The Prosurvival IKK-Related Kinase IKKε Integrates LPS and IL17A Signaling Cascades to Promote Wnt-Dependent Tumor Development in the Intestine

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Abstract

Constitutive Wnt signaling promotes intestinal cell proliferation, but signals from the tumor microenvironment are also required to support cancer development. The role that signaling proteins play to establish a tumor microenvironment has not been extensively studied. Therefore, we assessed the role of the proinflammatory Ikk-related kinase Ikke in Wnt-driven tumor development. We found that Ikke was activated in intestinal tumors forming upon loss of the tumor suppressor Apk. Genetic ablation of Ikke in β-catenin-driven models of intestinal cancer reduced tumor incidence and consequently extended survival. Mechanistically, we attributed the tumor-promoting effects of Ikke to limited TNF-dependent apoptosis in transformed intestinal epithelial cells. In addition, Ikke was also required for lipopolysaccharide (LPS) and IL17A-induced activation of Akt, Mek1/2, Erk1/2, and Msk1. Accordingly, genes encoding proinflammatory cytokines, chemokines, and anti-microbial peptides were downregulated in Ikke-deficient tissues, subsequently affecting the recruitment of tumor-associated macrophages and IL17A synthesis. Further studies revealed that IL17A synergized with commensal bacteria to trigger Ikke phosphorylation in transformed intestinal epithelial cells, establishing a positive feedback loop to support tumor development. Therefore, TNF, LPS, and IL17A-dependent signaling pathways converge on Ikke to promote cell survival and to establish an inflammatory tumor microenvironment in the intestine upon constitutive Wnt activation. Cancer Res; 76(9); 2587–99. ©2016 AACR.

Introduction

Colorectal cancer results from multiple genetic mutations and inflammatory processes (1). Somatic mutations associated with 80% of colorectal cancer cases target the adenomatous polyposis coli (APC) tumor suppressor gene, which leads to β-catenin activation, followed by additional mutations in K-Ras, PI3K3CA, and TP53 among others as tumors develop (2, 3).

The majority of colorectal cancer cases have no preexisting inflammation but nevertheless displays tissue infiltration by inflammatory cells, which is referred to as “tumor-elicited inflammation” (4, 5). Those infiltrates include CD4+ T-helper 1 (Th1) and CD8+ cytotoxic T cells (CTL), tumor-associated macrophages (TAM), and T-helper interleukin 17 (Th17) cells. The tumor-promoting functions of TAMs and T lymphocytes are mediated through the secretion of cytokines. TAMs produce IL23, which enhances tumor-promoting inflammatory processes through IL17A synthesis by Th17 cells and also suppresses the adaptive immune surveillance by reducing CD8+ CTL cell infiltration in tumors (4, 6–8). In turn, IL17A triggers MAPKs and NF-κB activations in intestinal epithelial cells (IEC) to support early tumor growth (9).

The establishment of a tumor microenvironment relies on transcription factors such as NF-κB (10, 11). IκB-kinase (Ik) β-dependent NF-κB activity in IECs promotes cell survival and...
drives the expression of proinflammatory cytokines in myeloid cells to link inflammation to cancer (12). In addition, NF-κB signaling in IECs also cooperates with β-catenin to facilitate the crypt stem cell expansion (13).

Both NF-κB and Stat3 transcription factors are activated by cytokines through parallel signaling pathways in solid tumors (14). Similar to NF-κB, Stat3 controls the expression of genes involved in cell survival, proliferation, and immunity. IL6, whose expression relies on NF-κB on xenoma propryoid myeloid cells, protects premalignant IECs from apoptosis through Stat3 activation in a model of colitis-associated cancer (15, 16). IL23 signaling also promotes Stat3 phosphorylation in Apc-mutated IECs through IL17A production by Th17 cells (7).

Constitutive β-catenin activation and/or Apc loss in the intestinal epithelium cause the loss of epithelial barrier function, an early event in intestinal tumorigenesis (7). As a result, commensal bacteria infiltrate the stroma and lead to tumor-associated inflammation (17). Bacterial products are sensed by Toll-like receptors (TLR) such as TLR4, which promotes colitis-associated cancer (18).

TLR signaling triggers IKKβ/NF-κB activation, leading to synthesis of proinflammatory cytokines and the phosphorylation of IKK-related kinases TBK1 and IKKε to induce type I interferon synthesis through IRF3 (19–21). IKKe is believed to play key roles in cancer by targeting multiple substrates, many of which act in NF-κB–dependent pathways (22–25). Both TBK1 and IKKε also directly phosphorylate AKT/protein kinase B (26, 27). So far, it remains to be demonstrated that IKKe acts as an oncogenic kinase in vivo.

Here we report that LPS and IL17A-dependent signaling pathways converge to Ikke to promote Wnt-dependent tumor development in IECs in vivo. These pathways drive the expression of proinflammatory cytokines, anti-microbial peptides, and chemokines, the latter recruiting macrophages to support IL23 and cytokines through parallel signaling pathways in solid tumors (14). Similar to NF-κB, Stat3 controls the expression of genes involved in cell survival, proliferation, and immunity. IL6, whose expression relies on NF-κB on xenoma propryoid myeloid cells, protects premalignant IECs from apoptosis through Stat3 activation in a model of colitis-associated cancer (15, 16). IL23 signaling also promotes Stat3 phosphorylation in Apc-mutated IECs through IL17A production by Th17 cells (7).

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Materials and Methods

Mouse models

Villin-Cre-ER<sup>T2</sup> Cnm1<sup>+/lox(ex3)</sup> (β-catenin<sup>−/−</sup>) mice were previously described (28, 29). Villin-Cre-ER<sup>T2</sup>Cnm1<sup>+/lox(ex3)</sup> mice were gavaged 5 consecutive days with 1 mg tamoxifen (Sigma) to induce β-catenin activation in enterocytes as described previously (30). Both Apc<sup>−/−</sup> and Ikke<sup>−/−</sup> mouse strains were from Jackson Laboratories (Bar Harbor, ME). For antibiotics treatments, 0.5 g ciprofloxacin, 1 g ampicillin, and 0.5 g metronidazole per liter were added in the drinking water 1 week before tamoxifen administration. All mice used were 8 to 16 weeks old when started with experiments (except for the Apc<sup>−/−</sup>/lox<sup>−/−</sup> survival experiments) and littermate controls were used. All procedures were approved by the local Ethical Committee of the University of Liege.

Bone marrow transplantation

Bone marrow transplantation and bone marrow cell isolation were done as described previously (30). Minor changes are described in the Supplementary data section.

Ex vivo organoid cultures

Intestinal crypts from Apc<sup>+/−</sup>/Ikkε<sup>−/−</sup> and Apc<sup>+/−</sup>/Ikkε<sup>+/+</sup> mice were isolated and cultured as described (31). Stimulations of ex vivo organoid cultures with IL17A and LPS were carried out as described (9).

Determination of proliferation and apoptosis

Mice were injected intraperitoneal with 100 mg/kg BrdU (Sigma) 90 minutes before their sacrifice and paraffin sections of duodenum tissues were stained using anti-BrdU antibody (RPN201; Amersham Biosciences/GE Healthcare) to quantify proliferating nuclei. Proliferative rates were determined by the ratio of average of positive cells in 10 crypts or by the ratio of positive cells to total cells in three proliferative cryptic area (where individual crypts could no longer be identified) per sample. Apoptotic cells in a given tissue section were determined histologically by TUNEL assay using an ApoAlert DNA Fragmentation Assay Kit (BD Biosciences Clontech).

Cell culture

SW480, HCT116, and HT-29 cells were obtained from ATCC in 2009. These cells were characterized by ATCC, using a comprehensive database of short tandem repeat (STR) DNA profiles. Frozen aliquots of freshly cultured cells were generated and experiments were done with resuscitated cells cultured for less than 6 months. Cell culture reagents, cytokines, and kinase inhibitors are described in the Supplementary data.

Lentiviral cell infection

Infections of Lenti-X 293T cells (Clontech) using lentiviral constructs described in the Supplementary data were carried out as previously described (32).

Protein expression, histological analysis, and immunoprecipitations

Isolation of enterocytes and Western blot analyses were performed as described previously (30). Paraffin sections (4 μm) and Western blots were stained using antibodies described in the Supplementary data section. For Immunoprecipitations, anti-TANK, -NAP1 and -IgG (negative control) antibodies were coupled covalently to a mixture of Protein A/G-Sepharose (see the Supplementary data for details). Immunoprecipitations were done as previously described (33).

Quantitative real-time PCR and RNA-seq expression analyses

Total RNAs were extracted and subjected to real-time PCR analyses as described (32). Primer sequences are available on request. Gene expression profiling of tumor tissues was carried out by RNA-Seq analysis. Both sample preparations and sequencing were performed at the GIGA transcriptomic facility (GIGA, University of Liege, Liege, Belgium). Methods to check total RNAs integrity, to carry out RNA-Seq expression analyses and for data analysis are described in the Supplementary Data.

In situ hybridization

Sample tissues were fixed with the standard procedures using 4% PFA (1 hour) and sucrose (15% 6 hours; 30% o/n) at 4°C and frozen in OCT freezing medium by the use of supercooled iso-propanol-dry ice mixture and stored at −80°C up to 6 month.
Frozen samples were cut 5 to 10 μm with a cryostat microtome at -20 °C on superfrost slides. In situ hybridization was carried out using the protocol provided by the manufacturer (RNAscope Multiplex Assay System; Advanced Cell Diagnostics Inc.).

FACS analyses
Control or Ikkε-depleted SW480 cells were pretreated or not with the pan-caspase inhibitor Z-VAD-FMK (Promega; 20 μmol/L) for 1 hour and subsequently untreated or stimulated with TNF (100 ng/mL)/cytochalasin (CHX; 50 μg/mL) for up to 8 hours. The quantification of apoptosis was done as previously described (32).

Statistical analysis
Data are expressed as mean ± SEM. Differences were analyzed by Student t-test or log-rank test (for Kaplan–Meier survival graphs of animal models) using Prism5 (GraphPad Software). The P values ≤ 0.05 (covering 95% confidence intervals) were considered significant (30).

Results
Wnt-driven tumor development in the intestine relies on Ikkε
We investigated whether Ikkε inactivation impacts on tumor formation in the Apc<sup>−/−</sup>mouse model, which spontaneously develops adenocarcinomas due to constitutive Wnt signaling (34). Inactivating Ikkε in Apc<sup>−/−</sup> mice significantly enhanced survival (226 days vs. 143 days, P < 0.001 in Apc<sup>−/−</sup>-Ikkε<sup>−/−</sup> and Apc<sup>−/−</sup>-Ikkε<sup>+/−</sup> mice, respectively) due to a decreased tumor incidence in distinct parts of the intestine (Fig. 1A–D). As a result, Apc<sup>−/−</sup>-Ikkε<sup>−/−</sup> mice did not suffer from anemia and splenomegaly as less dramatic (Fig. 1E and F, respectively). Ikkε deletion slightly impaired cell proliferation in tumors but not in normal intestinal crypts in Apc<sup>−/−</sup> mice (Fig. 1G). Consistently, pErk1/2 levels and, to some extent, cell proliferation as assessed by BrdU staining, were decreased in Apc<sup>−/−</sup>-Ikkε<sup>−/−</sup>–/– mice (Fig. 1H). Ikkε did not control cell proliferation in a cell-autonomous manner as ex vivo organoids generated with intestinal crypts from Apc<sup>−/−</sup>-Ikkε<sup>−/−</sup>–/– and Apc<sup>−/−</sup>-Ikkε<sup>+/−</sup> mice showed similar cell growth (Supplementary Fig. S1A). Ikkε phosphorylation on serine 172 was higher in intestinal tumors than in normal adjacent tissues from Apc<sup>−/−</sup>-Ikkε<sup>−/−</sup>–/– mice, as were protein levels of Tank, one of the IKK-related kinase TBK1 was potentiated upon Ikkε deficiency, suggesting a compensatory mechanism (Supplementary Fig. S3). Enhanced cell apoptosis was also seen upon TNF/CHX stimulation in other Ikkε-deficient colon cancer cell lines showing constitutive Wnt signaling, namely in p53-mutated HT29 and in p53-proficient HCT116 cells (Supplementary Fig. S4A–S4C). Therefore, Ikkε protects from TNF-dependent apoptosis through p53- and NF-κB–independent mechanisms in transformed IECs.

LPS- and IL17A-dependent pathways converge to Ikkε in colon cancer-derived cell lines
We next characterized the Ikkε-dependent pathways in colon cancer cells. Constitutive phosphorylation of ERK1/2 relied on Ikkε in differentiated HT-29 cells (Supplementary Fig. S5A). Moreover, Lipopolysaccharide (LPS)-induced phosphorylation of ERK1/2 was defective in Ikkε-depleted SW480 cells (Supplementary Fig. S5B). Thus, our data link Ikkε to ERK1/2 activation in transformed IECs.

IL17A signals in transformed IECs and Ikkε is activated by IL-17A in airway epithelial cells (9, 35, 36). Therefore, we assessed if IL17A promotes Wnt-dependent tumor development through Ikkε. IL-17A alone or in combination with LPS triggered Ikkε phosphorylation in ex vivo organoid cultures of transformed IECs (Supplementary Figs. S6A and 4A, respectively). Ikkε deficiency in ex vivo organoid cultures from Apc<sup>−/−</sup> mice as well as in SW480 cells impaired Akt, MEK1, p38 and ERK1/2 activation upon stimulation with both LPS and IL17A (Fig. 4A and B, respectively). Ikkε constitutively bound TANK but not with NAP1, another scaffold protein, in unstimulated or IL17A-treated SW480 cells (Supplementary Fig. S6B). TANK deficiency also severely impaired Akt, MEK1/2, ERK1/2 and p38 activation in cells stimulated with both LPS and IL17A (Supplementary Fig. S7). Therefore, the Ikkε–TANK complex integrates LPS- and IL17A–dependent cascades to activate multiple kinases.

Ikkε establishes a proinflammatory signature in the intestine upon constitutive Wnt signaling
To identify target genes induced through Ikkε, RNA-Seq analysis was done using total RNAs from duodenal samples of...
Figure 1.
Loss of Ikkε impairs tumor development in Apc^{min} mice. A, extended survival upon Ikkε deficiency in the Apc^{min} model. A, Kaplan-Meier survival graph is shown for Apc^{min/Ikkε+/—} (n = 34) and Apc^{min/Ikkε—/—} (n = 15) mice (***, P < 0.001; log-rank test). B, decreased tumor incidence in 4 months old Apc^{min/Ikkε+/—} (n = 7) versus Apc^{min/Ikkε—/—} (n = 19) mice. Data are mean ± SEM, n ≥ 7 for each genotype (*, P < 0.05 and ***, P < 0.001; Student t test). C, representative pictures of duodenum from 4 months old Apc^{min/Ikkε+/—} and Apc^{min/Ikkε—/—} mice. D, Ikkε deficiency impairs tumor development. Distribution of intestinal tumors in 4 months old mice of the indicated genotype (D, duodenum; J, jejunum; I, ileum; C, colon). Data are mean ± SE, n ≥ 7 for each genotype (*, P < 0.05 and ***, P < 0.001; Student t test). E, Ikkε deficiency reduces anemia in Apc^{min} mice. Blood hemoglobin (HGB) levels in 4 months old mice of the indicated genotype were quantified. Data are mean ± SE, n ≥ 5 for each genotype (*, P < 0.05; Student t test). F, Ikkε deficiency limits splenomegaly in Apc^{min} mice. Representative pictures of the spleen from 4 months old mice of the indicated genotype. G, Ikkε deficiency impacts on cell proliferation in tumors but not in normal crypts in the Apc^{min} model. The BrdU proliferation index in tumors and normal crypts of 4 months old Apc^{min/Ikkε+/—} and Apc^{min/Ikkε—/—} mouse tumors is shown (left and right, respectively). Data are mean ± SEM, n ≥ 3 for each genotype (**, P < 0.05; Student t test). n.s., nonsignificant. H, Ikkε promotes Erk1/2 activation in the Apc^{min} model. H&E staining, BrdU, and pErk1/2 immunohistological analyses of tumors of mice of the indicated genotype are shown.
**Ikke Promotes Wnt-Driven Tumor Development**

Figure 2. Ikke promotes tumor development in the β-catenin−/− tumor initiation model through Akt, MeK1, and ErK1/2 activations. A, Ikke expression in IECs in the β-catenin−/− model. In situ hybridization for Ikke mRNA in duodenal highly proliferating cryptic sections from β-catenin−/− mice, 22 days after first tamoxifen injection (green channel is for sample probe (Ikke), red channel is for negative control probe (DapB), and blue channel is for DAPI). Single dot signals are seen in Ikke-expressing epithelial (E) cells and, to a lesser extent, in inflammatory cells (arrows). LP, lamina propria. B, extended survival upon Ikke deficiency in the β-catenin−/− model. A Kaplan–Meier survival graph for β-catenin−/−/Ikke−/− (n = 7), β-catenin+/−/Ikke−/− (n = 7), β-catenin−/−/Ikke+/− (n = 13) mice after induction of tumorigenesis via 5 days tamoxifen injections is illustrated (*, P < 0.05; log-rank test). Data are mean ± SEM, n ≥ 5 for each genotype. C, impaired activation of multiple pathways upon Ikke deficiency in the β-catenin−/− model. Exports of duodenal epithelium from β-catenin−/−/Ikke−/− and β-catenin+/−/Ikke−/− mice, 0 and 22 days after the first tamoxifen injection were subjected to Western blotting using the indicated antibodies. D, impaired ErK1/2 activations upon Ikke deficiency in the β-catenin−/− model. H&E and BrdU stainings and Tank, pErK1/2 and pStat3 IHC analysis of duodenal epithelium from the indicated mice, 22 days after the first tamoxifen injection are showed.
Figure 3.
IkKe protects from TNF-dependent cell death in transformed intestinal epithelial cells. A, IkKe expression protects from cell death in vivo. TUNEL stainings (left) and cell death index as quantified by the number of TUNEL+ cells per field (right) of 4 months old Apc+/min/Ikkε+/+ or Apc+/min/Ikkε−/− small intestinal tumors. ***, P < 0.001 by Student t test, n ≥ 2 per genotype. B, IkKe protects from TNF/CHX-dependent apoptosis in colon cancer cells. Control or IkKe-deficient SW480 cells were pretreated or not with Z-VAD-FMK (20 μmol/L) for 1 hour, followed by a treatment with TNF (100 ng/mL) and CHX (50 μg/mL). FACS analyses were done to quantify apoptotic cells. The histogram shows FACS data from three independent experiments (Student t test; **, P < 0.01). C, IkKe deficiency enhances caspase-3/8 activation upon stimulation with TNF and CHX in colon cancer cells. Control or IkKe-depleted SW480 cells were treated with TNF (100 ng/mL) and CHX (50 μg/mL) and cell extracts were subjected to Western blotting.
numerous chemokines whose expression required Ikkε upon constitutive Wnt activation in the intestine. Indeed, mRNA levels of Cxcl12 (also referred to as SDF-1) and Cxcl11 were severely downregulated upon Ikkε inactivation (Fig. 5B). A chemokine/cytokine protein array confirmed the decreased expression of chemokines, including Cxcl9, Cxcl11, Cxcl12, G-CSF, and cytokines (IL7 and IL17A) in whole duodenal extracts from β-catenin+/−Ikkε−/− mice (Fig. 5C). In contrast, IL1r−/− mice also survived longer than control mice transplanted with bone marrow from Apc+/−Ikkε−/− mice transplanted with bone marrow from Ikkε−/− mice (Fig. 5H). Cd4, Cd8a, and Cd68 mRNA levels were also downregulated in these conditions (Fig. 5G). Therefore, Ikkε in EECs promotes the recruitment of macrophages to the tumor stroma through the expression of macrophage-attracting chemokines.

Cell autonomous Ikkε-dependent expression of inflammatory markers in IECs triggers the recruitment of macrophages to the tumor stroma

Consistent with a role of Ikkε in chemokines production, the number of macrophages infiltrating the tumor stroma of β-catenin+/−Ikkε−/− mice was significantly decreased, as evidenced by anti-F4/80 and CD163 immunofluorescence (IF) analysis (Fig. 5E and F). Reduced expression of both F4/80 and CD163 upon Ikkε deficiency was also revealed through real-time PCR analysis (Fig. 5G). Yet, Ikkε deletion did not impact on macrophages polarization as both M1 and M2 markers were similarly downregulated in duodena of β-catenin+/−Ikkε−/− mice (Fig. 5H). Cd4, Cd8a, and Cd68 mRNA levels were also downregulated in these samples (Fig. 5G). Therefore, Ikkε in IECs promotes the recruitment of macrophages to the tumor stroma through the expression of macrophage-attracting chemokines.

To assess whether Ikkε expression in hematopoietic cells also contributed to intestinal tumor development, bone marrow cells from β-catenin+/−Ikkε−/+ or β-catenin+/−Ikkε−/− mice were isolated and transplanted intravenously to irradiated β-catenin+/− or β-catenin+/−Ikkε−/− mice. Mice were kept for a month for the regeneration of immune cells before tamoxifen administration (Supplementary Fig. S10). Irradiated β-catenin+/−Ikkε−/− mice transplanted with bone marrow from Ikkε−/− mice survived longer than β-catenin+/−Ikkε−/+ mice transplanted with bone marrow from Ikkε−/− mice (33.5 days versus 27 days, respectively), which confirms the contribution of Ikkε in Wnt-driven tumor development (Supplementary Fig. S10). β-catenin+/−Ikkε−/+ mice transplanted with bone marrow from Ikkε−/− mice also survived longer (34.6 days), suggesting a contribution of Ikkε expression in

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**Figure 4.** Impaired activation of multiple oncogenic pathways upon Ikkε deficiency in transformed intestinal epithelial cells. A and B, Ikkε promotes Akt, p38, and Erk1/2 activation upon stimulation with both LPS and IL17A in IECs showing constitutive Wnt signaling. Ex vivo organoid cultures from Apc+/−Ikkε−/− and Apc+/−Ikkε−/− mice (A) or control and Ikkε-depleted SW480 cells (B) were treated or not with both LPS (1 ng/ml) and IL17A (50 ng/ml). Cell extracts were subjected to Western blotting.
Figure 5.
Ikkε controls intestinal proinflammatory gene expression and myeloid cell infiltration upon constitutive Wnt signaling. A, defective interferon signature and immune response upon Ikkε deficiency in β-catenin mice. A, gene set enrichment analysis of RNA-Seq expression data obtained with total RNAs from duodenal samples of the indicated mice is illustrated. Right, heatmap expression analysis from RNAseq data. (Continued on the following page.)
hematopoietic cells in the observed phenotype. Yet, irradiated β-cat^+/Ikk^−/− mice transplanted with bone marrow from Ikk^−/− or Ikk^+/− mice also showed a similar survival advantage (34 and 33.5 days, respectively) compared to irradiated β-cat^+/-Ikk^−/− mice transplanted with bone marrow from Ikk^−/− mice, which also highlighted the key contribution of Ikk^−/− expression in transformed IECs (Supplementary Fig. S10). These data highlight the contribution of Ikk^− in both IECs and hematopoietic cells (possibly through IL17A production through an Ikk-dependent pathway in Th17 cells) to support Wnt-driven tumor development in the intestine.

Ikk^− controls the expression of intestinal antimicrobial factors upon constitutive Wnt signaling

GSEA analysis also identified an enrichment of intestinal antimicrobial factors among Ikk^− target genes in β-cat^−/− mice (Fig. 6A). Indeed, both Reg3β/γ and Aang, whose mRNA levels increased in transformed IECs from β-cat^−/− mice, were downregulated upon Ikk^− inactivation (Fig. 6A and B). The number of Paneth cells, the major source of antimicrobial factors, was intact in β-cat^−/−Ikk^−/− mice (Supplementary Fig. S11). Yet, the number of visible antimicrobial factor releasing granules in each Paneth cell was severely decreased in intestinal crypts from β-cat^−/−Ikk^−/− mice (Supplementary Fig. S11). Therefore, Ikk^− interferes with Paneth cell differentiation. Of note, the goblet cell marker Muc1, whose expression increased upon constitutive Wnt signaling, was also decreased in Ikk^−-deficient IECs from β-cat^−/− mice (Fig. 6B). Moreover, mRNA levels of fucosyltransferase 2 (Fut2), an enzyme produced by innate lymphoid cells, which promotes epithelial fucosylation in the intestinal tract to protect from Salmonella typhimurium infection (37), also severely increased upon Wnt activation but decreased in the absence of Ikk^− (Fig. 6B). In addition, Ikk^− was required for complement C3 expression in transformed IECs (Supplementary Fig. S12A). Therefore, Ikk^− provides an inflammatory signature in IECs upon Wnt-dependent tumorigenesis in a cell-autonomous manner.

As LPS-dependent expression of complement C3 and activation of C/Ebpf in mouse embryonic fibroblasts requires the transcriptional induction of Ikk^− through NF-κB (38), we assessed C/Ebpf expression in primary IECs from Ikk^−/+ or Ikk^−/− mice subjected or not to LPS stimulation. Complement C3 expression was strongly induced by LPS at the mRNA level and Ikk^− deletion impaired its expression, especially after 4 and 8 hours of LPS stimulation in IECs (Supplementary Fig. S12B). Consistently, C/Ebpf protein levels were also decreased in Ikk^−-deficient IECs, with or without LPS stimulation and in IECs from β-cat^−/−Ikk^−/− mice compared to β-cat^−/−Ikk^+/− mice (Supplementary Fig. S12C and S12D). Thus, Ikk^− promotes C3 expression, by regulating C/Ebpf levels in normal and transformed IECs.

Commensal bacteria promote Ikk^− activation in tumors from β-cat^−/− mice and the expression of inflammatory markers and antimicrobial factors

Gut microbiota promotes tumor development in Apc^−/− mice (39). Moreover, commensal bacteria trigger TLR-dependent signaling pathways that converge on Ikk^− (19). To assess whether bacterial products trigger TLRs-dependent Ikk^− activation to provide the inflammatory tumor microenvironment, we subjected β-cat^−/−Ikk^−/− mice to broad-spectrum antibiotics (Abs) to deplete commensal bacteria. Data from feces of β-cat^−/−Ikk^−/− mice validated the efficiency of antibiotics (Supplementary Fig. S13). Abs treatment prolonged mouse survival, probably by interfering with Ikk^−, Akt, Msk1, and Stat3 activations (Fig. 7A and B). Therefore, bacterial products trigger the Ikk^− dependent activation of oncogenic pathways during Wnt-driven tumor development. We next assessed mRNA levels of pro-inflammatory cytokines and chemokines in whole duodenum from control versus Abs-treated β-cat^−/−Ikk^−/− mice. Most candidate genes whose expression was decreased in β-cat^−/−Ikk^−/− mice also showed reduced expression in Abs-treated β-cat^−/−Ikk^−/− mice (Fig. 7C). Also, similar to Ikk^− deficiency, the expression of multiple Paneth cells markers significantly decreased upon Abs treatment in β-cat^−/− mice (Fig. 7D). These data identified key Ikk^−-dependent oncogenic pathways triggered by bacterial products that provide an inflammatory tumor microenvironment in the intestine showing constitutive Wnt signaling.

Discussion

Here we define Ikk^− as a LPS- and IL17A-activated kinase acting upstream of multiple pathways in transformed IECs, leading to the establishment of a proinflammatory environment in two mouse models of Wnt-driven intestinal tumorigenesis. Ikk^− is also acting as a pro-survival kinase by limiting TNF- and caspase-8-dependent apoptosis in IECs showing constitutive Wnt signaling.

Ikk^− counteracts TNF-dependent cell death, similarly to the prosurvival Ikkβ but through NF-kB-independent mechanisms as IκBζ degradation and p65 phosphorylation by TNF remained intact in IκK-deficient IECs. It is likely that the phosphorylation of multiple unknown Ikk^− substrates will provide prosurvival signals.

(Continued.) Candidate genes up- or downregulated are illustrated in red or green, respectively. Experimental conditions are: 1 and 2, duodenal samples from β-cat^−/−Ikk^−/− mice at day 0 or 22 days after tamoxifen injection, respectively; 3 and 4, duodenal samples from β-cat^−/−Ikk^−/− mice at day 0 or 22 days after tamoxifen injection, respectively. n = 3 for each genotype. B, defective chemokine production in Ikk^−-deficient β-cat^−/− mice. Real-time PCR analyses were carried out with total RNAs isolated from whole mucosa of β-cat^−/−Ikk^−/− and β-cat^−/−Ikk^+/− mice, 22 days after the first tamoxifen injection. Data represent fold difference of Ct values from β-cat^−/−Ikk^−/− versus β-cat^−/−Ikk^+/− mice. Data are mean ± SEM, n ≥ 4 for each genotype. C, decreased protein levels of pro-inflammatory cytokines in Ikk^−-deficient β-cat^−/− mice. A chemokine protein array was conducted with protein extracts from duodenal tissues of the indicated mice, 22 days after the first tamoxifen injection. The graph shows relative fold expression. D, Ikk^− promotes CXCL expression upon stimulation by both LPS and IL17A in transformed IECs. Total RNAs extracted from ex vivo organoid cultures from Apc^−/−Ikk^+/− and Apc^−/−Ikk^−/− mice were treated or not with the indicated ligand(s) for up to 5 hours. The abundance of CXCL mRNA levels in untreated Apc^−/−Ikk^−/− mice was set to 1 and its level in other experimental conditions were relative to that after normalization with Gpdh. Data from triplicates (means ± standard deviations) are shown (***P < 0.001; **P < 0.01; *P < 0.05). E and F, Ikk^− promotes the infiltration of F4/80^+ (E) and CD68^+ (F) myeloid cells to highly proliferating crypts in β-cat^−/− mice 22 days after the first tamoxifen injection. Below, infiltrated F4/80^+ (E) and CD68^+ (F) myeloid cells were quantified as number of cells per field (per mm). Data are mean ± SEM, n = 3. G and H, decreased expression of intestinal cell (G) and M1/M2 markers (H) in Ikk^−-deficient β-cat^−/− mice. Real-time PCR analysis was carried out with total RNAs isolated from whole mucosa of β-cat^−/−Ikk^−/− and β-cat^−/−Ikk^+/− mice, 22 days after the first tamoxifen injection. Data shown represents fold difference of Ct values from β-cat^−/−Ikk^−/− versus β-cat^−/−Ikk^+/− mice. Data are mean ± SEM, n ≥ 4 for each genotype.
In addition to a prosurvival role, Ikkɛ acts as an oncogenic kinase by stimulating the recruitment of proinflammatory cells to support Wnt-driven tumorigenesis. Our bone marrow transplantation experiments highlight a dual function for Ikkɛ expression in both IECs and bone marrow-derived cells. This dual role is required to sustain a proinflammatory loop that supports tumor development, a loop initiated by Ikkɛ expression in transformed IECs (Supplementary Fig. S14). Th17 cells known to produce IL1β may critically rely on Ikkɛ to maintain this loop. Indeed, the key role of Ikkɛ in IL1β-driven Th17 maintenance supports this hypothesis (40). Removing Ikkɛ in transformed IECs or in bone marrow-derived cells disrupts this proinflammatory loop and tumor development is consequently delayed. Whether Ikkɛ expression in cancer-associated fibroblasts also provide oncogenic signals deserves further investigation using conditional knock-out mouse models.

Ikkβ is another proinflammatory molecule but mechanisms by which Ikkβ drives Wnt-dependent tumor initiation in the intestine are partially distinct. Ikkɛ is an Akt-activating kinase in Apc-mutated IECs whereas Ikkβ is not. Similarly, Erk1/2 is regulated by Ikkɛ but not by Ikkβ. Therefore, Ikkɛ provides a proinflammatory signature in transformed IECs, at least through some specific pathways distinct from those controlled by Ikkβ. Previous in vitro studies showed that Ikkɛ targets several substrates acting in NF-κB-activating cascades (23, 41). We show here that the oncogenic potential of Ikkɛ in transformed IECs mainly results from its capacity to provide a tumor microenvironment rather than from enhancing pro-proliferative cascades in a cell-autonomous manner.
Figure 7.
Gut microbiome promotes Ikkε activation and the expression of inflammatory markers and Paneth cell antimicrobial factors in tumors from β-cat−/− mice. A, antibiotics (+) treatment of β-cat−/− mice extends survival. A, Kaplan-Meier survival graph for β-cat−/− /Ikkε−/−, β-cat−/− /Ikkε+/+, or β-cat−/− /Ikkε−/+ mice treated with Abx (ciprofloxin (0.5 g/L), ampicillin (1 g/L), and metronidazole (0.5 g/L)) after induction of tumorigenesis via 5 days tamoxifen injections is illustrated. Data are mean ± SEM, n ≥ 6 for each genotype. B, microbiota promotes Ikkε, Akt, Msk1, and Stat3 phosphorylations in β-cat−/− mice. Extracts from duodenal tissue of the indicated mice after induction of tumorigenesis were subjected to Western blotting. C and D, proinflammatory cell/gene markers and Paneth cell secreted antimicrobial factors whose expression is Ikkε dependent in duodenal tissues of β-cat−/− mice also show lower levels of expression in Abx-treated Ikkε-sufficient animals. Real-time PCR analysis was carried out with total RNAs isolated from whole mucosa (C) or IECs (D) of the indicated mice 22 days after the first tamoxifen injection. Data from three independent experiments (means ± standard deviations) were plotted as in Fig. 5B (n ≥ 4 for each genotype).

Our data provide an in vivo demonstration that Ikkε promotes Akt activation in transformed IECs. The transcriptional program induced through the Ikkε-Akt pathway in Apc-mutated IECs remains unclear. One candidate could be Retn1b, which is upregulated in colon cancer, and protects against parasitic helminth infections by maintaining the colonic barrier function (42–44). Retn1b expression is induced through IL23 and Akt in intestinal goblet cells (45). Because Akt activation is Ikkε dependent in Apc-mutated IECs, Retn1b expression may be induced through this pathway. It is likely that CREB1, whose phosphorylation occurs through Akt and Msk1 (46), contributes to the induction of numerous Ikkε target genes. Similarly, C/EBPβ is another transcription factor acting downstream of Ikkε that drives the expression of proinflammatory molecules such as complement C3.

Constitutive Stat3 activation cooperates with NF-κB to promote cell survival and proliferation in the intestine (14). The defective Stat3 phosphorylation profile seen upon Ikkε inactivation results from an impaired recruitment of macrophages in the tumor stroma rather than an epithelial cell-autonomous effect of Ikkε on Stat3. This defect causes decreased levels of Stat3-activating cytokines such as IL6 in whole duodenum from β-cat−/− /Ikkε−/− mice.

Multiple cytokines and chemokines show an Ikkε-dependent expression in our model of Wnt-driven tumor initiation. One of
them is IL17A whose production was decreased upon Ikkβ deficiency. Once synthesized, IL17A can establish a positive loop by re-activating Ikke in transformed IECs. Consistently, IL17A or Ikkβ deficiency in ApcΔF508; Min mice similarly delays tumor development and also corrects splenomegaly (47). Therefore, signals from two distinct families of receptors, IL17RA and TLRs, converge to Ikke to promote Wnt-dependent tumor development in the intestine. Few candidates such as IL1Rx were upregulated in duodenum of β-catenin Ikkβ−/− mice, as similarly showed in a model of arthritis (48). As IL1rx antagonizes the function of IL1β, Ikke may potentiate IL1β signaling by limiting IL1rx expression.

The recruitment of macrophages in the intestinal tumor stroma, but not their polarization, requires Ikke. This is in sharp contrast with Ikke whose kinase activity is required for Wnt-driven intestinal tumor development by negatively regulating the recruitment of Interferon γ (IFNγ)-producing M1-like myeloid cells (30). Therefore, Ikke re-establishes an inflammatory signature to promote Wnt-driven tumor development through mechanisms distinct from those implying Ikkkα and Ikββ.

Disclosure of Potential Conflicts of Interest

L.C. Heukamp has ownership interest in a NEO New Oncology and reports receiving a commercial research grant from Roche, Boehringer, and MSD. No potential conflicts of interest were disclosed by the other authors.

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