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Article

Interleukin-11 Is the Dominant IL-6 Family Cytokine during Gastrointestinal Tumorigenesis and Can Be Targeted Therapeutically

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SUMMARY

Among the cytokines linked to inflammation-associated cancer, interleukin (IL)-6 drives many of the cancer "hallmarks" through downstream activation of the gp130/STAT3 signaling pathway. However, we show that the related cytokine IL-11 has a stronger correlation with elevated STAT3 activation in human gastrointestinal cancers. Using genetic mouse models, we reveal that IL-11 has a more prominent role compared to IL-6 during the progression of sporadic and inflammation-associated colon and gastric cancers. Accordingly, in these models and in human tumor cell line xenograft models, pharmacologic inhibition of IL-11 signaling alleviated STAT3 activation, suppressed tumor cell proliferation, and reduced the invasive capacity and growth of tumors. Our results identify IL-11 signaling as a potential therapeutic target for the treatment of gastrointestinal cancers.

INTRODUCTION

The onset and progression of cancer is facilitated by complex interactions between neoplastic cells and the heterogeneous stromal cell populations that are present in the tumor microenvironment (Egeblad et al., 2010; Hanahan and Coussens, 2012). While this is best documented for gastrointestinal (GI) cancers associated with persistent inflammation, the tumor microenvironment can also promote the growth of sporadic cancers arising from tumor intrinsic oncogenic mutations (Grivennikov et al., 2012). Studies utilizing knockout mice have begun to unravel the complex interplay between the neoplastic and stromal cells and have highlighted pivotal roles for inflammatory cytokines. In turn, these cytokines collectively promote cancer hallmark capabilities,

Significance

STAT3 activation is linked to poor survival in patients with cancer and is thought to arise primarily from elevated IL-6. Accordingly, inhibitors of IL-6 signaling are in clinical trials for a number of epithelial cancers. Using mouse models of inflammation-associated and sporadic gastrointestinal cancers, we have discovered that the IL-11/STAT3 signaling axis is a more potent driver of tumor progression than IL-6. Pharmacologic inhibition of IL-11/STAT3 in mouse models of gastrointestinal cancer and human tumor cell line xenografts inhibited the invasive capacity of neoplastic cells and reduced tumor growth. Importantly, IL-11 inhibition had no impact on hematopoiesis, an undesirable side effect of systemic STAT3 inhibition. Our data provide support for the clinical development of IL-11 signaling antagonists for the treatment of epithelial cancers.

including proliferation, angiogenesis, and metastasis, while simultaneously inducing resistance of neoplastic cells to death stimuli and immune destruction (Hanahan and Weinberg, 2011).

The interleukin (IL)-6 family of cytokines is defined by the shared use of the gp130 receptor β-subunit. Included within this family are IL-6, recognized for its role as a systemic acute phase mediator (Heinrich et al., 1990), and IL-11, which promotes platelet production (Teramura et al., 1992). More recently, both of these cytokines have been linked to the development of epithelial cancers (Matsuo et al., 2003; Nakayama et al., 2007). While activated myeloid cells are thought to produce most of the IL-6 in the tumor microenvironment, autocrine IL-6 signaling in neoplastic epithelial cells has also recently been documented (Gao et al., 2007; Grivennikov et al., 2009). IL-11 on the other hand is produced by cancer-associated fibroblasts (CAFs) in patients with colorectal cancer (CRC) and by myeloid cells and can be upregulated in cancer cells as part of an autocrine signaling loop (Calon et al., 2012; Ernst et al., 2008; Schwitalla et al., 2013; Shin et al., 2012). Although the engagement of the gp130 receptor by either IL-6 or IL-11 induces transient activation of Janus kinases (JAK) and the latent transcription factor STAT3, tissue responses are dependent on either the presence of soluble IL-6 receptor (R) or expression of the membrane-associated IL-6Rα and IL-11Rα receptors (Becker et al., 2004; Heinrich et al., 1998).

Excessive STAT3 activation is a feature of the majority of solid cancers and is frequently associated with elevated cytokine expression, including IL-6 and IL-11 (Ernst et al., 2008; Grivennikov et al., 2009). However, some epithelial malignancies are also associated with activating somatic mutations in the genes encoding STAT3, gp130, and associated JAK1/2, as well as epigenetic silencing of the *SOCS3* gene, which encodes a critical negative regulator for gp130 cytokine signaling (Casanova et al., 2012; He et al., 2003; Rebouissou et al., 2009). In GI cancers, excessive STAT3 activation is also linked to tumor invasion and nodal metastasis and predicts poor patient survival (Deng et al., 2010; Kim et al., 2009; Morikawa et al., 2011). We have previously shown that epithelial STAT3 activation in mice promotes inflammation-associated gastric cancer (GC) and colitis-associated colorectal cancer (CAC) (Bollrath et al., 2009; Ernst et al., 2008). Furthermore, genetic reduction of STAT3 expression diminished tumor burden in these models, and suggested that pharmacologic targeting of the gp130/STAT3 signaling pathway may confer significant therapeutic benefits in the treatment of these malignancies.

The concept of combating tumor progression through pharmacologic inhibition of growth-promoting cytokines is emerging as a therapeutic opportunity that bypasses the difficulties of targeting intracellular signaling molecules and transcription factors. To date, much focus has been placed on antagonizing the activity of IL-6, with clinical trials for ovarian, renal, prostate, and breast cancers underway (Guo et al., 2012; Puchalski et al., 2010). Here, we investigate the role of the related cytokine IL-11 in GI tumorigenesis.

RESULTS

Increased IL-11 Expression in Human GI Cancers Is Associated with Excessive STAT3 Activation

Although elevated IL-6 expression is linked to excessive STAT3 activation associated with poor survival in patients with CRC, less is known about the role of IL-11 in these cancers (Esfandi et al., 2006; Morikawa et al., 2011). We therefore compared the expression of *IL6* and *IL11* in a panel of 14 primary CRC samples (Figure 1A) and in 16 primary human GC samples (Figure S1A available online). We found that both cytokines were consistently elevated in tumor tissue compared to unaffected GI tissues from the same patients. To examine the relationship between epithelial STAT3 activation and the expression of inflammatory cytokines associated with tumorigenesis, we assessed pTyr-STAT3 staining as a marker of activated STAT3, in additional CRC samples, and found that only *IL11* mRNA expression significantly correlated with high epithelial STAT3 activation (Figures 1B and S1B). Within this high STAT3 patient cohort, we found no correlation between *IL6* and *IL11* expression (not shown). Because we consistently observed heterogeneous STAT3 activation within tumor samples, we characterized the location of pTyr-STAT3 positive cells within a panel of 59 resected primary CRC samples. We observed strong pTyr-STAT3 staining in the tumor core in 39% (23 of 59) of samples. When we scored invasive regions of these tumors separately, we found strong pTyr-STAT3 staining in 61% (36 of the 59; Figure 1C) of samples, suggesting a potent trigger for STAT3 activation near the invasive front. Similarly, in a panel of GC samples, we observed high levels of epithelial STAT3 activation in 26% (33 of 125) of the specimens (Figure S1C). Collectively, these observations suggest that IL-11-mediated STAT3 activation may be associated with epithelial tumorigenesis and invasion of neoplastic cells into the submucosa.

IL-11 Signaling Is a Dominant Driver of Inflammation-Associated Colon Cancer in Mice

To compare the role of IL-6 and IL-11 during CRC, we induced CAC in mice. In the CAC model (Figure 2A), a single injection of the alkylating mutagen azoxymethane (AOM) leads to sporadic induction of a number of mutations, including mis-sense mutations in *Ctnnb1* in intestinal epithelial cells, resulting in stabilization of the corresponding β-catenin protein and aberrant activation of the Wnt-signaling pathway (Neufert et al., 2007; Tanaka et al., 2005). Subsequent repetitive administration of the luminal irritant dextran sulfate sodium (DSS) promotes "flares" of inflammation, akin to those observed in chronic ulcerative colitis, which augment colonic tumor development in mice (Wirtz et al., 2007). Similar to our observation in human CRC, we detected increased IL-11 and pTyr-STAT3 protein levels in the distal colonic tumors of wild-type (WT) mice on day 72 of the CAC protocol (Figure 2B). Because the progression of CAC-induced colonic neoplasias is mediated by STAT3 (Bollrath et al., 2009; Grivennikov et al., 2009), we compared the requirement for the STAT3 activating cytokines IL-6 and IL-11 during this process. Using serial endoscopy, we detected macroscopic tumors in WT and $Il6^{KO}$ mice by day 47, whereas tumor onset was substantially delayed in $Il11ra1^{KO}$ and $Il6^{KO}$;$Il11ra1^{KO}$ compound mutant mice (Figure 2C). Although the absence of IL-6 signaling resulted in a lower frequency of colonic adenomas in $Il6^{KO}$ mice compared to WT mice (Grivennikov et al., 2009), tumors were almost completely absent in the colons of $Il11ra1^{KO}$ mice at autopsy, irrespective of the presence of IL-6 (Figures 2C–2E). Consistent with a dominant role for IL-11-mediated STAT3 activation in tumor formation we observed a reduction

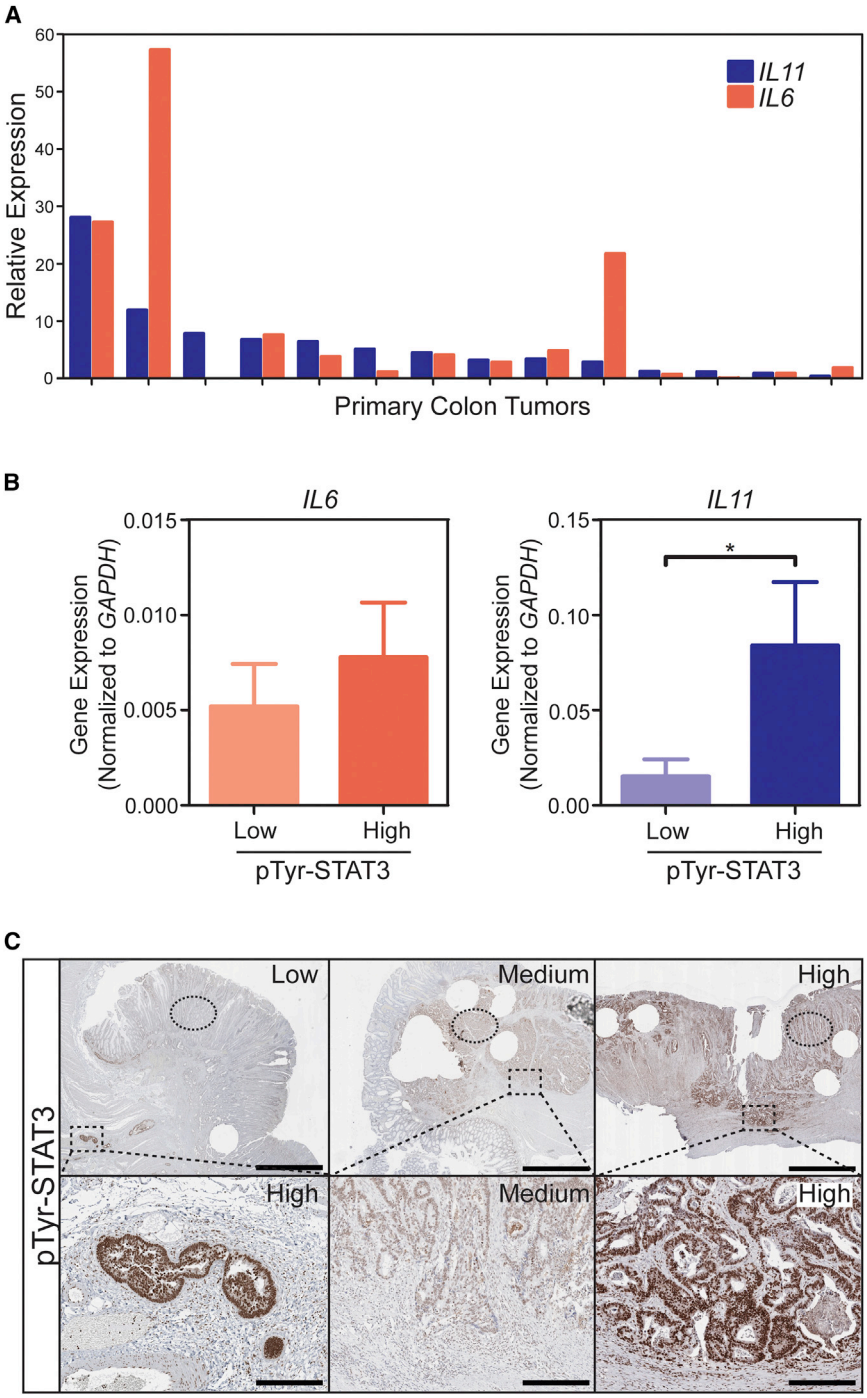

Figure 1. Elevated *IL11* Expression in Human CRC Is Associated with Excessive STAT3 Activation

(A) *IL6* and *IL11* mRNA expression in 14 individual human CRC samples. Data were normalized to *GAPDH* expression and are shown as fold change compared to unaffected colon tissue from the same patients.

(B) STAT3 activation in 46 human CRC samples was determined by immunohistochemistry for pTyr-STAT3 to classify samples with low (24 samples) or high (22 samples) STAT3 activation followed by analysis for *IL6* and *IL11* mRNA expression. Data were normalized to *GAPDH* expression and are shown as mean ± SEM (*$p < 0.05$).

(C) Representative pTyr-STAT3 immunohistochemistry depicting STAT3 activation in the core region (oval) or invasive front (rectangle) of representative human CRC samples. The bottom row depicts higher magnifications of the boxed areas. Scale bars: 3 mm (top row); 300 μm (bottom row). See also Figure S1.

in pTyr-STAT3 levels within isolated colonic epithelial cells of *Il11ra1*^{KO} mice following a single injection of AOM and one cycle of DSS (Figure S2A). Similarly, distal colonic tumors from *Il11ra1*^{KO} and *Il6*^{KO};*Il11ra1*^{KO} mice showed reduced levels of pTyr-STAT3 and reduced expression of the downstream target protein BCL-2, compared to WT and *Il6*^{KO} mice (Figure 2F).

To further validate the requirement for IL-11 signaling in the CAC model, we utilized homozygous *gp130*$^{F/F}$ mutant mice that expressed gp130(Y757F) (Tebbutt et al., 2002). gp130(Y757F) cannot interact with gp130's negative regulator SOCS3, leading to enhanced IL-6 family cytokine-mediated STAT3 activation (Figure S2B). First, we confirmed that IL-11 expression was elevated in the CAC tumors of *gp130*$^{F/F}$ mice (Figure S2C). We next inhibited IL-11 or IL-6 signaling genetically in *gp130*$^{F/F}$ mice and revealed a dominant role for IL-11 driven STAT3 activation in promoting CAC-induced tumorigenesis (Figures S2D–S2G). Moreover, the reduction in tumor burden observed in *Il11ra1*^{KO} and *gp130*$^{F/F}$;*Il11ra1*^{KO} mice coincided with a reduction in the submucosal inflammation compared to tumor-bearing WT and *gp130*$^{F/F}$ mice (Figures 2E and S2F). Collectively, these observations establish IL-11 signaling as a driver of inflammation-associated tumorigenesis that is more profound than that previously ascribed to IL-6.

Colonic Tumorigenesis Is Not Dependent on IL-11-Responsive Hematopoietic Cells

Physical disruption of the mucosal barrier by DSS exposes innate immune cells of the submucosa to luminal antigens and provides a stimulus for the production of pro-inflammatory cytokines that can fuel epithelial tumor progression (Hanahan and Coussens, 2012). To determine whether the tumor-promoting function of IL-11 was mediated by cells of hematopoietic origin, we generated reciprocal bone marrow chimeras. Following complete engraftment, recipient mice were subjected to only the first two DSS cycles of the CAC protocol (Figure 2A) and were then monitored by serial endoscopy. We found that tumor burden in lethally

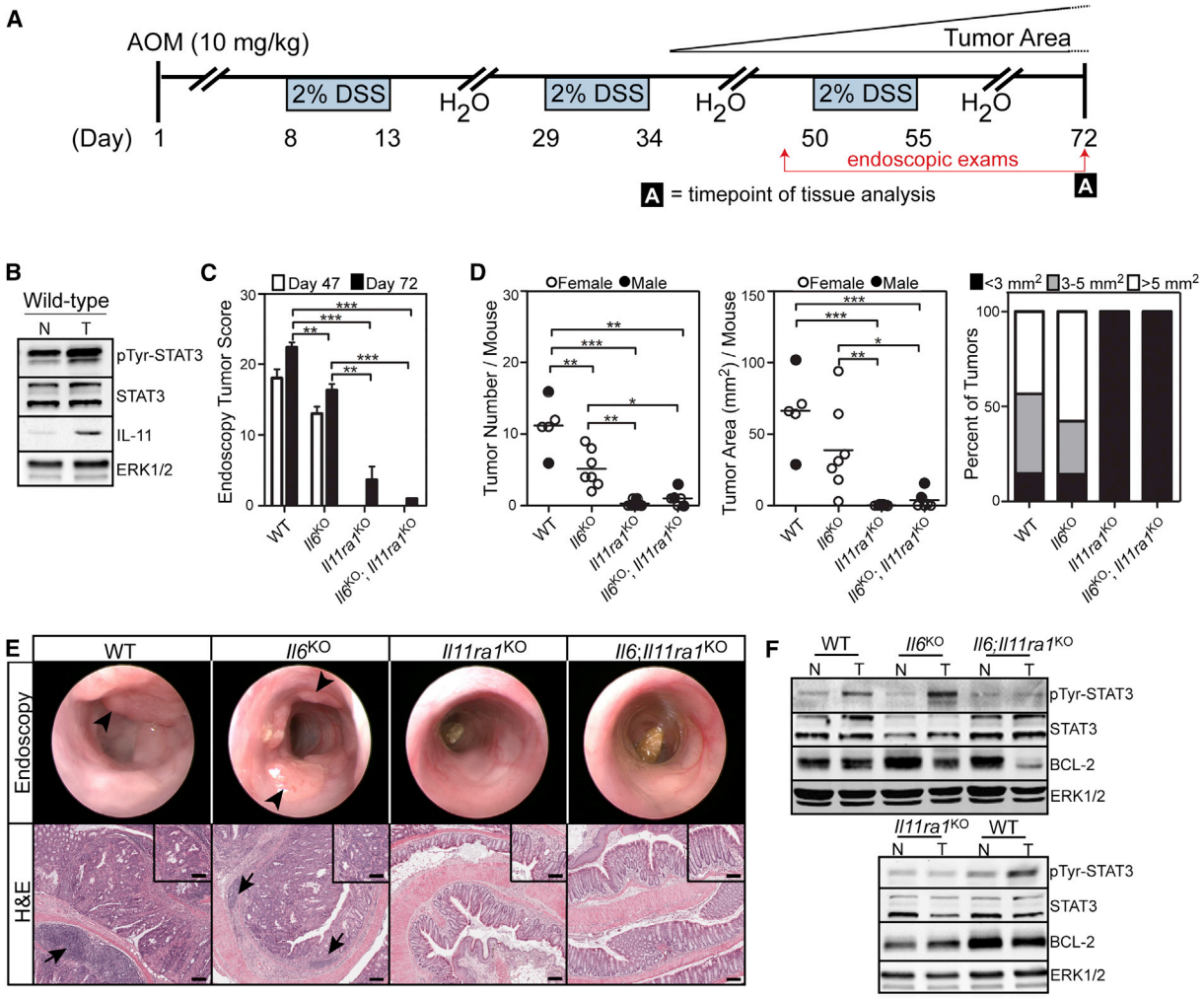

Figure 2. IL-11 Is the Dominant IL-6 Family Cytokine during CAC in Mice

(A) Schematic representation of the CAC model. A single injection of AOM is followed by repeated administration of DSS provided ad libitum in the drinking water. The formation of distal colonic tumors is then monitored by endoscopy at the indicated time points.

(B) Representative immunoblot analysis of distal colonic tumor (T) and adjacent nontumor (N) tissue from WT mice collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

(C) Tumor burden of mice of the indicated genotypes was scored by endoscopy at day 47 and 72 of the CAC model; data are presented as mean ± SEM ($n \geq 4$ mice per cohort; $^{**}p < 0.01$, $^{***}p < 0.001$).

(D) Colonic tumor burden from individual mice of the indicated genotypes at autopsy on day 72. Horizontal lines refer to mean values ($n \geq 5$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(E) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) on day 72 of the CAC model. Representative images of corresponding hematoxylin and eosin stained (H&E) sections (bottom row) with regions of inflammatory cell infiltration (arrows) in the tumor microenvironment. Scale bars: 200 μm; inset: 130 μm.

(F) Representative immunoblot analysis of colonic tumors (T) and adjacent nontumor (N) tissues for pTyr-STAT3 and BCL-2 from mice of the indicated genotypes collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

See also Figure S2.

irradiated WT recipients reconstituted with *Il11ra1*^{KO} bone marrow was comparable to that of their WT littermates reconstituted with WT bone marrow (Figures 3A and 3B). In contrast, loss of IL-11 signaling in the nonhematopoietic cells of recipient *Il11ra1*^{KO} hosts rendered mice resistant to CAC. To examine whether loss of IL-11 signaling altered the recruitment of immune cells into the tumor microenvironment, we stained colons for CD45-positive hematopoietic cells, F4/80-positive macrophages (Figures 3C and 3D), Gr1-positive neutrophils, and CD3-positive lymphocytes (Figure S3A). This revealed that tumor-associated submucosal immune cell infiltrates occurred irrespective of the capacity of the hematopoietic cells to respond to IL-11. Likewise, we observed that the expression of many inflammatory cytokines in tumors and unaffected colonic mucosa were not affected by the absence of IL-11-responsive hematopoietic cells (Figure S3B). In contrast, *Il11ra1* ablation in the nonhematopoietic compartments of the recipient hosts led to a reduction in submucosal inflammation

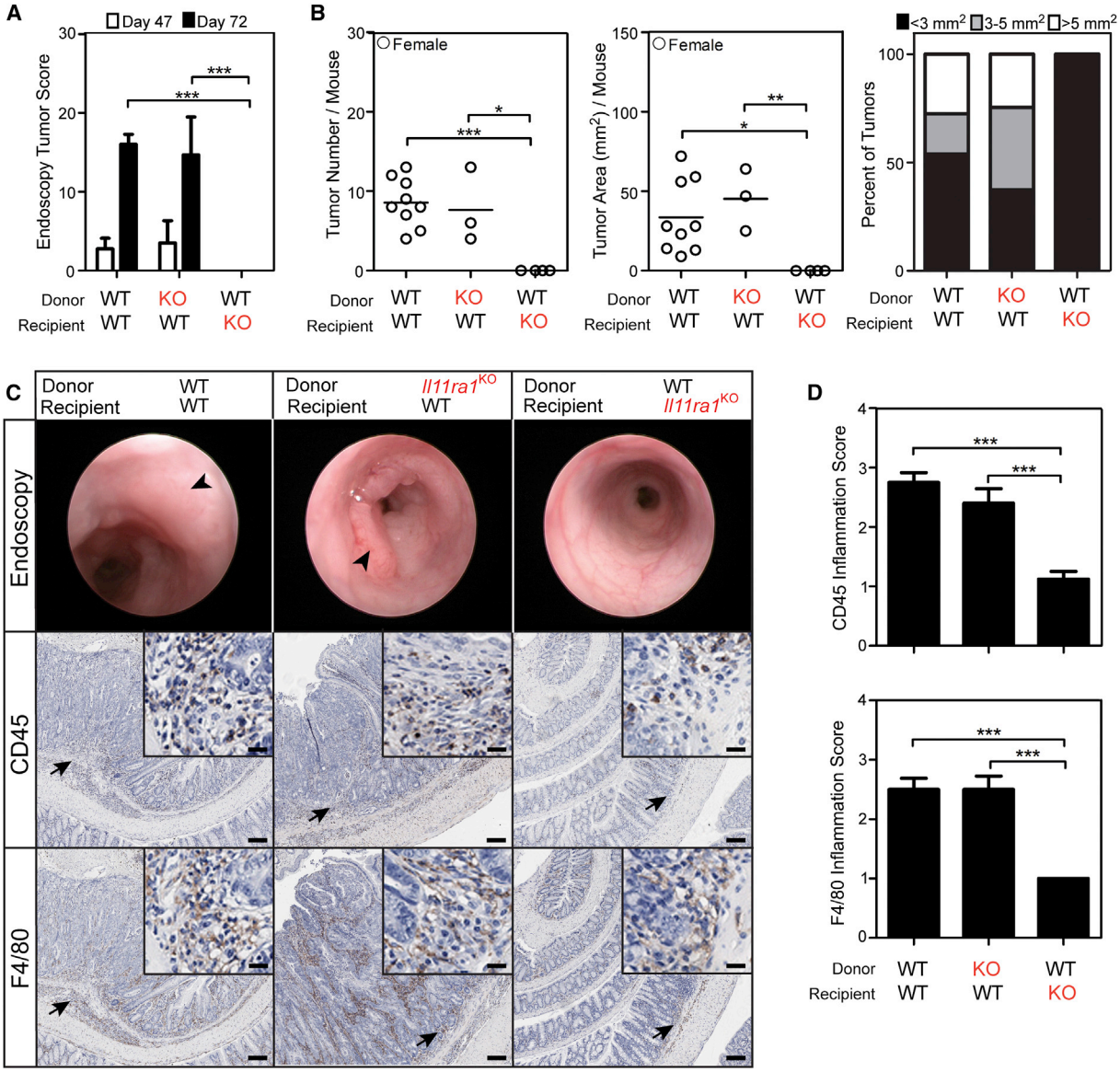

Figure 3. IL-11-Responsive Hematopoietic Cells Are Dispensable for Tumorigenesis

(A) Tumor burden of reciprocal WT and *Il11ra1*^{KO} (KO) bone marrow chimeras was scored by endoscopy at day 47 and 72 of the CAC model and is depicted as mean ± SEM (n ≥ 4 mice per cohort; ***p < 0.001).

(B) Colonic tumor burden in individual reciprocal WT and *Il11ra1*^{KO} (KO) bone marrow chimeras at autopsy on day 72 of the CAC model. Horizontal lines refer to mean values (n ≥ 3 mice per cohort; *p < 0.05, **p < 0.01, ***p < 0.001).

(C) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) on day 72 of the CAC model. Representative immunohistochemical staining for the pan-hematopoietic marker CD45 (middle row) and the macrophage marker F4/80 (bottom row) on adjacent sections of distal colonic tumors from mice of the indicated genotype. Arrows indicate submucosal cell infiltrates. The insets depict higher magnification areas. Scale bars: 200 μm; inset: 30 μm.

(D) Quantification of inflammatory cell infiltrates detected in the submucosa of the sections in (C). Scores are presented as mean ± SEM (n ≥ 3 mice per cohort; ***p < 0.001).

See also Figure S3.

(Figures 3C and 3D) and associated *Tnf* expression, while the expression of other inflammatory cytokines was not altered (Figure S3B). Our results suggest that tumorigenesis depends on IL-11 signaling and correlates with the severity of inflammation and that IL-11 mediates these effects through nonhematopoietic cells.

Sporadic Intestinal Tumorigenesis Requires IL-11 Signaling

The vast majority of sporadic human CRCs arise as a consequence of somatic mutations in components of the Wnt/β-catenin pathway in the absence of chronic inflammatory conditions. To examine the role of cytokines that signal via gp130 receptor

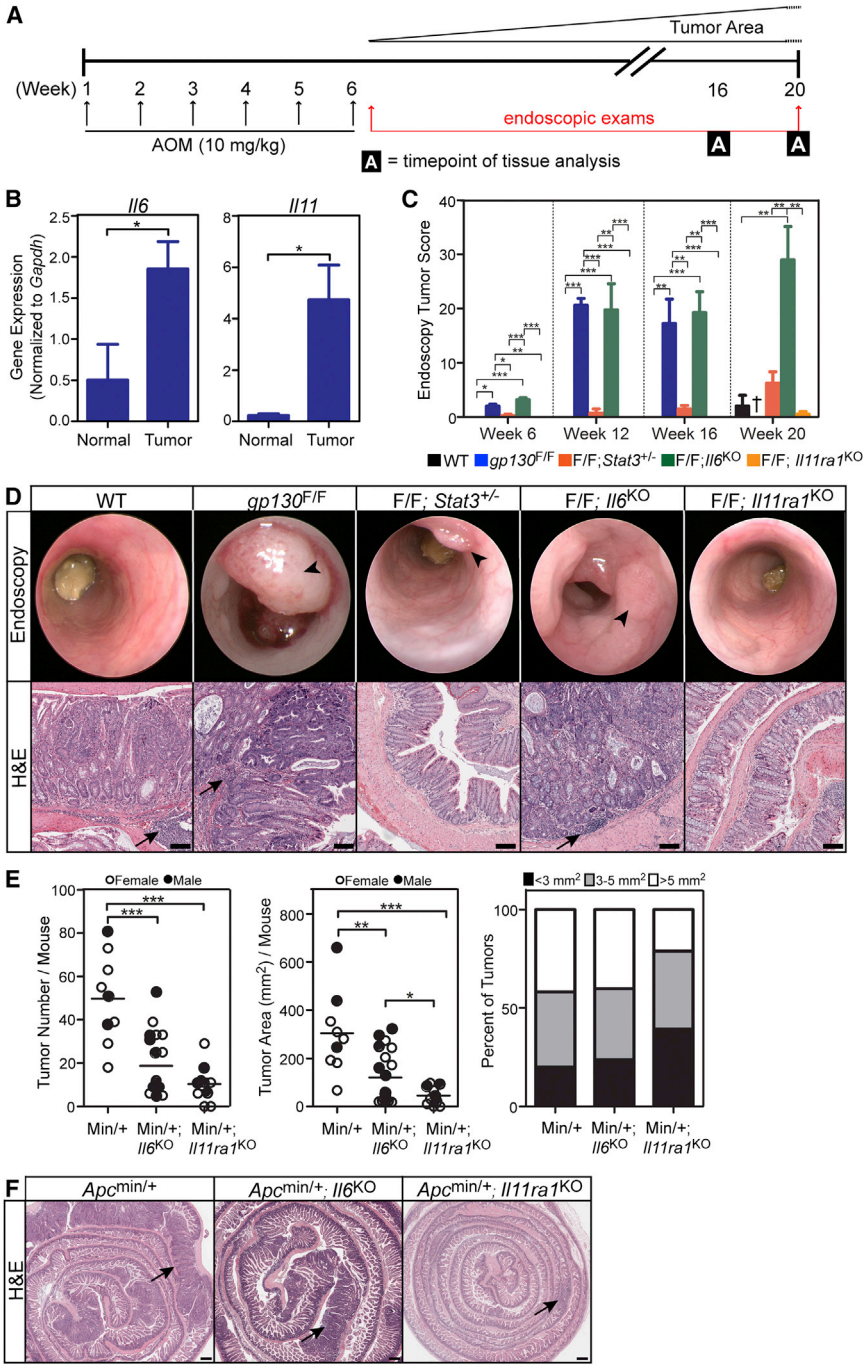

Figure 4. Loss of IL-11/STAT3 Signaling Delays the Onset of Sporadic CRC in Mice

(A) Schematic representation of the sporadic CRC model. The spontaneous formation of distal colonic tumors in mice injected weekly with AOM is monitored by endoscopy.

(B) *Il6* and *Il11* mRNA expression in distal colonic tumor and adjacent nontumor tissue from *gp130*$^{F/F}$ mice collected at week 16 of the sporadic CRC model. Data were normalized to *Gapdh* expression and are shown as mean relative expression ($2^{\wedge}dCT$) ± SEM ($n \geq 3$ mice per cohort; $^{*}p < 0.05$).

(C) Tumor burden of individual mice of the indicated genotype was scored by endoscopy at the indicated time and is presented as mean ± SEM ($n \geq 4$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). †Moribund *gp130*$^{F/F}$ mice that were euthanized by 18 weeks for ethical compliance.

(D) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) at weeks 18–20 of the sporadic CRC model. Representative images of H&E tissue sections (bottom row) with regions of inflammatory cell infiltration (arrows) in the tumor microenvironment. Scale bars: 200 μm.

(E) Colonic tumor burden in individual *Apc*$^{min/+}$ compound mutant mice at 150 days of age. Horizontal lines refer to mean values ($n \geq 9$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(F) Representative images of H&E sections of the distal small intestine from compound mutant *Apc*$^{min/+}$ mice of the indicated genotypes. Scale bars: 200 μm.

See also Figure S4.

complexes in the absence of overt inflammation, we challenged WT and *gp130*$^{F/F}$ mice repeatedly with AOM (Figure 4A). Because the distal colonic tumors of *gp130*$^{F/F}$ mice showed increased *Il6* and *Il11* expression compared to unaffected adjacent tissue (Figure 4B), we carried out serial endoscopy to assess tumor progression in *gp130*$^{F/F}$ mice with impaired IL-6, IL-11, or STAT3 signaling. In this model of the early stages of sporadic CRC observed in humans, the colonic tumor latency in *gp130*$^{F/F}$ mice was reduced to 20 weeks, compared to over 35 weeks in WT controls (Figure 4C). We consistently observed reduced tumor burden in *gp130*$^{F/F}$;*Il11ra1*^{KO} and *gp130*$^{F/F}$;*Stat3*$^{+/-}$ mice, compared to the *gp130*$^{F/F}$ mice (Figures 4C, 4D, and S4A). This coincided with reduced expression of the STAT3 target gene *Socs3* in the remaining small tumors of *gp130*$^{F/F}$;*Il11ra1*^{KO} mice (Figure S4B), where we surmise that other cytokines may account for the residual expression of *Socs3*. Although pan-inflammatory disease is reduced in *gp130*$^{F/F}$;*Il6*^{KO} mice (Ernst et al., 2008) and associated with prolonged survival compared with their *gp130*$^{F/F}$ littermates, the colonic tumor burden was comparable between the two cohorts (Figures 4C and 4D). These observations are consistent with previous findings that gastric tumorigenesis is not the cause of premature death in the *gp130*$^{F/F}$ mice and collectively confirm a dominant role for IL-11 signaling during sporadic CRC.

Given the dependency of AOM-induced tumorigenesis on activating mutations in *Ctnnb1*, we next examined the cellular distribution of β-catenin in neoplastic crypts. As expected, we observed nuclear β-catenin staining, indicative of active Wnt-signaling, in the cells of emerging tumors and the proliferative intestinal stem cell region of crypts of *gp130*$^{F/F}$ and

gp130$^{F/F}$;*Il11ra1*^{KO} mice (Figure S4C). This was supported by comparable expression of the Wnt-target genes *Cd44*, *Axin2*, and *Myc* between mice of the different genotypes and suggests that alteration of IL-11/STAT3 signaling does not alter Wnt-signaling (Figure S4D). Although our AOM-only CRC model lacks the flares of inflammation arising from DSS administration, limiting STAT3 signaling in *gp130*$^{F/F}$;*Il11ra1*^{KO} or *gp130*$^{F/F}$;*Stat3*$^{+/-}$ mice reduced tumor-associated submucosal inflammation (Figure 4D) and expression of markers associated with inflammation (*Cox2*, *Tnf*), invasion (*Mmp9*), and proliferation (*Ccnd1*; Figure S4D).

Our observations suggest that interference with IL-11/STAT3 signaling may be able to limit Wnt/β-catenin-driven intestinal tumorigenesis. To further validate this hypothesis, we next employed the *Apc*$^{min/+}$ mouse model of familial adenomatous polyposis syndrome, where spontaneous intestinal dysplasia develops from loss of heterozygosity of the *Apc* gene (Su et al., 1992). Reminiscent of our findings in the CAC and sporadic CRC AOM-only models (Figures 2 and 4), we observed elevated expression of *Il6* and *Il11* in *Apc*$^{min/+}$ tumors compared to adjacent unaffected tissue (Figures S4E and S4F). We confirmed that genetic ablation of *Il6* reduced tumor numbers and burden (Baltgalvis et al., 2008), and show that tumorigenesis was even further reduced in *Apc*$^{min/+}$;*Il11ra1*^{KO} mice (Figures 4E and 4F). Collectively, these results suggest that IL-11 signaling is required for the development of intestinal tumors that share aberrant activation of the Wnt/β-catenin pathway as an underlying initiating oncogenic event.

IL-11 Signaling Can Be Targeted Therapeutically

We reasoned that a reduction in GI tumor burden following partial genetic inhibition of the IL-11 signaling pathway was required to justify the development of therapeutics against this pathway. To this end, we utilized *gp130*$^{F/F}$ mice as a validated model of spontaneously arising intestinal-type GC. While the gastric tumors that form in the distal antrum of *gp130*$^{F/F}$ mice have abnormally elevated expression of both IL-6 and IL-11 (Figure S5A), the strict dependence of gastric tumor growth on IL-11 signaling (Ernst et al., 2008) makes it an ideal model to test anti-IL-11 therapies. We found that at all stages of gastric tumor development, *gp130*$^{F/F}$ mice lacking one allele of *Stat3* or *Il11ra1* had consistently reduced tumor burden compared to their control *gp130*$^{F/F}$ littermates (Figures S5B and S5C). These results suggest that partial inhibition of the IL-11/STAT3 signaling axis is sufficient to cause a significant reduction in tumor burden.

To test the effects of an antagonist of IL-11 signaling in a model of GI cancer, we systemically treated *gp130*$^{F/F}$ mice with mIL-11 Mutein, a potent IL-11 signaling antagonist that has a 20-fold higher affinity than IL-11 for binding to IL-11Rα (Lee et al., 2008). We found that the IL-11 antagonist reduced pTyr-STAT3 levels in the gastric tumors and adjacent hyperplastic antrum tissue (Figure 5A). We next explored the therapeutic potential of prolonged systemic mIL-11 Mutein administration and found that regardless of the stage of disease, age, and sex of the mice, mIL-11 Mutein treatment significantly reduced overall tumor burden and gastric epithelial hyperplasia (Figures 5B–5D). Successful treatment with different doses of the mIL-11 Mutein for 4 weeks was not associated with weight loss (Figures S5D and S5E) or changes in hematopoietic composition, including platelet counts (Figure S5F), and did not compromise hemostasis (Figures S5G and S5H).

To explore whether mIL-11 Mutein conferred a transient or a long-lasting therapeutic benefit, we randomly assigned *gp130*$^{F/F}$ mice that were treated with mIL-11 Mutein for 4 weeks to either a cohort for immediate analysis or a cohort for a 4-week treatment-free follow-up period. We found that gastric tumor burden in the follow-up cohort was increased compared to the mIL-11 Mutein treatment-only cohort (Figures 5E–5G), suggesting that continuous inhibition of IL-11 signaling is required to block tumors.

To investigate the mechanism by which mIL-11 Mutein reduces tumor burden, we quantified the numbers of PCNA-positive proliferative and Apop-tag-positive apoptotic epithelial cells in the gastric tumors (Figures 6A and S6A). The decrease in PCNA-positive cells observed in mIL-11 Mutein-treated animals coincided with reduced expression of the cell-cycle regulators Cyclin D1, D2, and D3 (Figure 6B). Surprisingly, we found that mIL-11 Mutein treatment was also associated with increased expression of the pro-apoptotic protein BIM, rather than a reduction in the STAT3 target prosurvival protein BCL-2 (Figure 6C). Moreover, mIL-11 Mutein treatment diminished submucosal inflammation, reflected by the reduced infiltration of CD45-positive hematopoietic and F4/80-positive macrophage cells and reduced expression of the inflammatory mediators KC and IL-1β (Figures 6D, S6B, and S6C).

IL-6 and IL-11 Activate Similar Gene Expression Signatures but Target Different Cells

Because both IL-6 and IL-11 are secreted by epithelial and CD45-positive hematopoietic cells in the gastric tissue of *gp130*$^{F/F}$ mice (Figures S6D and S6E), we performed microarray analysis to explore the underlying mechanism for the requirement of IL-11 signaling for gastric tumor promotion. We compared gene expression in gastric tumors of *gp130*$^{F/F}$ mice following a single injection of recombinant human IL-6 or IL-11. The gene expression profiles of the IL-6- and IL-11-treated samples were separately compared to the PBS-vehicle-treated control samples, resulting in two lists of responsive genes (Table S1). Comparison of these lists revealed an extensive overlap of responsive genes between the two cytokines (i.e., *Socs3*, *Vegfa*, *Pim3*, *Reg3b*) with a notable bias for a more profound response to IL-6 (Table S1; Figure S6F). As expected, functional classification analysis showed that genes on either list belong to 13 common functional gene clusters (Figure S6G). However, we also identified several genes that were specifically regulated by only one of the two cytokines (Figure S6F). We found that some of the IL-6-specific genes were associated with immune cells (i.e., *Dusp6, Junb, Cish/Socs1*), while those specific for IL-11 were associated with epithelial activities (i.e., *Spp1/Osteopontin*). The small difference in gene signatures associated with different cellular functions suggested that IL-6 and IL-11 may target different cell populations. To explore this possibility, we adoptively transferred *gp130*$^{F/F}$;*Il11ra1*^{KO} bone marrow into lethally irradiated *gp130*$^{F/F}$ mice with early gastric tumors. We found that the tumor burden in these mice remained similar to that of *gp130*$^{F/F}$ littermates reconstituted with *gp130*$^{F/F}$ bone marrow (Figure 6E). Collectively, our data suggest that IL-6 and IL-11 activate similar gene expression signatures, with gastric

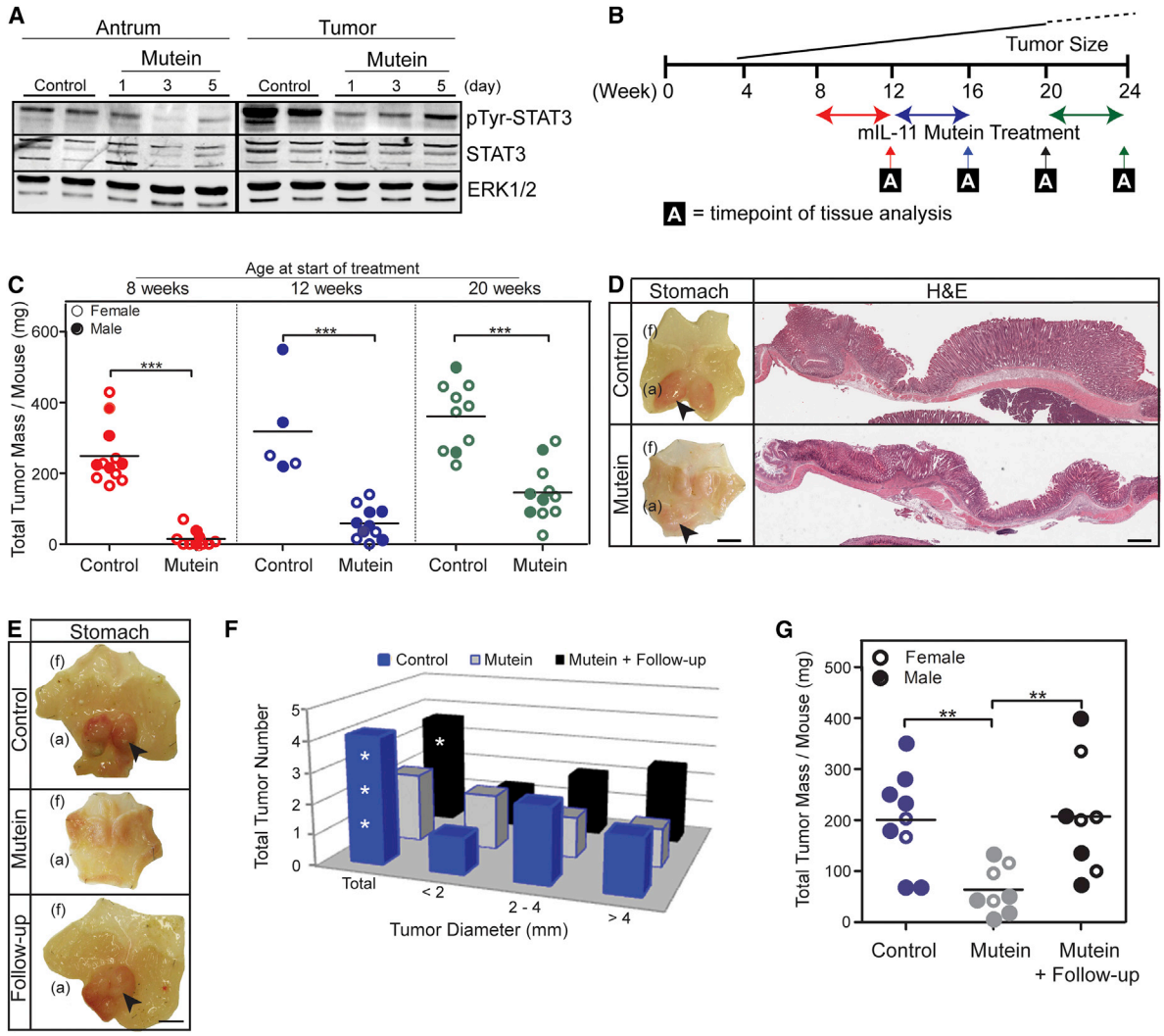

Figure 5. Pharmacologic Inhibition of IL-11 Signaling Inhibits Gastric Tumorigenesis

(A) Representative immunoblot analysis for activated STAT3 (pTyr-STAT3) in gastric antral and tumor tissue from individual 12-week-old *gp130*$^{F/F}$ mice treated with mIL-11 Mutein or a vehicle control on days 1, 3, and 5. ERK1/2 was used as a loading control.

(B) *gp130*$^{F/F}$ mice with established gastric tumors were treated with mIL-11 Mutein or a vehicle control for 4 consecutive weeks. Treatment commenced at 8 (red), 12 (blue), or 20 weeks (green) of age.

(C) Total gastric tumor mass of individual control- and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice of the treatment age group indicated in (B). Horizontal lines refer to mean values ($n \geq 5$ mice per cohort; ***$p < 0.001$).

(D) Representative whole mounts of stomachs (left) and corresponding H&E longitudinal sections (right) from 16-week-old control and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice. Stomachs were opened along the greater curvature and pinned-out with the lumen facing the viewer (f = fundus, a = antrum). Scale bars: 6 mm (whole mount); 500 μm (H&E).

(E–G) Sixteen-week-old *gp130*$^{F/F}$ mice were analyzed after 4 consecutive weeks of mIL-11 Mutein treatment or after an additional 4-week treatment-free follow-up period. Representative whole mounts of stomachs of *gp130*$^{F/F}$ mice of the indicated treatment group (E); f, fundus; a, antrum; scale bar: 6 mm. In each mouse, individual tumors were classified according to their size (F) and the combined mass of resected tumors was determined (G). Horizontal lines and histograms refer to mean values ($n \geq 8$ per mice cohort; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S5.

tumorigenesis likely to arise from cells in the glandular epithelium that may be biased toward IL-11 responsiveness.

mIL-11 Mutein Therapy Suppresses CAC Tumor Progression

Having demonstrated that IL-11 signaling can be therapeutically targeted in the *gp130*$^{F/F}$ model of spontaneous GC (Figure 5), we next examined whether mIL-11 Mutein could confer a therapeutic benefit in the absence of predetermined mutations in *gp130*$^{F/F}$ mice. Therefore, we treated WT mice with established CAC tumors for 4 weeks with mIL-11 Mutein (Figure S7A). Serial endoscopy revealed that mIL-11 Mutein treatment led to a cytostatic effect on existing colonic tumors (Figure 7A), which was confirmed at autopsy by a significant reduction in tumor

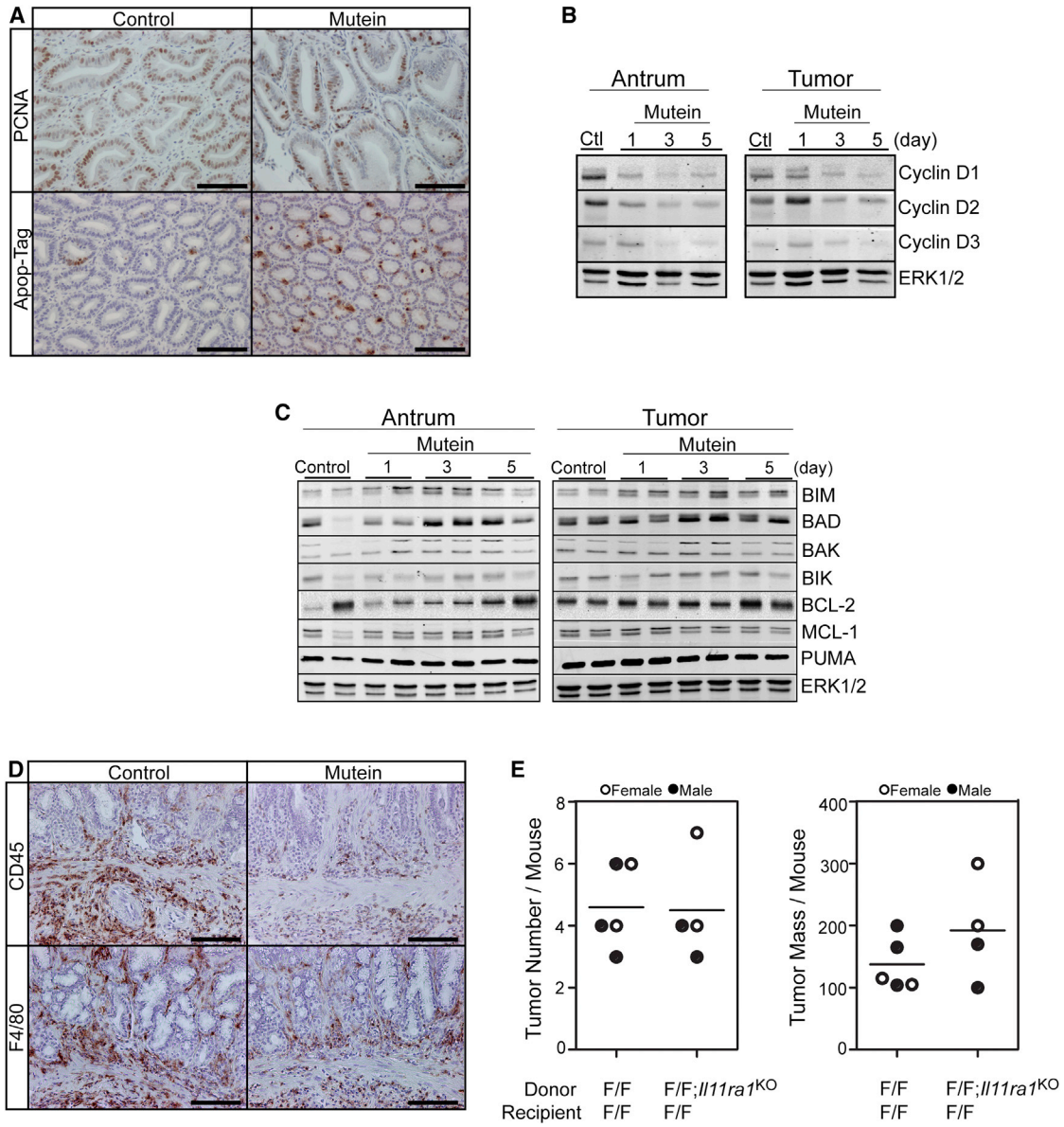

Figure 6. Mutein Treatment Reduces Tumor Cell Proliferation and Triggers Cell Death

(A) Representative immunohistochemical staining for proliferation (PCNA) and cell death (Apop-Tag) markers on adjacent gastric tumor sections from control- and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice at 12 weeks of age. Scale: 200 μm.

(B and C) Immunoblot analysis of gastric antral and tumor tissue from individual 12-week-old *gp130*$^{F/F}$ mice treated with mIL-11 Mutein or a vehicle control (Ctl) on days 1, 3, and 5 and analyzed for the expression of cyclin (B) or apoptosis-related proteins (C). ERK1/2 was used as a loading control.

(D) Representative immunohistochemical stainings for the pan-hematopoietic marker CD45 and the macrophage marker F4/80 on adjacent gastric tumor sections from control and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice at 12 weeks of age. Scale bars: 200 μm.

(E) Total gastric tumor numbers (left) and mass (right) of *gp130*$^{F/F}$ bone marrow chimeras at 14 weeks of age. Horizontal lines refer to mean values ($n \geq 4$ mice per cohort).

See also Figure S6 and Table S1.

multiplicity and size compared to vehicle-treated animals (Figures 7B and 7C). We also confirmed that mIL-11 Mutein significantly alleviated CAC-induced tumor burden in *gp130*$^{F/F}$ mice (Figures S7B and S7C). Importantly, we show that in *gp130*$^{F/F}$ mice, mIL-11 Mutein treatment reduced neoplastic colonic epithelial proliferation and increased apoptosis (Figures S7D and S7E). Interestingly, *gp130*$^{F/F}$ mice display elevated colonic tumor BCL-2 expression compared to WT mice (Figure 2F), which was not altered following Mutein treatment (Figure S7F). Similar to our results from the GC model (Figure 6), we attribute the therapeutic benefit of mIL-11 Mutein in colonic tumors to a reduction in tumor-associated STAT3 activation and increased expression of pro-apoptotic BIM (Figures 7D and S7F). Taken together, these results indicate that inhibition of IL-11 signaling

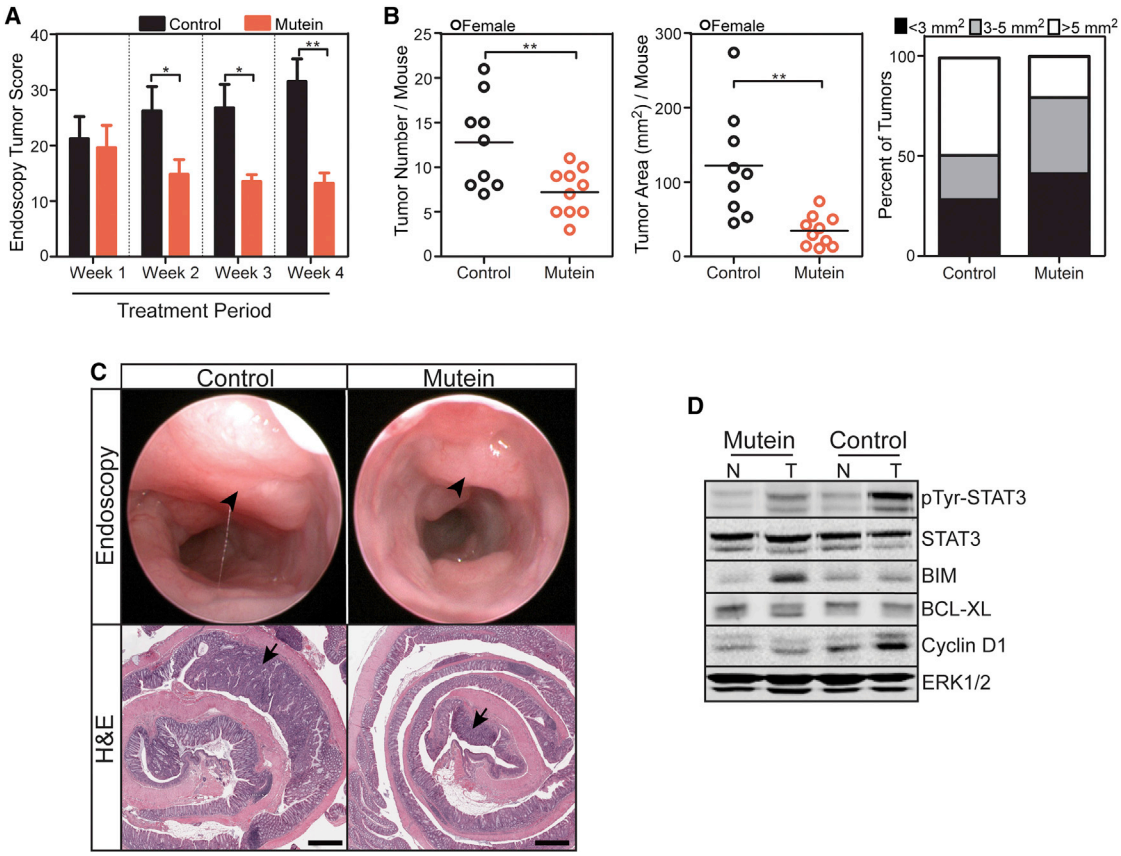

Figure 7. Therapeutic Inhibition of IL-11 Signaling Reduces Tumor Burden in CAC-Challenged WT Mice

(A) CAC-challenged WT mice were treated with mIL-11 Mutein for 4 consecutive weeks commencing on day 46 of the CAC model (refer to Figure S7A). Tumor burden was scored by serial endoscopy at the indicated time points and is presented as mean ± SEM ($n \geq 4$ mice per cohort; *$p < 0.05$, **$p < 0.01$).

(B) Colonic tumor burden in individual control and mIL-11 Mutein-treated WT mice at autopsy on day 72. Horizontal lines refer to mean values ($n \geq 9$ mice per cohort; **$p < 0.01$).

(C) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) and corresponding H&E tissue sections (bottom row) with tumors and associated inflammatory cells (arrows) in control and mIL-11 Mutein-treated WT mice at day 72 of the CAC protocol. Scale bars: 200 μm.

(D) Representative immunoblot analysis of distal colonic tumor (T) and adjacent nontumor (N) tissue from control and mIL-11 Mutein-treated WT mice collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

See also Figure S7.

promotes anti-tumor effects in a well-established model of human CRC.

mIL-11 Mutein Therapy Slows the Growth of Human GI Cancer Cells in Xenografts

To investigate whether mIL-11 Mutein could also impede the growth of human GI cancers, we used two representative human GI cancer derived cell lines, DLD1 and MKN28. DLD1 cells harbor mutations in *APC, PIK3CA, TP53*, and *KRAS*, which are frequently observed in CRC (Trainer et al., 1988), whereas MKN28 cells have mutations in *TP53*, prevalent in GC (Matozaki et al., 1992). In response to stimulation with recombinant human IL-11, both cell lines displayed dose-dependent activation of STAT3 (pTyr-STAT3 staining; Figures 8A and S8A). Because mIL-11 Mutein cross-reacts with the human IL-11Rα, we pretreated these cell lines with mIL-11 Mutein and found a dose-dependent inhibition of hIL-11-mediated STAT3 activation (Figures 8B and S8B). Given that we observed increased STAT3 activation at the invasive front of human tumors (Figure 1C) and because IL-11 has been implicated in the invasion and metastasis of epithelial cancer cells (Calon et al., 2012; Lay et al., 2012), we next examined whether mIL-11 Mutein treatment could inhibit the invasive potential of human GI cancer cells in vitro. We found that recombinant hIL-11 promoted cell migration in a transwell assay and that inhibition of IL-11 signaling by pretreatment with mIL-11 Mutein blocked the invasive capacity of DLD1 and MKN28 cells (Figures 8C and S8C). To assess whether the growth of human cancer cells in vivo was susceptible to inhibiting the activity of IL-11 that is produced by both tumor cells and the surrounding stroma (Figure S8D), we established subcutaneous xenografts of DLD1 tumor cells in immune-deficient BALB/c nude mice. Once tumors became palpable, we treated mice with mIL-11 Mutein for 4 consecutive weeks. We observed significantly reduced tumor growth in mIL-11 Mutein-treated animals compared to vehicle-treated controls, resulting in an ~50% decrease in tumor mass at autopsy (Figures 8D and 8E). Immunohistochemical analysis of the tumors revealed a significant reduction of activated STAT3

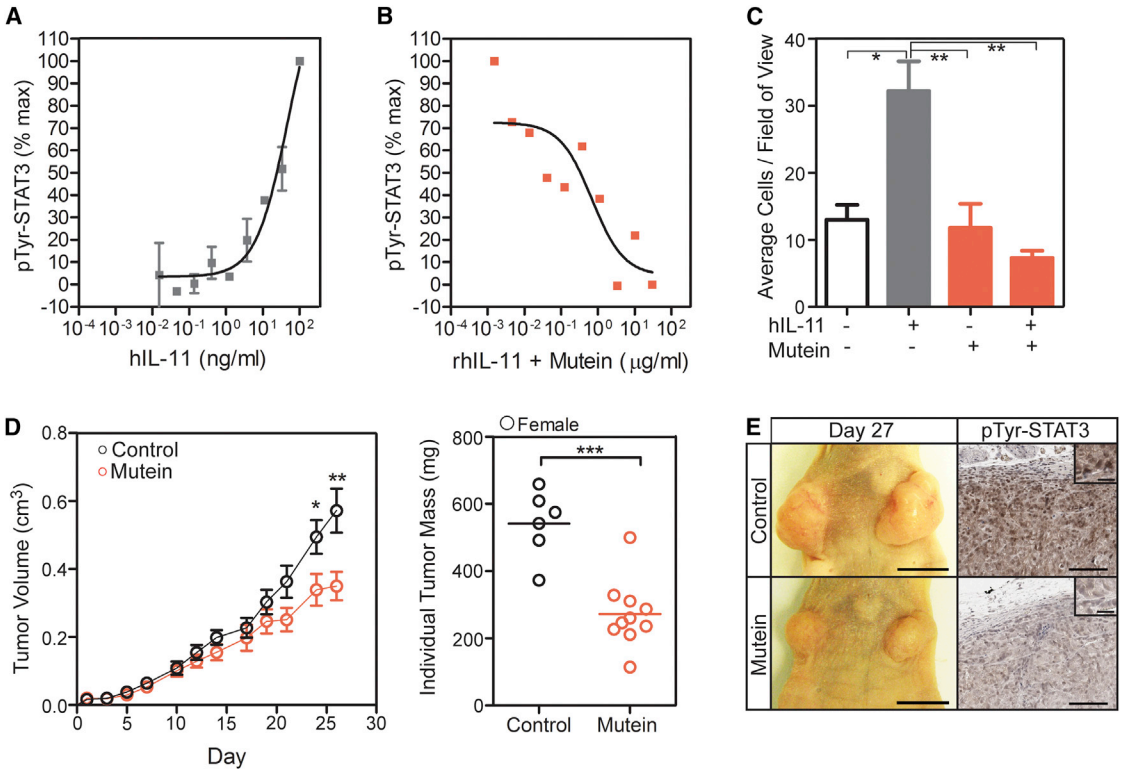

Figure 8. Therapeutic Inhibition of IL-11 Signaling Reduces the Growth of Human CRC Cells in Immune-Deficient Mice

(A and B) pTyr-STAT3 in DLD1 cells stimulated with increasing concentrations of recombinant hIL-11 (A) or stimulated with 20 ng/ml of hIL-11 following pretreatment with increasing concentrations of mIL-11 Mutein (B). pTyr-STAT3 was determined by flow cytometry. Data in (A) are means ± SEM.

(C) DLD1 cells pretreated with mIL-11 Mutein (100 μg/ml) for 60 min were combined with recombinant hIL-11 and Matrigel as indicated. The mixture was added to the top chambers of transwell plates (2×10^5 cells/well) with IL-11-deficient medium in the bottom chambers. Cells that migrated to the bottom chamber were enumerated 24 hr later. Results represent mean ± SEM (*$p < 0.05$, **$p < 0.01$).

(D) BALB/c nude mice were injected subcutaneously with 5×10^6 DLD1 cells on contralateral sides. When palpable tumors had formed 3 days later, the mice were treated three times per week over 24 days with mIL-11 Mutein (10 mg/kg) or a vehicle control. Tumor volumes were calculated from serial caliper measurements, and the total tumor mass of each individual tumor was determined at autopsy. Horizontal lines refer to mean values. Data are means ± SEM ($n \geq 3$ mice per treatment group; *$p < 0.05$, **$p < 0.01$).

(E) Representative photographs of DLD1 xenograft tumors on day 27 (left; scale bars: 11 mm) and corresponding immunohistochemical stains for pTyr-STAT3 (right; scale bars: 100 μm). Insets represent higher magnifications (scale bars: 25 μm).

See also Figure S8.

(pTyr-STAT3 staining; Figure 8E) consistent with our proposed role for IL-11-mediated STAT3 signaling in the promotion of GI cancers. Collectively, our data confirm that therapeutic inhibition of IL-11 signaling inhibits the progression of human GI tumors.

DISCUSSION

Emerging therapeutic strategies for epithelial cancers have explored cytokine inhibition as an alternative to pharmacologic targeting of signal transducing kinases and intracellular transcription factors. A major focus has been placed on cytokines that suppress tumor cell apoptosis through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), including IL-1β and tumor necrosis factor-α (Greten et al., 2004; Popivanova et al., 2008). Meanwhile, STAT3-activating cytokines are also attracting therapeutic interest, due to the prevalence of STAT3 activation in many types of human cancers, and the capacity of STAT3 to promote epithelial tumor progression and to suppress the host's antitumor immune response (Becker et al., 2004; Bollrath et al., 2009; Yu et al., 2007). In particular, antibodies that neutralize IL-6 (i.e., siltuximab) or block IL-6Rα (i.e., tocilizumab) are in clinical trials for ovarian, prostate, and renal cancers (Guo et al., 2010; Karkera et al., 2011). Our data argue that despite its overlapping expression with IL-6, IL-11 is the dominant STAT3-activating cytokine required for the progression of GI cancers.

Although the contribution of inflammatory cells to GI tumorigenesis is well established (Rutter et al., 2004), the beneficial impact of interfering with specific hematopoietic cell types appears to vary between different types of cancer. Genetic ablation of T and B cells (Boulard et al., 2012), macrophages (Oguma et al., 2008), mast cells (Gounaris et al., 2007), and components of the TLR/Myd88-signaling cascade (Rakoff-Nahoum and Medzhitov, 2007; Tye et al., 2012) reduced GI tumor burden in mice. Here, we show that the progression of GI tumors is not influenced by the capacity of hematopoietic cells to respond to IL-11, but instead requires the response of nonhematopoietic cells, such as the neoplastic epithelium itself. However, our

analysis does not preclude the possibility that bone marrow-derived cells are a critical source for IL-11, which in turn may account for the capacity of myeloid cells to promote invasiveness of epithelial tumors (Coussens et al., 2000). Consistent with this, we observed reduced IL-11 expression in NF-κB signaling-defective myeloid cells, which suppressed invasion of AOM-induced tumors in *Tp53* knockout mice (Schwitalla et al., 2013).

At this stage, it is not clear if the subtle difference in gene expression signatures observed between tumors from IL-6- and IL-11-challenged $gp130^{F/F}$ mice accounts for IL-11-dependent tumor development or whether tumors arise from a distinct (epithelial) cell population that selectively expresses IL-11Rα, but not the IL-6Rα. We favor the latter scenario, because the administration of Hyper-IL-6, a designer cytokine comprised of IL-6 fused to the IL-6Rα cytoplasmic tail, bypassed the requirement for membrane bound IL-11Rα and increased CAC tumor burden is an outcome not observed following IL-6 administration (Grivennikov et al., 2009). Consistent with an IL-6 "trans-signaling" mechanism, transgenic mice expressing sgp130-Fc, which competes with membrane gp130 for binding of IL-6 complexed to the cleaved IL-6Rα cytosolic domain, are less susceptible to CAC-induced tumorigenesis (Becker et al., 2004; Matsumoto et al., 2010). Although "trans-signaling" may also expand the function of IL-11 to IL-11Rα-deficient tumor cells, cleavage of the extracellular domain of IL-11Rα has not been observed in vivo. Given that IL-11 expression is also augmented in response to oncogenic RAS signaling (Shin et al., 2012), our results suggest that inhibition of IL-11 signaling may restore responsiveness to epidermal growth factor receptor (EGFR) therapy in the broad range of epithelial cancers that are characterized by excessive EGFR expression. This seems plausible, because cetuximab and other EGFR inhibitors show efficacy for the treatment of cancers without K-RAS mutations (Quesnelle et al., 2007), while resistance to EGFR inhibition can be overcome by simultaneous STAT3 suppression (Sen et al., 2012).

We predict that the reliance of the GI tract on IL-11/STAT3 signaling for tumor progression evolved from an intestinal repair mechanism to provide protection against the continuous inflammation caused by mechanical abrasion of the epithelium, which is observed in STAT3 signaling-deficient $gp130^{\Delta Stat}$ mice (Tebbutt et al., 2002). By exposing the injured epithelium to platelets, and other IL-11-producing CD45-positive cells, the mucosal cell layer can be regenerated from IL-11Rα expressing mucosal (stem) cells, in part through a STAT3-dependent mechanism. In the newly emerging epithelium, STAT3 would not only engage anti-apoptotic and proliferative gene programs, but also simultaneously amplify IL-11 expression (Boerma et al., 2007; Deutscher et al., 2006; Ernst et al., 2008; Gibson et al., 2010; Orazi et al., 1996). Incidentally, inhibition of IL-11 signaling increased expression of the pro-apoptotic protein BIM in a manner akin to that observed in response to therapeutic inhibition of the STAT3-target VEGF-A (Naik et al., 2011). The functional link between the epithelial repair response and the extent of local inflammation therefore requires an IL-6 family cytokine with a tissue specific response like IL-11, rather than the broad systemic activity elicited by IL-6 that can also exacerbate intestinal inflammation. Indeed, in the absence of overt colitis, the inflammatory microenvironment associated with sporadic *Apc* or *Tp53* mutations confers a growth-promoting advantage to adenomas (Grivennikov et al., 2012; Schwitalla et al., 2013). IL-11 may also facilitate the survival of advanced cancers with multiple mutations and increase their metastatic capacity (Calon et al., 2012). The latter effect can be further exacerbated by CAFs, which are an additional source of IL-11 (Calon et al., 2012) as well as by tumor hypoxia that is associated with increased IL-11 production (Onnis et al., 2013).

Direct therapeutic targeting of STAT3 has proven difficult, although inhibition of upstream activators, including JAK2, has shown more promise (Hedvat et al., 2009). Unfortunately, systemic inhibition of STAT3 or JAK2 in mice or humans (Ernst et al., 2008; Santos et al., 2010) is often associated with thrombocytopenia. Here, we show that a peptide-based IL-11 antagonist is well tolerated with no adverse effects on platelet numbers, despite the capacity of IL-11 to stimulate megakaryopoiesis (Musashi et al., 1991). These observations are consistent with the phenotype of mice lacking the IL-11 receptor α chain, which exhibit normal hematopoiesis at steady state and during response to chemoablative or hemolytic stress (Nandurkar et al., 1997). However, our results bring into question the clinical use of recombinant IL-11 for the treatment of thrombocytopenia in patients undergoing chemotherapy or to reconstitute epithelial barrier integrity in patients with inflammatory bowel disease (Cantor et al., 2003; Danese, 2012).

By identifying IL-11 as a functionally dominant inducer of neoplastic STAT3 activity in the GI epithelium, our studies establish a molecular entity that can be readily targeted by pharmacologic agents. Therapeutic interference with IL-11 signaling, therefore, could potentially selectively suppress the STAT3-associated cancer hallmark capabilities that collectively promote disease progression of some of the most prevalent epithelial cancer types in humans. It remains to be established whether the therapeutic inhibition of IL-11/STAT3 signaling in turn exposes compensatory pathways in tumor cells that can be further exploited to induce tumor cell death.

EXPERIMENTAL PROCEDURES

Clinical Material

All human tissues were obtained with informed patient consent and analyzed in accordance with approval from the ethics committee of the Technical University of Munich, the Peter MaCallum Cancer Center, and the Melbourne Health Human Ethics Committee (2010.154; 2012.25). Tumor and adjacent normal (noncancerous) tissues were analyzed.

Mice and Treatments

All animal procedures were approved and conducted in accordance with the Animal Ethics Committee of the Ludwig Institute for Cancer Research and the Walter and Eliza Hall Institute, Australia. Mice lacking functional alleles for IL-11Rα ($Il11ra1^{KO}$) and IL-6 ($Il6^{KO}$), homozygous for the gp130(Y757F) knockin mutation ($gp130^{F/F}$), or heterozygous for *Apc* ($Apc^{min/+}$) have been described previously (Ernst et al., 2008; Jenkins et al., 2005; Moser et al., 1990). The $Apc^{min/+}$ compound mutant mice were on a C57Bl/6 genetic background, whereas others were on a 129/sv x C57Bl/6 genetic background. Littermate controls were used for comparison when possible. BALB/c nude animals were purchased from the Australian Research Services. To minimize variation in gut microflora, all animals were bred in the same room and housed on the same rack in a specific pathogen-free barrier facility at the Ludwig Institute for Cancer Research, Australia.

CAC was induced in 129/sv × C57Bl/6 mice by intraperitoneal injection of AOM (10 mg/kg; Sigma). One week later, animals were provided drinking water

ad libitum containing 2% DSS (w/v; MP Biomedicals, molecular weight 35,000–50, 000 kDa) for 5 days, followed by 2 weeks of normal drinking water, which was repeated for two or three cycles as indicated. For CRC, animals received intraperitoneal injections of AOM (10 mg/kg) once weekly over 6 consecutive weeks. Tumor onset and progression in the distal colon was monitored by endoscopy as described previously (Becker et al., 2006).

Xenografts were established in 6-week-old nude BALB/c mice by subcutaneous injection of 5 × 10^6 DLD1 cells. Tumor volume was measured every second day with calipers and calculated using the formula: 0.52*(Tumor Length*(Tumor Width)∧2)/1,000. For therapeutic mIL-11 Mutein treatments, mice received intraperitoneal injections three times a week over 4 consecutive weeks with 40 mg/kg mIL-11-Mutein or a PEG-vehicle control (unless otherwise indicated). The affinity, specificity, and bioactivity of mIL-11-Mutein has been described previously (Lee et al., 2008).

Statistics

All data are representative of at least two independent experiments. Comparisons between values from two groups were performed using Student's t tests (two tailed) and multiple groups by ANOVA (Bonferroni post-hoc): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional experimental information is available in the Supplemental Experimental Procedures.

ACCESSION NUMBER

The NCBI Gene Expression Omnibus repository number for the complete array data presented in this paper is GSE43800.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.06.017.

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