**IKKα 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 IκB kinase α (IKKα) as a central regulator of a tumoricidal microenvironment during intestinal carcinogenesis. Mice deficient in IKKα kinase activity are largely protected from intestinal tumor development that is dependent on the enhanced recruitment of interferon γ (IFNγ)-expressing M1-like myeloid cells. In IKKα mutant mice, M1-like polarization is not controlled in a cell-autonomous manner but, rather, depends on the interplay of both IKKα 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 IKKα 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 [IFNγ] 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)-κB activation leads to the establishment of a protumorigenic inflammatory microenvironment of various malignancies (Bollangyo and Greten, 2009). NF-κB is tightly controlled by the IκB-kinase (IKK) complex, which consists of two catalytic subunits, namely the IKKα and IKKβ proteins, as well as the regulatory subunit IKKγ (Chariot, 2009). The classical NF-κB
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 IKKβ-IκBα-dependent NF-κB signaling, alternative NF-κB activation depends solely on IKKα (Valabhapurapu and Karin, 2009). Moreover, IKKα comprises a nuclear localization signal and can therefore also confer important nuclear functions (Chariot, 2009). Whereas, in most malignancies, IKKβ-dependent NF-κB signaling clearly promotes tumorigenesis, the role of IKKα in this context is more complex. Inhibition of IKKα 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 IKKα 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 IKKα 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 CTNNB1 mutations that cause persistent activation of the Wnt pathway (Fearon, 2011). We could recently demonstrate that proinflammatory IKKβ-dependent NF-κB 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-κB activation controls development of epithelial-mesenchymal transition (EMT) and myeloid cell recruitment in TpS3-deficient invasive carcinomas (Schwitalla et al., 2013b). In contrast, IKKα directly phosphorylates β-catenin, thus increasing its abundance to promote cyclin D1 expression (Albanese et al., 2003), and in colorectal cancer cells, an active IKKα isoform was described (Margalef et al., 2012). However, functional genetic evidence supporting a cell autonomous or nonautonomous role of IKKα and/or the alternative NF-κB activation pathway in colorectal carcinogenesis is lacking.

RESULTS

Impaired IKKα Activation Suppresses Intestinal Tumorigenesis

To functionally examine the role of IKKα during early intestinal tumorigenesis, we employed IkkαAA/AA knockin mice, which contain alanines instead of serines in the activation loop of IKKα and express therefore a nonactivatable form of this kinase (Cao et al., 2001). Whereas IkkαAA/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. IkkαAA/AA mice displayed regular numbers and distribution of goblet cell tubes, 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, IkkαAA/AA mice and littermate controls were repetitively challenged with the procarcinogen azoxymethane (AOM), which is commonly used to induce adenoma growth in the distal colon of rodents. Expression of mutant IKKα markedly reduced number of adenomas (>75%) when animals were analyzed 20 weeks after the first carcinogen exposure (Figure 1A). IkkαAA/AA mice developed only few relatively small tumors that displayed slower proliferation rates (Figures 1B and 1C). Instead, in IkkαAA/AA mice multifocal low-grade intraepithelial neoplasia was frequently observed. To confirm the IkkαAA/AA-dependent antitumorigenic effect in a genetic model of adenomatous polyposis, we crossed IkkαAA/AA mice to ApcMin/+ mice and monitored their survival. Similarly, loss of IKKα function conferred a protective effect and prolonged survival of ApcMin/+ mice significantly (median survival of 236.5 days in homozygous IkkαAA/AA mutants versus 184.5 days in heterozygous IkkαAA/+ mutant mice; p < 0.0001; Figure 1D). Accordingly, when we analyzed 4-month-old ApcMin/+ animals, tumor incidence and size as well as proliferation of tumor epithelia was significantly decreased in IkkαAA/AA mutant mice (Figures 1E–1H; data not shown). Furthermore, consistent with lower tumor burden, anemia—usually developing in ApcMin/+ mice as tumorigenesis progresses—was normalized in ApcMin/+/*kkαAA/AA compound mutants (Figures 11 and 1J). Collectively, these results suggested that IkkαAA/AA mediated antiproliferative effects during early tumor stages, which led to marked tumor suppression in both models of intestinal tumorigenesis.

IkkαAA/AA Mice Block Tumor Cell Proliferation Independently of Alternative NF-κB Activation

Tamoxifen-inducible β-cat−/− 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−/− 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 IKKα 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 IKKα activation prolonged survival of β-cat−/− mutant animals (Figure 2F). Interestingly, loss of NF-κB2/p100 did not affect survival, indicating that IKKα acted independently of the alternative NF-κB activation pathway (Hayden and Ghosh, 2004). In line with this notion, we also did not observe any differences in p100 processing in β-cat−/−/*kkαAA/AA IEC (data not shown).

Prolonged Survival of Ikkα Mutant β-cat−/−. Mice Depends on IFNγ

To further explore the underlying IKKα-controlled proproliferative mechanism, we performed a microarray analysis comparing RNA isolated from wild-type, IkkαAA/AA, β-cat−/−, or β-cat−/−/*kkαAA/AA IEC 15 days after the first tamoxifen administration. A total of 732 genes were significantly differentially expressed. In IEC from β-cat−/−/*kkαAA/AA compared to β-cat−/−. mice, a general downregulation of Wnt-dependent transcripts rather than control of particular gene subsets was observed. These different
transcription profiles supposedly reflected the observed differences in IEC morphology between the two genotypes (Figure 2A), but not distinct IKKα-controlled signaling events. Indeed, knockdown of IKKα 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). Therefore, we focused our attention on the group of transcripts that were markedly upregulated in IEC from β-catenin-/IKKαAA/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 of immune and inflammatory genes in the β-catenin-/IKKαAA/AA group of transcriptome.
of type I and II IFN targets in β-catenin-/-/IkkαAA/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 IFNγ in whole mucosa of β-catenin-/-/IkkαAA/AA mice (Figure 3D). Moreover, immunoblot analysis confirmed activation of tyrosine-phosphorylated Stat1(Y701) as well as upregulation of Nos2 and IRF-1 in IKKα mutant IEC (Figure 3E). Because IFNγ/Stat1 signaling is known to suppress IEC proliferation in a paracrine type II interferon-dependent manner.

**IkkαAA/AA Myeloid Cells Rather Than T or NK Cells Comprise the Source of IFNγ**

The most common IFNγ-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+IFNγ+ or CD8+IFNγ+ T cells between β-catenin-/- and β-catenin-/-/IkkαAA/AA mice when animals were analyzed 15 days after the first tamoxifen administration (Figure 5B). More importantly, loss of Ifng, but not blocking type I interferon signaling by Ifnar deletion, completely prevented IKKα-mediated survival advantage (Figure 4C). Collectively, these data provided clear evidence that mutant IKKα suppressed IEC proliferation in a paracrine type II interferon-dependent manner.
from either wild-type or IkkαAA/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-Foxn1nu) that lack T cells did not revert the survival advantage of IkkαAA/AA mutant β-catenin 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-catenin+/IkkαAA/AA mice (Figure 5C), indicating that neither CD4+, CD8+ T cells, nor NK cells were responsible for the IFNγ-mediated survival extension of IkkαAA/AA mutant β-catenin 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, Il12, Nos2, Cxcl9, Cxcl10, and Cxcl11) are considered tumoricidal, whereas M2 macrophages (characterized by high levels of Arg1, Mr1, Ccl7, 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...
was observed in IKKα 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 β-cat<sup>-/-</sup>/Ikkα<sup>AA/AA</sup> mice, where they could be found interspersed in between IEC. In contrast, infiltration of F4/80<sup>+</sup> and Gr-1<sup>+</sup> myeloid cells was limited to the villus stroma in IKKα wild-type-expressing β-cat<sup>-/-</sup> mice (Figures 6E–6H). Importantly, immunofluorescence confirmed that indeed both macrophages and neutrophils expressed IFNγ (Figures 6I and 6J), indicating that an enhanced recruitment of M1-like myeloid cells was responsible for the IFNγ-dependent survival advantage of β-cat<sup>-/-</sup>/Ikkα<sup>AA/AA</sup> mice.

Enhanced Recruitment of Myeloid Cells Depends on IKKβ Activation in β-cat<sup>-/-</sup>/Ikkα<sup>AA/AA</sup> IEC

Myeloid-cell-recruiting chemokines such as Cxcl1, Cxcl2, Cxcl5, and Ccl2 are controlled by classical NF-κB activation (Grivennikov et al., 2010). To functionally confirm that indeed both macrophages and neutrophils expressed IFNγ (Figures 6I and 6J), indicating that an enhanced recruitment of M1-like myeloid cells was responsible for the IFNγ-dependent survival advantage of β-cat<sup>-/-</sup>/Ikkα<sup>AA/AA</sup> mice.

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et al., 2013a). Similarly, deletion of lkkβ prolonged survival of Ctnnb mutants (Figure 7F). However, consistent with a lack of ltfg induction, β-cat−/−/lkkαAA/AA animals were no longer protected in the absence of lkkβ and their survival was now comparable to that of β-cat−/−/lkkαAA/IEC mice (Figure 7F). Thus, these data further supported the importance of IFNγ-expressing myeloid cells for the survival of β-cat−/−/lkkαAA/AA mice and confirmed that M1-like polarization did not occur in a cell autonomous manner in Ikkα 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 (Grivennikov 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 mutated tumor cells. Although Ikkα 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 Ikkα comprises a central regulatory element in the suppression of M1-like myeloid cell controlled microenvironment rather than directly stimulating tumor cell proliferation. Elevated IFNγ levels in lkkαAA/AA mucosa are most likely responsible for growth arrest of initiated epithelial cells. However, considering the particular intraepithelial localization of myeloid cells in β-cat−/−/lkkαAA/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 Ikkα 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-κB-dependent secretion of chemokines in lkkαAA/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 IFNγ 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 IFNγ 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-κB activation in IEC. Considering that Ikkα 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 IFNγ upregulation is associated with improved survival (Grenz et al., 2013), Ikkα may represent a valuable therapeutic target for CRC therapy or prevention. If, however, putative Ikkα 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 Ikkα because simultaneous overlapping Ikkβ inhibition would most likely prevent the beneficial effects of selective Ikkα inhibition.

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

Depending on the type of malignancy, Ikkα can provide both tumor-promoting and tumor-suppressive mechanisms that are in most instances cell autonomous. Ikkα 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,
NIK-dependent IKKα 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 IKKα 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, IKKα 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, IKKα kinase inactivation culminates in the recruitment of tumor-promoting inflammatory macrophages and depletes 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 IkkaAA/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 β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> mice depend on IKKβ-dependent NF-κB activation in IEC

(A) Real-time PCR analysis of indicated genes in β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup>, β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> mice whole mucosa samples 15 days after first tamoxifen application. Data are mean ± SE; n ≥ 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 β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> and β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> mice 15 days after first tamoxifen administration. The scale bar represents 50 μm.

(F) Kaplan-Meier survival graph of β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> (n = 11; red line) and β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> mice (n = 6; blue line). Survival of β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> and β-cat<sup>−/−</sup>/Ikk<sup>α</sup>/Ikk<sup>β</sup> mice shown as comparison (dashed gray lines).
**EXPERIMENTAL PROCEDURES**

**Mice**

Ikke^AA/AA (Cao et al., 2001), Ikke^AA/AA (Liu et al., 2008), Ikke^AA/AA (Greten et al., 2004), Nfkβ2^−/− (Paxian et al., 2002), and β-caten^−/− (Schwittalla et al., 2013a) mice have been recently described. C57BL/6G貂^-^MⅧ, Ifng^−/−, and Nu-Foxn1^−/− mice were purchased from the Jackson Laboratories, and Ihnr^−/− 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/6G貂^-^MⅧ, mice, Ikke^AA/AA animals had been backcrossed to a C57BL/6 background for eight experiments. Generations using β-caten^−/− 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. Azoxy methane (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 × 10^6 bone marrow cells from ikke^AA/AA mice or ikke^AA/AA 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 (Bollihath et al., 2009; Schwittalla et al., 2013a). The following antibodies were used: anti-β-caten (IMG136A; Imgenex), anti-β-caten (05-535; Upstate), anti-β-caten (A4700; Sigma), anti-β-caten (UBI 6734; United Bio Research), anti-phospho-STAT1 (9171; Cell Signaling Technology), anti-STAT1 (SC-346; Santa Cruz Biotechnology), anti-β-caten (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-caten (af-1496).

**Histological Procedures and Flow Cytometry**

Standard immunohistochemical procedures were performed using the following antibodies: anti-bromodeoxyuridine (BrdU) antibody (RPN201; GE Healthcare), anti-β-caten (Abcam; ab199749; clone EPR646); CD68 (Fisher Scientific; MS-397; clone KP1), anti-β-caten (SC-788), anti-F4/80 (Caltag; MF 480043), anti-Gr-1 (eBioscience; 12-5931-85), and anti-IFN-γ (BD-554412). 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 (Invitrogen). T cell proliferation was analyzed by FACS analysis 6 hr 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 anti-body (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 ± SD. 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 rank test for Kaplan-Meier survival graphs using Prism4 (GraphPad Software) or SPSS Statistics 21. p values ≤ 0.05 were considered significant.

**ACCESION 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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