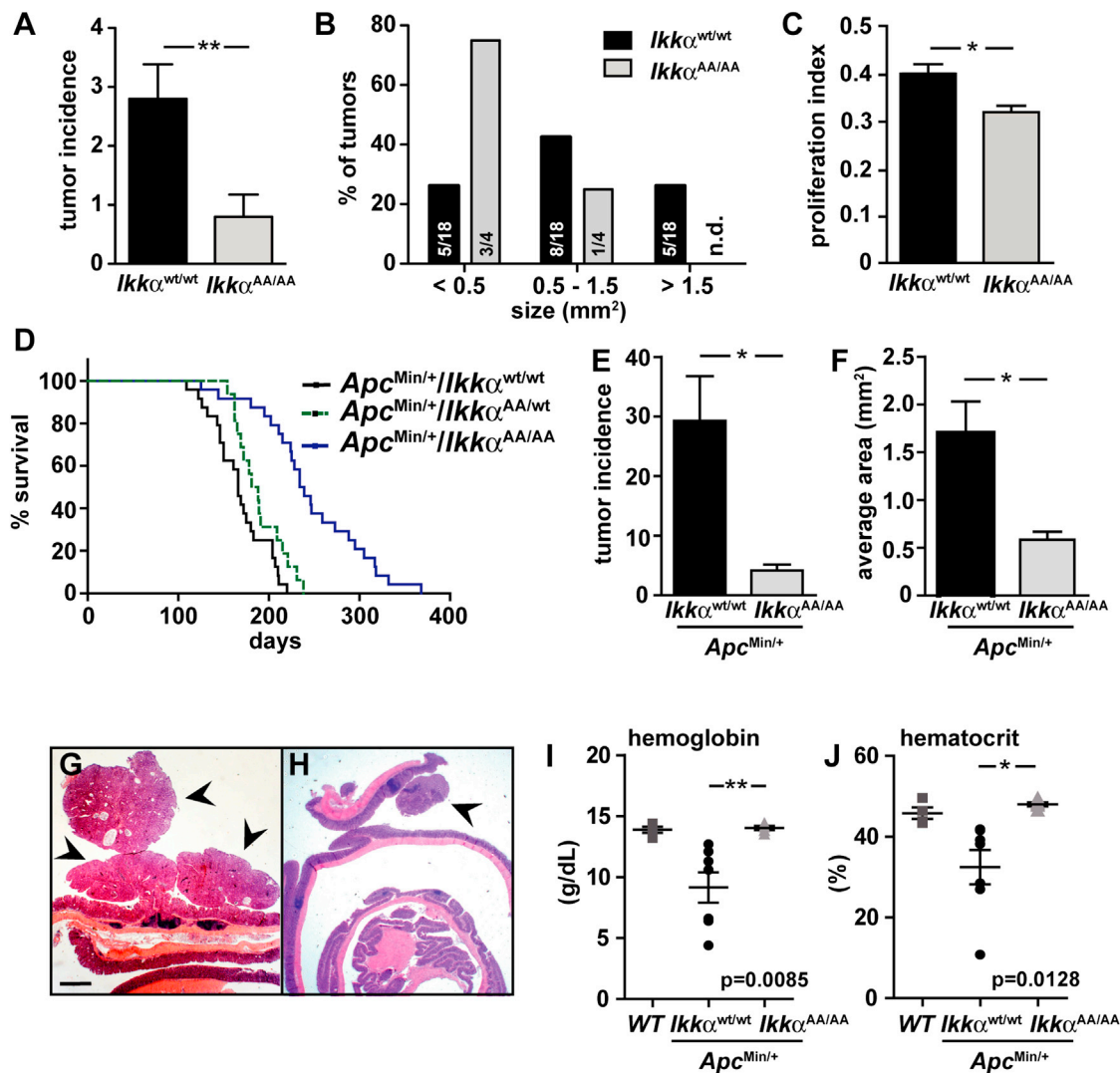


Figure 1. Block of IKK α Activation Impairs Development of Carcinogen and Genetically Induced Intestinal Tumor Models

(A) Tumor incidence of *Ikkα*^{WT/WT} and *Ikkα*^{AA/AA} mice 20 weeks after the first of six weekly AOM treatments. Data are mean ± SE; n = 5 mice of each genotype; **p < 0.01 by t test.

(B) Size distribution of AOM-induced tumors in *Ikkα*^{WT/WT} (n = 18) and *Ikkα*^{AA/AA} (n = 4) mice; n.d., not detectable; tumors of this size were not observed in *Ikkα*^{AA/AA} mice.

(C) BrdU proliferation index of *Ikkα*^{WT/WT} and *Ikkα*^{AA/AA} tumor epithelia. Data are mean ± SE; n ≥ 3 tumors of each genotype; *p < 0.05 by t test.

(D) Kaplan-Meier survival curve of *Apc*^{Min/+}/*Ikkα*^{WT/WT} (n = 24), *Apc*^{Min/+}/*Ikkα*^{WT/AA} (n = 16), and *Apc*^{Min/+}/*Ikkα*^{AA/AA} (n = 24); ***p < 0.0001 by log rank test.

(E) Tumor incidence in small intestine and colon of *Apc*^{Min/+}/*Ikkα*^{WT/WT} and *Apc*^{Min/+}/*Ikkα*^{AA/AA} mice at 4 months. Data are mean ± SE; n ≥ 6; *p < 0.05 by t test.

(F) Average tumor area in small intestine and colon of *Apc*^{Min/+}/*Ikkα*^{WT/WT} and *Apc*^{Min/+}/*Ikkα*^{AA/AA} mice at 4 months. Data are mean ± SE; n ≥ 15 tumors of each genotype; *p < 0.05 by t test.

(G and H) Representative hematoxylin and eosin (H&E)-stained sections of colons from *Apc*^{Min/+}/*Ikkα*^{WT/WT} (G) and *Apc*^{Min/+}/*Ikkα*^{AA/AA} (H) mice at 4 months; scale bar = 1 mm. Arrowhead indicates adenoma.

(I and J) Hemoglobin (I) and hematocrit (J) levels of *Apc*^{Min/+}/*Ikkα*^{WT/WT} and *Apc*^{Min/+}/*Ikkα*^{AA/AA} mice at 4 months. Data are mean ± SE; n ≥ 5 of each genotype; **p < 0.01 and *p < 0.05 by t test.

transcription profiles supposedly reflected the observed differences in IEC morphology between the two genotypes (Figure 2A), but not distinct IKK α -controlled signaling events. Indeed, knock-down of IKK α did not decrease β -catenin binding to its Tcf/Lef motif in human embryonic kidney 293 cells when transfected with a constitutively active β -catenin mutant (Figure S1). There-

fore, we focused our attention on the group of transcripts that were markedly upregulated in IEC from *β -cat^{c.a.}/Ikkα^{AA/AA}* mice. These could be classified into genes associated with immune response and inflammatory functions when sorted by their membership in KEGG pathways (Figure 3A). More specifically, gene set enrichment analysis (GSEA) indicated an enrichment

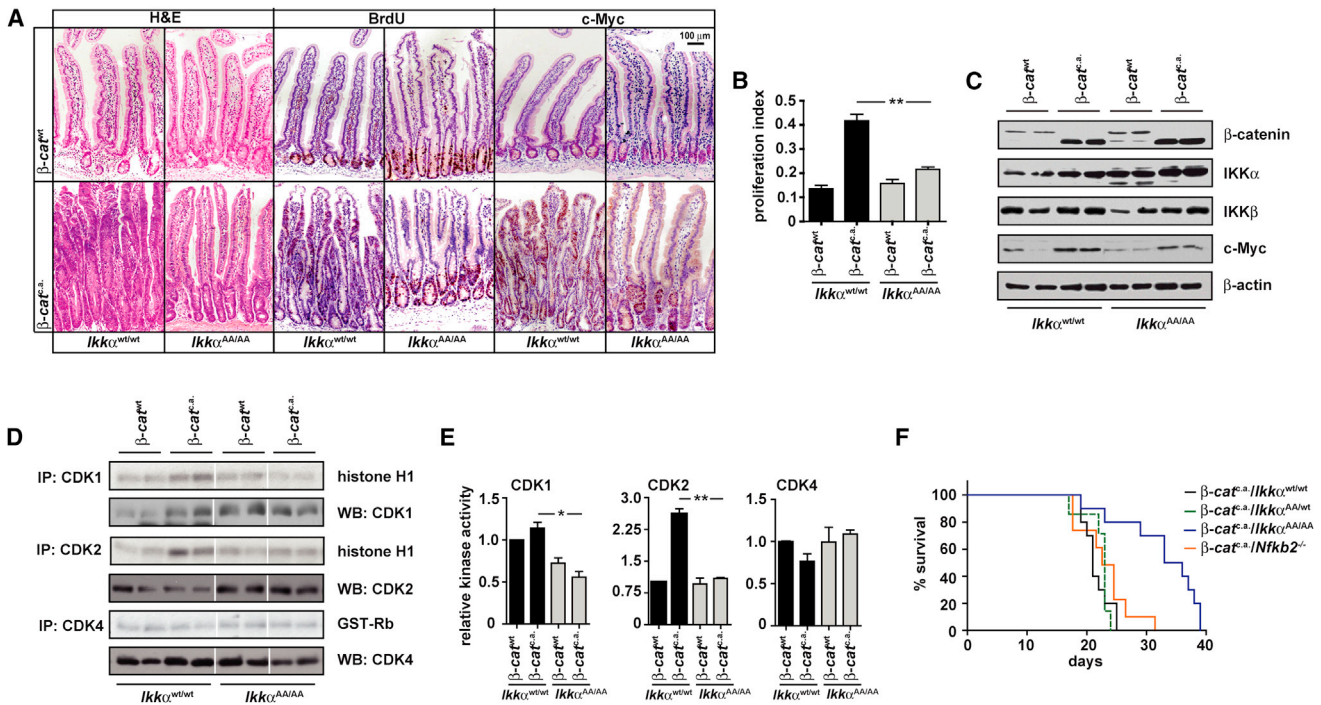


Figure 2. Reduced Proliferation in IKK α -Activation-Impaired Mice Is due to G1 Cell Cycle Arrest and Is Independent of Alternative NF- κ B Activation

(A) H&E staining as well as immunohistochemical analysis of BrdU and c-Myc in small intestine of wild-type, *Ikkα*^{AA/AA}, β -cat^{c.a.}, and β -cat^{c.a.}/*Ikkα*^{AA/AA} mice 15 days after the first tamoxifen application. The scale bar represents 100 μ m.

(B) BrdU proliferation index of epithelial cells in unchallenged wild-type and *Ikkα*^{AA/AA} mice as well as β -cat^{c.a.} and β -cat^{c.a.}/*Ikkα*^{AA/AA} mice 15 days after first tamoxifen administration. Data are mean \pm SE; n = 3 for all genotypes; **p < 0.01 by t test.

(C) Immunoblot analysis of β -catenin, IKK α , IKK β , and c-Myc in β -cat^{c.a.}/*Ikkα*^{WT/WT} and β -cat^{c.a.}/*Ikkα*^{AA/AA} mice IEC lysates at 0 or 15 days after first tamoxifen injection.

(D and E) Kinase assays (D), loading controls, and (E) loading-corrected relative kinase activities for endogenous CDK1, CDK2, and CDK4 in IEC from unchallenged wild-type and *Ikkα*^{AA/AA} mice as well as β -cat^{c.a.} and β -cat^{c.a.}/*Ikkα*^{AA/AA} mice 15 days after first tamoxifen application. Data are mean \pm SE; n = 2 for all genotypes; *p < 0.05 and **p < 0.01 by ANOVA followed by Bonferroni post hoc test for multiple data sets. GST, glutathione S-transferase; IP, immunoprecipitation; WB, western blot.

(F) Kaplan-Meier survival graph for β -cat^{c.a.}/*Ikkα*^{WT/WT} (n = 11), β -cat^{c.a.}/*Ikkα*^{WT/AA} (n = 7), β -cat^{c.a.}/*Ikkα*^{AA/AA} (n = 10), and β -cat^{c.a.}/*Nfkb2*^{-/-} (n = 8) mice; **p < 0.01 by log rank test.

See also Figure S1.

of type I and II IFN targets in β -cat^{c.a.}/*Ikkα*^{AA/AA} IEC (Figure 3B), including *Stat1*, *Irf1*, *Nos2*, *Oas1*, *Pkr*, and *Isg15*, which could be confirmed by real-time PCR (Figure 3C). This was paralleled by a marked upregulation of IFN γ in whole mucosa of β -cat^{c.a.}/*Ikkα*^{AA/AA} mice (Figure 3D). Moreover, immunoblot analysis confirmed activation of tyrosine-phosphorylated Stat1(Y701) as well as upregulation of Nos2 and IRF-1 in IKK α mutant IEC (Figure 3E). Because IFN γ /Stat1 signaling is known to suppress tumor cell proliferation, this raised the possibility that the decreased IEC proliferation in β -cat^{c.a.}/*Ikkα*^{AA/AA} mice was non-cell-autonomous and IFN γ dependent. To confirm this hypothesis, we either adoptively transferred *Ikkα*^{AA/AA} bone marrow to β -cat^{c.a.} mice or used conditional *Ikkα*^{F/F} mutants to specifically delete IKK α in β -cat^{c.a.} IEC (Liu et al., 2008). IEC-restricted deletion of IKK α only moderately protected β -cat^{c.a.} mice (Figure 4A). In contrast, adoptive transfer of *Ikkα*^{AA/AA} bone marrow extended survival of β -cat^{c.a.} mice almost to the same extent as it was otherwise seen in *Ikkα*^{AA/AA} whole-body

mutants (Figure 4B). More importantly, loss of *Irfng*, but not blocking type I interferon signaling by *Irfnar* deletion, completely prevented IKK α -mediated survival advantage (Figure 4C). Collectively, these data provided clear evidence that mutant IKK α suppressed IEC proliferation in a paracrine type II interferon-dependent manner.

IKK α ^{AA/AA} Myeloid Cells Rather Than T or NK Cells Comprise the Source of IFN γ

The most common IFN γ -expressing cell types in the lamina propria are T and natural killer (NK) cells. Surprisingly, we did not detect any difference in the number of mucosa-infiltrating CD3⁺ T cells by immunohistochemistry (Figure 5A). Moreover, fluorescence-activated cell sorting (FACS) analysis did not indicate changes in the number of CD4⁺IFN γ ⁺ or CD8⁺IFN γ ⁺ T cells between β -cat^{c.a.} and β -cat^{c.a.}/*Ikkα*^{AA/AA} mice when animals were analyzed 15 days after the first tamoxifen administration (Figure 5B). Moreover, when we differentiated naive T cells

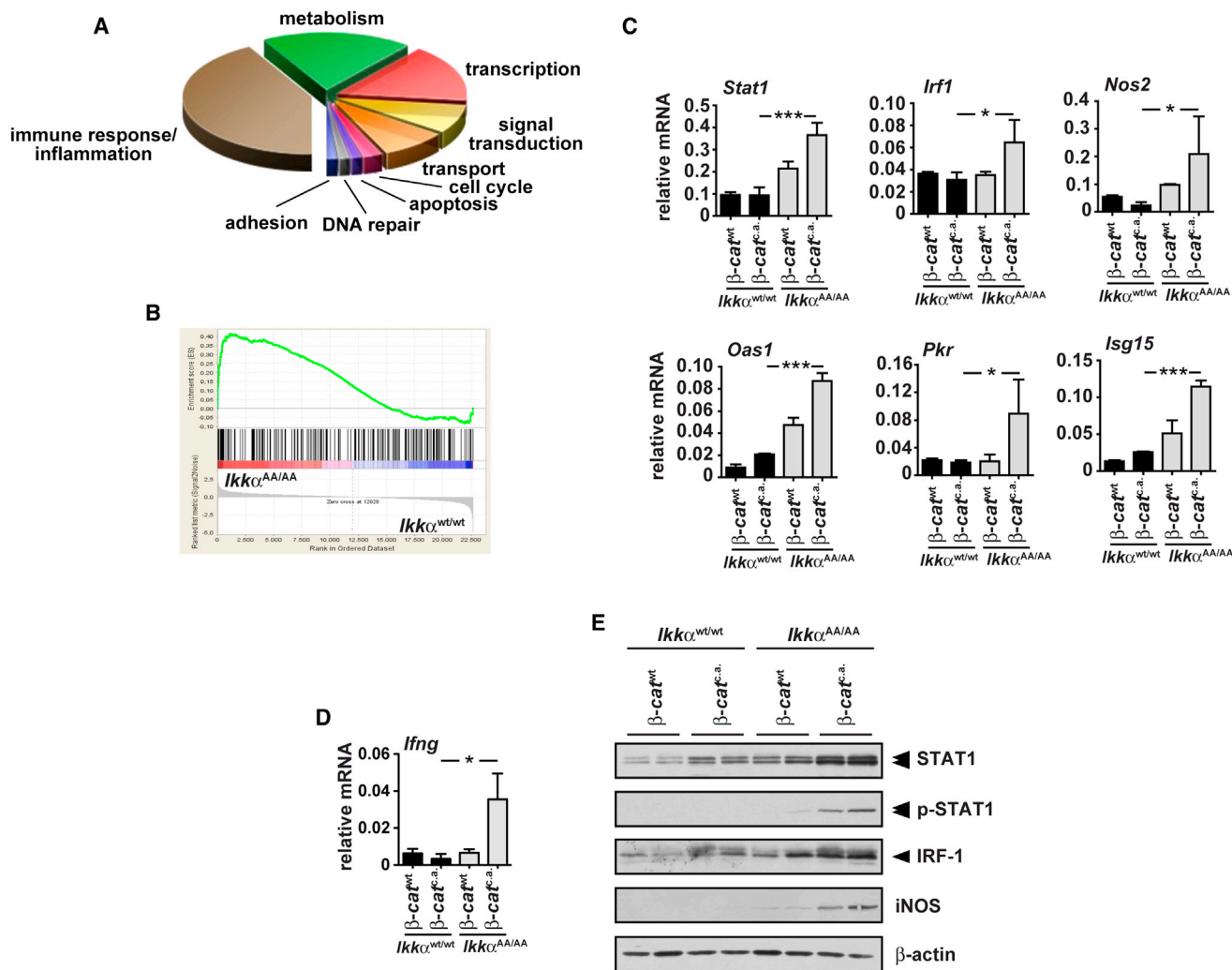


Figure 3. Generation of an IFN γ -Dominated Microenvironment in the Mucosa of β -cat^{c.a.}/*Ikk* α ^{AA/AA} Mice

(A) KEGG pathway analysis of the genes significantly upregulated in β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice IECs 15 days after first tamoxifen administration. (B) Gene set enrichment analysis (GSEA) comparing expression of all genes with a combined data set of interferon-regulated genes (Browne et al., 2001; Zhang et al., 2005; Bosco et al., 2010; Einav et al., 2005; Natsume et al., 2005; Reactome database; STKE database) in β -cat^{c.a.}/*Ikk* α ^{WT/WT} versus β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice IECs 15 days after first tamoxifen injection; normalized enrichment score = 2.03; ****p < 0.001. (C and D) Real-time PCR expression analysis of indicated genes in mucosa from wild-type, *Ikk* α ^{AA/AA}, β -cat^{c.a.}, and β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice 15 days after the first tamoxifen application. Data are mean \pm SE; n \geq 3 for all genotypes. *p < 0.05; ****p < 0.001 by ANOVA followed by Bonferroni post hoc test for multiple data sets. (E) Immunoblot analysis of IFN γ downstream targets p-STAT1, STAT1, IRF1, and iNOS in small-intestinal IEC from wild-type, *Ikk* α ^{AA/AA}, β -cat^{c.a.}, and β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice 15 days after the first tamoxifen application.

from either wild-type or *Ikk* α ^{AA/AA} animals into either Th₁ or Th₂ cells ex vivo, we were not able to determine any significant changes between the two genotypes (Figure S2A). Consequently, athymic nude mice (NU-*Foxn1*^{nu}) that lack T cells did not revert the survival advantage of β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice (data not shown). In addition, also depletion of NK cells using α -asialo-GM-1 antibody (reduction of >90% of splenic DX5⁺ cells was confirmed by FACS; data not shown) did not affect survival of β -cat^{c.a.}/*Ikk* α ^{AA/AA} mice (Figure 5C), indicating that neither CD4⁺, CD8⁺ T cells, nor NK cells were responsible for the IFN γ -mediated survival extension of *Ikk* α ^{AA/AA} mutant β -cat^{c.a.} mice.

During carcinogenesis, polarization of myeloid cells into M1 or M2 macrophages or in the case of neutrophils into N1 or N2 has been suggested to play an important role for tumor development (Sica and Mantovani, 2012). Depending on the prevailing polarization profile, M1 macrophages (typically expressing *Ifng*, *Tnfa*, *Ii12*, *Nos2*, *Cxcl9*, *Cxcl10*, and *Cxcl11*) are considered tumoricidal, whereas M2 macrophages (characterized by high levels of *Arg1*, *Mrc1*, *Ccl17*, *Ccl22*, *Ym1*, and *Fizz1*) have been suggested to promote tumorigenesis (Sica and Mantovani, 2012). Considering the lack of T and NK cell involvement in the pronounced expression of *Nos2* and *Ifng*, we speculated that instead a general shift in macrophage polarization toward M1 could have been

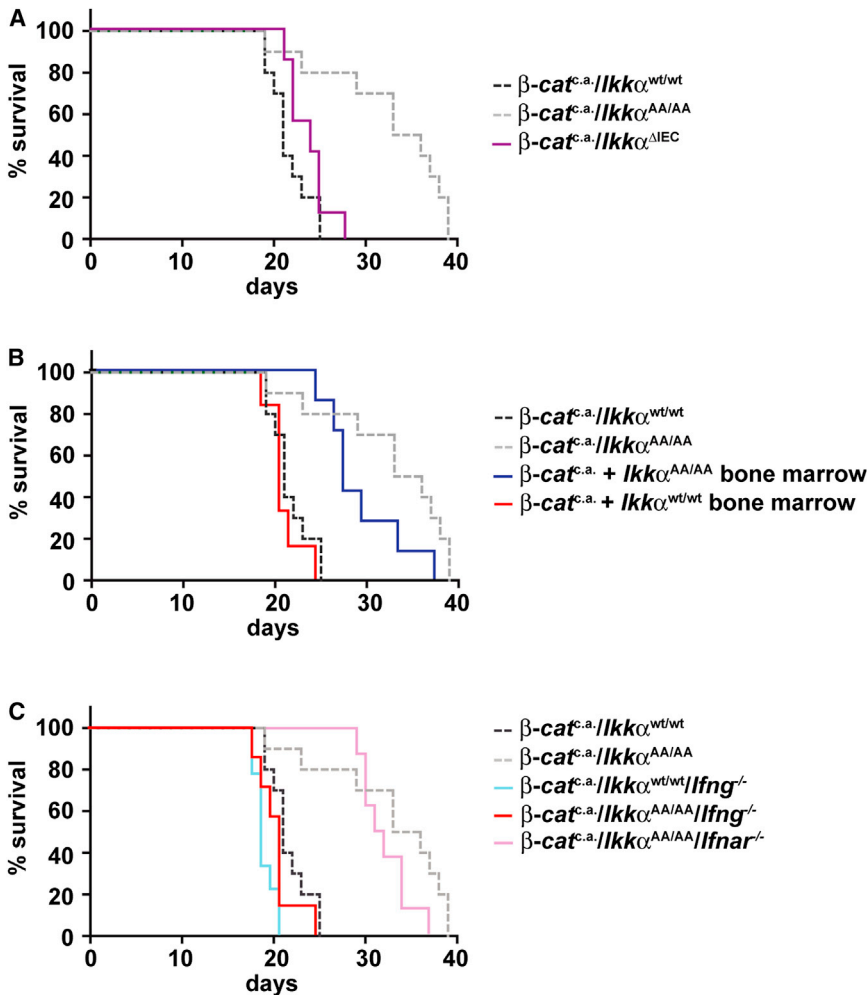


Figure 4. Improved Survival of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ Mice Depends on $\text{IFN}\gamma$

(A) Kaplan-Meier survival graph of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\Delta\text{IEC}}$ mice (violet line). Survival of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}$ and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice shown as comparison (dashed gray lines; $p = \text{not significant [n.s.]}$).

(B) Kaplan-Meier survival graph of lethally irradiated $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}$ mice transplanted with either $Ikk\alpha^{\text{WT/WT}}$ ($n = 6$; red line) or $Ikk\alpha^{\text{AA/AA}}$ ($n = 7$; blue line) bone marrow; *** $p < 0.001$ by log rank test.

(C) Kaplan-Meier survival graph of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}/Ifng^{-/-}$ ($n = 9$; light blue line), $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}/Ifng^{-/-}$ ($n = 5$; red line; ** $p < 0.005$), and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}/Ifnar1^{-/-}$ mice ($n = 8$; pink line; $p > 0.05$) mice. Survival of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}$ and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice shown as comparison (dashed gray lines).

responsible for this. Indeed, besides *Nos2* and *Ifng*, expression of several other genes encoding M1 markers were elevated in the lamina propria of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice 15 days after the first tamoxifen administration, whereas only *Ccl17* among M2-associated genes was downregulated (Figure 6A). To examine whether $Ikk\alpha^{\text{AA/AA}}$ mutant macrophages per se would reveal an M1 phenotype, we stimulated bone-marrow-derived macrophages (BMDM) under M1- or M2-polarizing conditions. Interestingly, ex vivo $Ikk\alpha^{\text{AA/AA}}$ macrophages did not reveal an enhanced M1 polarization profile compared to control BMDM and did not show a preferred polarization (Figure 6B). Moreover, also *Ifng* expression was indifferent when BMDM of either genotype were stimulated with a combination of IL-12 and IL-18 (Figure S2C), thus ruling out a cell autonomous regulation in the generation of $Ikk\alpha^{\text{AA/AA}}$ M1-like cells in vivo.

Elevated expression levels of myeloid-recruiting chemokines such as *Cxcl1*, *Cxcl2*, *Cxcl5*, and *Ccl2* (Figure 6C) led to enhanced recruitment of macrophages, neutrophils, and dendritic cells into the lamina propria of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mutants, as determined by real-time PCR of genes encoding surface markers *Emr1*, *Ly6g*, and *Itgax* (Figure 6D) as well as by FACS analysis (data not shown). Only *Cxcl1* and *Cxcl2* upregulation

was observed in $Ikk\alpha$ mutant epithelia (Figure 6C), indicating that *Cxcl5* and *Ccl2* were derived from infiltrating immune cells. Interestingly, apart from enhanced myeloid cell recruitment, also localization of both macrophages and neutrophils was distinct in $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice, where they could be found interspersed in between IEC. In contrast, infiltration of F4/80^+ and Gr-1^+ myeloid cells was limited to the villus stroma in $Ikk\alpha$ wild-type-expressing $\beta\text{-cat}^{\text{c.a.}}$ mice (Figures 6E–6H). Importantly, immunofluorescence confirmed that indeed both macrophages and neutrophils expressed $\text{IFN}\gamma$ (Figures 6I and

6J), indicating that an enhanced recruitment of M1-like myeloid cells was responsible for the $\text{IFN}\gamma$ -dependent survival advantage of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice.

Enhanced Recruitment of Myeloid Cells Depends on IKK β Activation in $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ IEC

Myeloid-cell-recruiting chemokines such as *Cxcl1*, *Cxcl2*, *Cxcl5*, and *Ccl2* are controlled by classical NF- κ B activation (Grivnickov et al., 2010). To functionally confirm that indeed the enhanced recruitment of M1-like myeloid cells was responsible for the extended survival in $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice, we reasoned that loss of IKK β -dependent NF- κ B activation in IEC should block recruitment of myeloid cells in $Ikk\alpha$ mutant $\beta\text{-cat}^{\text{c.a.}}$ animals. Thus, we intercrossed floxed *Ikk β* mutants (*Ikk β ^{F/F}*) with $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice to generate $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}/Ikk\beta^{\Delta\text{IEC}}$ as well as $\beta\text{-cat}^{\text{c.a.}}/Ikk\beta^{\Delta\text{IEC}}$ compound mutants. Expectedly, IKK β deletion decreased *Cxcl1* and *Ccl2* as well as *Emr1*, *Ly6g*, and *Itgax* expression and prevented *Ifng* upregulation (Figure 7A). Immunofluorescence confirmed diminished F4/80^+ and Gr-1^+ cell infiltration into mucosa of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}/Ikk\beta^{\Delta\text{IEC}}$ animals (Figures 7B–7E). We recently demonstrated that loss of RelA/p65 expanded the life span of $\beta\text{-cat}^{\text{c.a.}}$ mice (Schwitalla

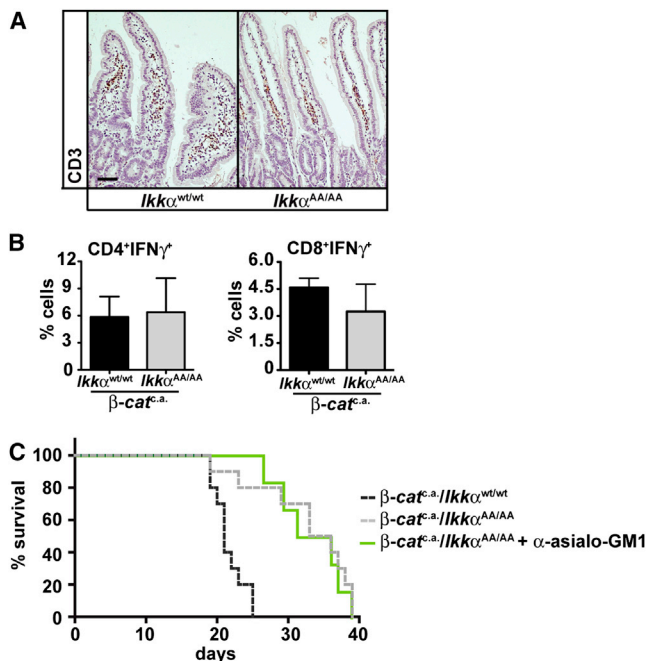


Figure 5. IKK α Mutant T Cells and NK Cells Do Not Contribute to Elevated IFN γ Expression in $\beta\text{-cat}^{\text{c.a.}}$ Mice

(A) Immunohistochemical analysis of CD3 in small intestine of $\beta\text{-cat}^{\text{c.a.}}$ and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice 15 days after the first tamoxifen application. The scale bar represents 100 μm .

(B) FACS analysis of CD4⁺IFN γ ⁺ or CD8⁺IFN γ ⁺ mucosa infiltrating lymphoid cells isolated from $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}$ and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice small intestinal mucosa 15 days after first tamoxifen administration. T cells were restimulated using PMA (20 ng/ml) and ionomycin (1 $\mu\text{g/ml}$) for 6 hr in the presence of Brefeldin A. Data are mean \pm SE; n = 2.

(C) Kaplan-Meier survival graph of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice injected with $\alpha\text{-asialo-GM1}$ antibody (n = 6; green line). Survival of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{WT/WT}}$ and $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice shown as comparison (dashed grey lines; p = n.s.).

et al., 2013a). Similarly, deletion of *Ikk β* prolonged survival of *Cttnb* mutants (Figure 7F). However, consistent with a lack of *Ifn γ* induction, $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ animals were no longer protected in the absence of IKK β and their survival was now comparable to that of $\beta\text{-cat}^{\text{c.a.}}/Ikk\beta^{\Delta\text{IEC}}$ mice (Figure 7F). Thus, these data further supported the importance of IFN γ -expressing myeloid cells for the survival of $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice and confirmed that M1-like polarization did not occur in a cell autonomous manner in IKK α mutant myeloid cells.

DISCUSSION

Cell plasticity is an important phenomenon during carcinogenesis that affects basically all cells in the tumor microenvironment (Hanahan and Weinberg, 2011). Whereas induction of EMT altering tumor cells themselves is an essential prerequisite for invasion and metastasis, polarization of infiltrating immune cells provides the microenvironment-dominating cytokine milieu, which ultimately controls behavior of resident stromal and tumor cells. Depending on the cytokine milieu, carcinogenesis is promoted or suppressed (Grivnickov et al., 2010). To decipher the

cellular and molecular mechanisms that shape the cytokine milieu bears great therapeutic potential, because cytokine-producing immune cells are unlikely to develop resistance mechanisms in contrast to mutagenized tumor cells. Although IKK α has been suggested to control various cell autonomous tumor-promoting mechanisms in CRC (Albanese et al., 2003; Margalef et al., 2012), here we provide evidence that IKK α comprises a central regulatory element in the suppression of M1-like myeloid cell controlled microenvironment rather than directly stimulating tumor cell proliferation. Elevated IFN γ levels in *Ikk α ^{AA/AA}* mucosa are most likely responsible for growth arrest of initiated epithelial cells. However, considering the particular intraepithelial localization of myeloid cells in $\beta\text{-cat}^{\text{c.a.}}/Ikk\alpha^{\text{AA/AA}}$ mice, we cannot rule out the possibility that these cells may also participate either in extracellular killing or phagocytosis of tumor cells as it was recently demonstrated using oncogenic HRAS(G12V)-transformed cells in zebrafish larvae (Feng et al., 2010).

Importantly, polarization of IKK α mutant myeloid cells depends on a complex interplay of IEC and infiltrating cells and is not a cell autonomously controlled process. Instead, it involves classical NF- κ B-dependent secretion of chemokines in *Ikk α ^{AA/AA}* IEC that triggers recruitment of myeloid cells, which in turn secrete cytokines that culminate in M1 polarization and fuel into a feedforward loop that drives IFN γ secretion. This is further supported by the fact that administration of IL-12-neutralizing antibodies is not sufficient to block this loop and to prevent IFN γ secretion (S.I.G. and F.R.G., unpublished data). However, prevention of myeloid cell infiltration and subsequent ablation of M1-like polarization can be achieved by inhibition of NF- κ B activation in IEC. Considering that IKK α expression correlates with poor prognosis in human Union for International Cancer Control stage II CRC (S.I.G. and F.R.G., unpublished data), whereas IFN γ upregulation is associated with improved survival (Grenz et al., 2013), IKK α may represent a valuable therapeutic target for CRC therapy or prevention. If, however, putative IKK α inhibitors were indeed at some point considered for CRC therapy, our data imply that such compounds are required to be highly specific inhibitors of only IKK α because simultaneous overlapping IKK β inhibition would most likely prevent the beneficial effects of selective IKK α inhibition.

We recently demonstrated that, during Wnt-initiated tumorigenesis, NF- κ B activation cooperates with β -catenin to control dedifferentiation of postmitotic epithelia and stem cell expansion (Schwitalla et al., 2013a). Although in IKK α mutant IEC chemokine expression is enhanced in an NF- κ B-dependent manner, we do not have any evidence that Wnt signaling is enhanced in *Ikk α ^{AA/AA}* IEC as well. This supports the notion that IKK α is responsible for the negative regulation of a very distinct set of NF- κ B target genes only (Lawrence et al., 2005; Schwitalla et al., 2013a).

Depending on the type of malignancy, IKK α can provide both tumor-promoting and tumor-suppressive mechanisms that are in most instances cell autonomous. IKK α controls expression of the inhibitor of metastasis maspin in breast and prostate cancer (Luo et al., 2007; Tan et al., 2011) and is required for ErbB2-induced mammary tumorigenesis. In the latter case,

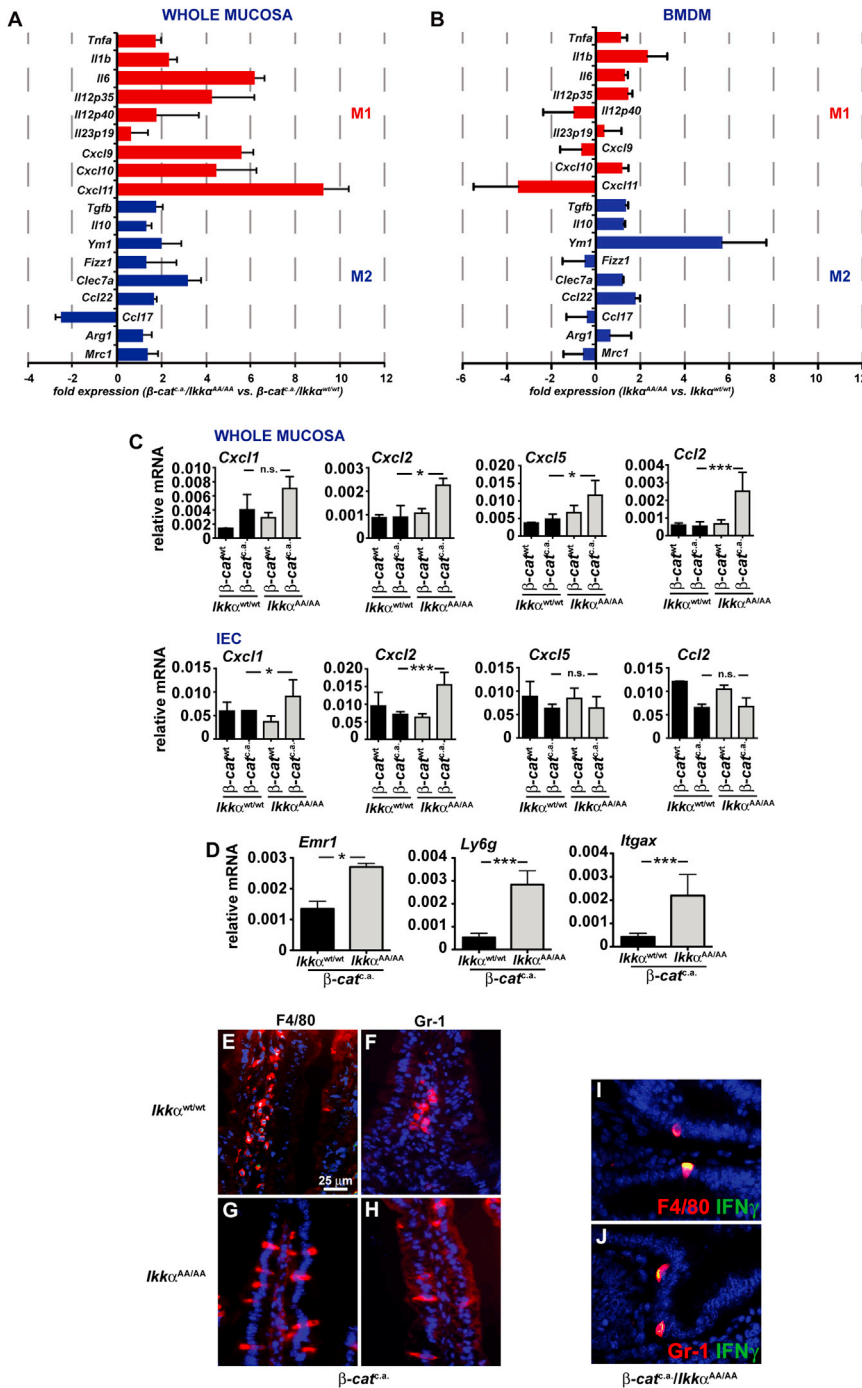


Figure 6. M1-like Polarized Myeloid Cells Are Source of IFN γ in β -cat^{c.a.}/*Ikka*^{AA/AA} Mice

(A) Real-time PCR expression analysis of M1 (red) and M2 (blue) macrophage markers in lamina propria samples from β -cat^{c.a.}/*Ikka*^{WT/WT} and β -cat^{c.a.}/*Ikka*^{AA/AA} mice 15 days after first tamoxifen administration. Data shown represent fold difference of Ct values from β -cat^{c.a.}/*Ikka*^{WT/WT} versus β -cat^{c.a.}/*Ikka*^{AA/AA} mice. Data are mean \pm SE; $n \geq 3$ for both genotypes.

(B) Real-time PCR expression analysis of M1 (red) and M2 (blue) macrophage markers in bone-marrow-derived macrophages (BMDM) from *Ikka*^{WT/WT} and *Ikka*^{AA/AA} mice that have been stimulated with a mix of IFN γ (5 ng/ml) and lipopolysaccharide (100 ng/ml; M1 markers) or IL-4 (10 ng/ml; M2 markers) for 4 hr. Data shown represent fold difference of Ct values from *Ikka*^{AA/AA} versus *Ikka*^{WT/WT} mice. Data are mean \pm SE; $n \geq 3$ for both genotypes.

(C) Real-time PCR expression analysis of chemokines *Cxcl1*, *Cxcl2*, *Cxcl5*, and *Ccl2* in whole mucosa samples or IEC from unchallenged wild-type and *Ikka*^{AA/AA} mice as well as β -cat^{c.a.} and β -cat^{c.a.}/*Ikka*^{AA/AA} mice 15 days after first tamoxifen application. Data are mean \pm SE; $n \geq 3$ for all genotypes. * $p < 0.05$, *** $p < 0.001$ by ANOVA followed by Bonferroni post hoc test for multiple data sets.

(D) RT-PCR expression analysis of myeloid cell markers *Emr1*, *Ly6g*, and *Itgax* in whole mucosa samples from unchallenged wild-type and *Ikka*^{AA/AA} mice as well as β -cat^{c.a.} and β -cat^{c.a.}/*Ikka*^{AA/AA} mice 15 days after first tamoxifen application. Data are mean \pm SE; $n \geq 3$ for all genotypes. * $p < 0.05$; *** $p < 0.001$ by t test.

(E–H) Immunofluorescence staining of myeloid cell markers F4/80 (E and G) and Gr-1 (F and H) in small intestine of β -cat^{c.a.}/*Ikka*^{WT/WT} and β -cat^{c.a.}/*Ikka*^{AA/AA} mice 15 days after first tamoxifen application. The scale bar represents 25 μ m.

(I and J) Coimmunofluorescence of F4/80 and IFN γ (I) as well as Gr-1 and IFN γ (J) in small intestine of β -cat^{c.a.}/*Ikka*^{WT/WT} and β -cat^{c.a.}/*Ikka*^{AA/AA} mice 15 days after first tamoxifen application.

See also Figure S2.

NIK-dependent IKK α activation regulates expansion of tumor-initiating cells by directly phosphorylating the cyclin-dependent kinase inhibitor p27 (Zhang et al., 2013). Additional tumor-promoting nuclear functions of IKK α include cell cycle regulation and chromosomal accessibility by phosphorylation of histone H3, AuroraB kinase, or the nuclear corepressor SMRT, which triggers its nuclear export with HDAC3 and its degradation (Chariot, 2009). In contrast, IKK α acts as a tumor suppressor in models of skin or

lung SCC (Liu et al., 2008; Xiao et al., 2013). Importantly, during development of lung SCC, IKK α kinase inactivation culminates in the recruitment of tumor-promoting inflammatory macrophages and depletion of macrophages prevents SCC formation (Xiao et al., 2013). This is in clear contrast to our findings presented here, yet the reason for this diverse macrophage activation profile in these two different tumor entities remains currently unclear. One could envision that specific alterations in the intestinal microbiome of *Ikka*^{AA/AA} mice may be involved in the tumor-suppressive M1 polarization of myeloid cells that only becomes apparent after barrier defect causing oncogene activation (Grivennikov et al., 2012).

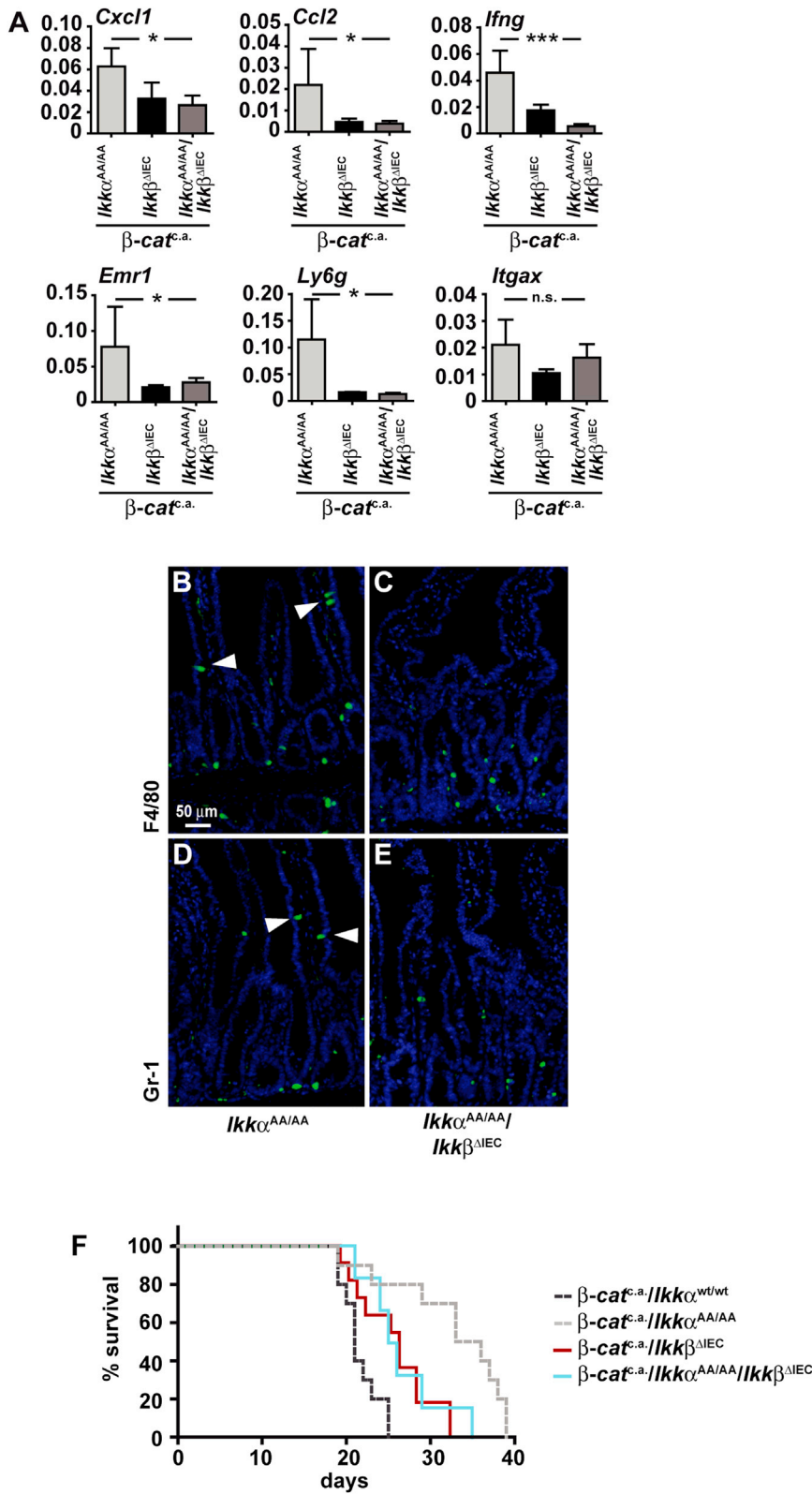


Figure 7. Enhanced Recruitment and Activation of Myeloid Cells in $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}* Mice Depend on IKK β -Dependent NF- κ B Activation in IEC

(A) Real-time PCR analysis of indicated genes in $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{WT/WT}*, $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}*, and $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}/*Ikk β ^{Δ IEC}** mice whole mucosa samples 15 days after first tamoxifen application. Data are mean \pm SE; $n \geq 3$ for all genotypes. * $p < 0.05$; *** $p < 0.001$ by ANOVA followed by Bonferroni post hoc test for multiple data sets.

(B–E) Immunofluorescent staining of F4/80 (B and C) and Gr-1 (D and E) in small intestine of $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}* and $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}/*Ikk β ^{Δ IEC}** mice 15 days after first tamoxifen administration. The scale bar represents 50 μm .

(F) Kaplan-Meier survival graph of $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk β ^{Δ IEC}* ($n = 11$; red line) and $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}/*Ikk β ^{Δ IEC}** mice ($n = 6$; blue line). Survival of $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{WT/WT}* and $\beta\text{-cat}^{\text{c.a.}}$ /*Ikk α ^{AA/AA}* mice shown as comparison (dashed gray lines).

EXPERIMENTAL PROCEDURES

Mice

Ikkα^{AA/AA} (Cao et al., 2001), *Ikkα^{F/F}* (Liu et al., 2008), *Ikkβ^{F/F}* (Greten et al., 2004), *Nfkb2^{-/-}* (Paxian et al., 2002), and *β-cat^{c.a.}* (Schwitalla et al., 2013a) mice have been recently described. C57BL/6Apc^{Min/+}, *Irfng^{-/-}*, and NU-Foxn1^{nu} mice were purchased from the Jackson Laboratories, and *Irfnar^{-/-}* mice were provided by F. Schmitz, TU Munich. To examine AOM-induced tumorigenesis, mice had been backcrossed to a FVB background for four generations, and littermate controls were used in all experiments. Before crossing to C57BL/6Apc^{Min/+} mice, *Ikkα^{AA/AA}* animals had been backcrossed to a C57BL/6 background for eight generations. Experiments using *β-cat^{c.a.}* mutants were performed on a mixed C57BL/6 × 129Sv × FVB background, and in all experiments, littermate controls were used. Tamoxifen (Sigma; 1 mg in an ethanol/sunflower oil mixture) was applied by oral gavage on 5 consecutive days. Azoxymethane (10 mg/kg; Sigma) was injected intraperitoneally (i.p.) once weekly for 6 weeks. In adoptive transfer experiments, recipient mice were irradiated (9 Gy) and 2 × 10⁶ bone marrow cells from *Ikkα^{AA/AA}* mice or *Ikkα^{WT/WT}* littermate controls were transferred by tail vein injection. Eight weeks after transplantation, tamoxifen administration was started. To deplete NK cells, 200 μg α-Asialo GM1 antibody (no. 986-10001; Wako) was injected i.p. every 4 days. All procedures were reviewed and approved by the Regierung von Oberbayern.

Protein Analysis

Isolation of enterocytes, immunoblot analysis, immune complex kinase assay, and DNA affinity precipitation assay were performed as described previously (Bollrath et al., 2009; Schwitalla et al., 2013a). The following antibodies were used: anti-IKKα (IMG136A; Imgenex), anti-IKKβ (05-535; Upstate), anti-β-actin (A4700; Sigma), anti-β-catenin (UBI 6734; United Bio Research), anti-phospho-STAT1 (9171; Cell Signaling Technology), anti-STAT1 (SC-346; Santa Cruz Biotechnology), anti-c-Myc (SC-788), anti-IRF-1 (SC-640), anti-NOS2 (SC-651), anti-Cdc2 (SC-54), anti-Cdk2 (SC-163), anti-Cdk4 (SC-260), and anti-β-catenin (SC-1496).

Histological Procedures and Flow Cytometry

Standard immunohistochemical procedures were performed using the following antibodies: anti-bromodeoxyuridine (BrdU) antibody (RPN201; GE Healthcare), anti-IKKα (Abcam; ab109749; clone EPR464), CD68 (Fisher Scientific; MS-397; clone KP1), anti-c-myc (SC-788), anti-F4/80 (Caltag; MF 480043), anti-Gr-1 (eBioscience; 12-5931-85), and anti-IFNγ (R&D Systems; BAF 485). For the isolation of lamina propria cells, intestines of mice were opened longitudinally, cleared of mucus, chopped into small pieces, and shook in Hank's balanced salt solution (Invitrogen) containing 30 mM EDTA (Sigma-Aldrich) at 37°C for 20 min followed by 30 s of heavy vortexing to detach the epithelial layer. The remaining tissue pieces were washed at least five times in PBS and then digested in RPMI (Invitrogen) containing 1 mg/ml collagenase I (Sigma-Aldrich) and 20 μg/ml DNase I (Sigma-Aldrich) for 90 min at 37°C while gently shaking. Liberated cells were then washed in RPMI containing 10% fetal calf serum (Biochrom) and 1% penicillin-streptomycin (Invitrogen). T cells were restimulated using phorbol myristate acetate (PMA) (20 ng/ml) and ionomycin (1 μg/ml) for 6 hr in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich). Flow cytometric analysis was performed on a Gallios flow cytometer (Beckman Coulter Genomics) or a FACSCalibur (Becton Dickinson), and results were analyzed using FlowJo software (Tree Star). The following fluorochrome-conjugated antibodies were used: fluorescein isothiocyanate-anti-CD4 (BD-557653), phycoerythrin (PE)-anti-CD4 (BD-553651), PE-anti-CD8 (BD-550798), and PE-anti-IFNγ (BD-554412).

RNA Analysis

Total RNA extraction, cDNA synthesis, real-time PCR and gene expression profiling, and GSEA were performed as described previously (Bennecke et al., 2010). Primer sequences are available on request. In GSEA, we matched various interferon-related gene sets from GSEA Software Database (Bosco et al., 2010; Browne et al., 2001; Einav et al., 2005; Natsume et al., 2005; Zhang et al., 2005), Reactome database (http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_25229), and STKE database to all tran-

scripts from the Affymetrix Mouse Genome 430A 2.0 Array, respectively. GSEA software is available from Broad Institute of MIT and Harvard University (<http://www.broadinstitute.org/gsea/index.jsp>). We acknowledge the use of GSEA software (Subramanian et al., 2005) to validate correlation between molecular pathways signatures in any phenotype of interest. For the analysis of gene sets, we modified default parameters as follows: permutation number to 1,000, collapse data set to gene symbols if "false," permutation type to gene_sets, and only gene sets with the size of 1–2,000 signatures were used for analysis.

In Vitro Antigen Presentation Assay

Bone-marrow-derived dendritic cells (BMDC) were differentiated from wild-type (WT) and *Ikkα^{AA/AA}* bone marrow in RPMI supplemented with 20 ng/ml GM-CSF for 7 days. On day 7, BMDCs were loaded with ovalbumin (0.5 mg/ml) at 37°C for 1 hr. T cells from spleens of OT II mice (Jackson Laboratories) were purified using a CD4⁺CD62L⁺ T cell isolation Kit (Miltenyi Biotech) and carboxyfluorescein succinimidyl ester (CFSE)-labeled (5 μM; 10 min; 37°C). In 96-well plates, 1 × 10⁴ BMDCs were cocultured with 1 × 10⁵ CFSE-labeled OT II cells for 72 hr. Subsequently, cultures were stimulated with PMA (20 ng/ml) for 6 hr, T cell proliferation was analyzed by FACS analysis on a FACS Calibur, and IFNγ production was determined by ELISA.

Directed Differentiation of Naive CD4⁺ T Cells

Purified naive T_H cells (CD4⁺CD62L⁺) were stimulated with anti-CD28 antibody (2 μg/ml; eBioscience) and anti-CD3ε-antibody (145-2C11; 5 μg/ml; eBioscience), which had been bound to cell culture plates by anti-Syrian hamster antibody (10 μg/ml; Jackson ImmunoResearch). Cells were directed into the T_{H1} line by treatment with IL-12 (2 ng/ml; R&D Systems). Anti-IL-4 antibody was added (10 μg/ml; BD Biosciences) to prevent differentiation toward the T_{H2} line. Differentiation toward the T_{H2} line was achieved using IL-4 (10 ng/ml; R&D Systems) in the presence of anti-IFN-γ antibody (10 μg/ml; eBioscience) to suppress differentiation into T_{H1} cells. Cells were collected for real-time PCR analysis, and RNA was isolated using TRI reagent (Sigma-Aldrich). To confirm successful differentiation, cells were tested for their capacity to produce IFN-γ (T_{H1}) or IL-4 (T_{H2}). For that purpose, cells were harvested after the 3-day stimulation, washed, and incubated without stimulus but in presence of IL-2 (10 ng/ml; R&D Systems) for 3 additional days. Afterward, the cells were collected, restimulated with plate-bound anti-CD3 antibody (see above) in the presence of Brefeldin A (10 μg/ml, Sigma-Aldrich), and intracellular cytokine staining was performed.

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis methods were standard two-tailed Student's t test for two data sets and ANOVA followed by Bonferroni post hoc test for multiple data sets or log rank test for Kaplan-Meier survival graphs using Prism4 (GraphPad Software) or SPSS Statistics 21. p values ≤ 0.05 were considered significant.

ACCESSION NUMBERS

Gene expression data have been deposited in the Gene Expression Omnibus database under accession number GSE51631.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.006>.

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