Gut microbiota accelerate tumor growth via c-jun and STAT3 phosphorylation in APCMin/+ mice

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Introduction

Chronic inflammation and colon cancer progression are irrefutably linked in inflammatory bowel disease patients since cancer susceptibility correlates with the extent and duration of colonic disease/mucosal inflammation. Accumulating evidence demonstrate that inflammatory cells in the tumor microenvironment and their products can enhance progression of cancer in conjunction with the supporting stromal cells. Moreover, inflammatory cells secrete a variety of pro-inflammatory molecules and growth factors that can stimulate tumor cell proliferation, survival, migration, apoptosis and angiogenesis (1). Notably, cytokines like interleukin (IL)-6 (2) and IL-23 (3) as well as factors acting downstream of the activation cascade of the transcription factor (TCF) nuclear factor-kappaB (NF-kB) have been implicated to play an important role in cancer development and dissemination (4).

Interestingly, NF-kB activation can result from components of gut microflora. Enteric microflora play a pivotal role in gut inflammation as demonstrated by the efficacy of antibiotics in ameliorating the severity of inflammation in inflammatory bowel disease patients (5). Furthermore, many animal models of colitis show that the intestinal inflammation and presence of invading inflammatory cells are abrogated when the animals are derived into germ-free (GF) environment, i.e. an environment devoid of microbiota (6).

The APCMin/+ mice carry a mutation in the tumor suppressor gene, adenomatous polyposis coli (APC) and demonstrate a consequent predisposition to multiple intestinal neoplasia (Min) (7). This mutation leads to aberrant activation of the Wnt/β-catenin signaling pathway in the colonic epithelium (8). This activation, however, requires additional signaling components that synergistically push the aberrant epithelial cell into accelerated cell growth. Two such cofactors have been described. While phosphorylation of the activator protein 1 (AP-1) TCF c-Jun can promote intestinal tumor growth in APCMin/+ mice through interaction with TCF-4 in a β-catenin-dependent fashion (9), phosphorylation of the TCF STAT3 has been connected to the increased tumor invasion and poorer prognosis of human colorectal adenocarcinoma (10).

In human colorectal cancers (CRCs) as well as in animal models of CRC, the compromised intestinal epithelial lining elicits severe inflammation frequently connected with bleeding and anemia. Common treatment regimes are to restore the levels of erythrocytes by use of erythropoietin (EPO). Although EPO is documented for its capacity to restore hemoglobin levels (11), its efficacy in correcting anemia in patients with advanced colorectal tumor growth is still controversial. This is in part due to the observation that EPO receptor expression is detectable in tumor tissues indicating that EPO-R expression is not restricted to erythrocytes alone.

Here we report that specific pathogen-free (SPF) APCMin/+ mice, when derived into GF conditions, display a drastic drop in colonic tumor incidence as well as reduced overall tumor load. In contrast, SPF APCMin/+ mice exhibit high tumor load, splenomegaly and anemia with a massive infiltration of inflammatory cells arising from a dysfunctional intestinal epithelial barrier at advanced ages. Moreover, colonic tumors from these mice display elevated levels of phosphorylated c-Jun and STAT3 (p-Tyr705), with infiltrating CD11b+ myeloid cells expressing nuclear p-STAT3 (Tyr-705).

Materials and methods

Animals and study design

The colony of C57BL/6J-APCMin/+ mice was maintained under SPF or (GF) environment at the Center For Gnotobiotic Research (CFGR), Karolinska Institutet. The studies were performed in agreement with Swedish ethical regulations. Murine EPO (BD Pharmingen) was administered for 7 days as daily intraperitoneal injections at the dose of 1000 U/kg. Control group of mice received injections of sterile phosphate-buffered saline (PBS). Mice were killed after either 1 day or 21 days following EPO treatment (Supplementary Figure S9A is available at Carcinogenesis Online). Sets of at least six age- and gender-matched mice were used in each single experiment with EPO treatment. For tumor counting, the whole intestine was fixed in PBS-buffered 4% formalin solution and cut longitudinally. Only clearly defined adenomas exceeding 0.8 mm, under dissection microscope, were counted as tumors.

Immunohistochemistry

Colonic tissue from APCMin/+ mice was fixed in 4% formaldehyde, cryopreserved in 20% sucrose and sectioned on a cryostat. In order to visualize p-STAT3 (Tyr-705) staining, sections were blocked in 10% donkey serum (Jackson Laboratory) in PBS containing 0.05% Tween20 for 45 min and then incubated overnight at 4°C using rabbit monoclonal p-STAT3 (Tyr-705) antibody (Cell Signaling) or control immunoglobulin G at 1:100 dilution. The slides were then washed in PBS containing 0.05% Tween20 and incubated with Alexa Fluor 594 anti-rabbit secondary antibody (Invitrogen). 4’-6-Diamidino-2-phenylindole was used for nuclear staining.

Western blot analysis

Samples for western blot were prepared as follows: intestine was washed in ice-cold PBS and then cut longitudinally. Adenomas were cut out, placed into lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X, 1 mM diethiothreitol) supplemented with complete ethylenediaminetetraacetic acid-free protease inhibitor (Roche) and phosphatase inhibitors (5 mM sodium fluoride and 0.4 mM sodium vanadate) before being disintegrated using sterile scissors. Intestinal mucosa was gently scraped using scalpel, homogenized and then lysed. Samples were analyzed using
sodium dodecyl sulfate gel electrophoresis and western blotting, using 50 μg of protein extracts per lane. Polyvinylidene difluoride membranes were incubated with anti-STAT3, anti-p-STAT3 (Ty-705) (Cell Signaling), anti-p-κB (Ser63/73) or anti-β-actin (Santa Cruz Biotechnology) antibodies and then incubated with peroxidase-conjugated secondary antibodies (Dako). To do assay STAT3 activation in APCMin+/C176 treated tumors, APCMin+/C176 mice with fecal occult blood were treated with curcumin (Sigma) by oral gavage for 5 days at a dose of 100 mg/kg. Control group of mice received sterile PBS. After harvesting, samples were processed for western blotting as outlined above.

**Real-time quantitative PCR**

Total RNA was extracted from purified splenic CD11b+ cells and mouse intestinal tissues using PicoPure RNA isolation kit (Arcturus) and RNeasy RNA isolation kit (Qiagen), respectively, according to manufacturers’ instructions. Complementary DNA synthesis was performed using Superscript II (Invitrogen). The following mouse primer sequences were used for real-time PCR quantification: tumor necrosis factor-α forward—5′-CAAATGGCCTCCTCCTCAT-3′ and reverse—5′-CTCTCCACCTTGCTGTTT-3′; IL-1β forward—5′-GCTGAAAGCTCTCCCCATCA-3′ and reverse—5′-GGCCACAGATTTTGTCGTG-3′; IL-23 (p19) forward—5′-GCACTTGTTGCTGATACA-3′ and reverse—5′-ATCTCTGCGTGAGGGATT-3′; Bcl-3 forward—5′-GAGTCCCTGAGCTGGCGGCTCCACATG-3′ and reverse—5′- AATATTCACTGCTGATCACTGCCCTCC-3′ and β-actin forward—5′-CTGATATCCCTCCTGCTG-3′ and reverse—5′-CCTGCAGCCCATGAGG-3′. Quantitative real-time PCR was performed in triplicates for each sample on an ABI 7500 Real-time PCR System (Applied Biosystems) using Power SYBR Green PCR Mastermix (Applied Biosystems). Data are presented as fold change of relative gene expression.

**Fluorescein isothiocyanate-dextran assay**

APCMin+/C176 (n = 5) and B6 mice (n = 6) were administered with 4.4 mg of fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight 4000; Sigma) in PBS via oral gavage. After 2 h and 40 min, blood samples were collected by cardiac puncture and measured for fluorescence using a FITC filter set in TECAN Infinite F200.

**Flow cytometry**

To remove erythrocytes, splenocyte suspension was lysed in erythrocyte lysis buffer, washed and counted. Bone marrow cells were prepared from femur and Tibia, washed and counted. Cells (0.5 x 10⁷) were used for each staining group. Cells were blocked with 10% mouse serum and then incubated with primary antibodies to GR1 (eBiosciences), Ter-119 (BD Pharmingen) and CD11b (AbD Serotec) molecules (diluted 1:100 in PBS/2% mouse serum) for 1 h. Thereafter, cells were washed and incubated with PBS/2% mouse serum containing streptavidin-Alexa-488 (Invitrogen) and anti-c-kit-PE (BD Pharmingen) for 15 min prior to fluorescence-activated cell sorting analysis.

**Purification of splenic macrophages**

Splenes of APCMin+/C176 mice were dissociated into single cell suspensions, passed through cell strainers (45 μm; Becton Dickenson) and counted. Thereafter, cells were incubated in ice-cold PBS containing CD11b-specific MicroBeads (Miltenyi Biotec GmbH) and 5 mM ethylenediaminetetraacetic acid to reduce cell aggregation. Incubation was performed for 30 min at 4°C. Cell purification was performed using macrophage separation columns according to producer’s recommendations. Fluorescence-activated cell sorting analysis of the CD11b+ cells was also performed after mass spectrometry separation columns column separation to check the purity of macrophages (Supplementary Figure S11 is available at Carcinogenesis Online). The purified CD11b+ cells were then cultured in RPMI-1640 medium supplemented with 1% fetal bovine serum and penicillin-streptomycin and stimulated with EPO (600 U/ml) for 1 h.

**Primary culture of colonic epithelial cells and colonic tumors**

Cols from wild-type mice were resected and placed in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 1% fetal bovine serum, L-glutamine and penicillin-streptomycin. After cutting the colon into short segments, the tissues were washed and digested at 37°C for 90 min with Dispase I (1 mg/ml) (Roche Diagnostics). The digested mucosa was then flushed several times with Dulbecco’s modified Eagle’s medium to release cells and crypts loosely attached after enzymatic digestion. Subsequently, the cells were plated in 24-well plates (~1.5 x 10⁷ cells per well) coated with a 1:1 solution of Dulbecco’s modified Eagle’s medium: Matrigel (Becton Dickenson) and allowed to settle for 30 min. Freshly isolated tumor specimens were minced with sterile scissors and processed as per the colonic epithelial cells. Viability of colonic epithelial and tumor cells was routinely >90%. Ex vivo cultures were stimulated with lipopolysaccharide (LPS) (5 μg/ml) or EPO (600 U/ml) for the indicated time.

In vitro stimulation assays

Mouse colonic carcinoma cell line CT26 was obtained from American Cell Type Culture Collection (ATCC-CRL-2638). Prior to stimulation, cells were starved on serum-free RPMI-1640 medium overnight. Thereafter, cells were stimulated with LPS (5 μg/ml) and/or murine EPO (100 U/ml) for the indicated time. Cell lysates were then prepared and analyzed using western blot.

**3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium proliferation assay**

CT26 cells were starved overnight in serum-free medium and then cultured in RPMI-1640 medium containing 0.1% fetal calf serum and the indicated treatments, in the presence and absence of c-Jun kinase inhibitor II (10 μg/ml) for 3 days. Proliferation was measured using AQeuousOne proliferation assay (Figure 1), demonstrating the pathogenic role of commensal bacteria in bone marrow and spleen (Supplementary Figure S1 is available at Carcinogenesis Online; Figure 3A). As gut microbiota have been suggested to perpetuate inflammation and thus tumor growth (12), we derived SPF APCMin+/C176 mice into GF conditions and monitored their tumor load. We found that GF APCMin+/C176 mice had a significant reduction (P < 0.05, Wilcoxon rank-sum test) in intestinal tumor load (Figure 1), demonstrating the pathogenic role of commensal bacteria in tumorigenesis. Moreover, in SPF APCMin+/C176 mice, the mucosal barrier damage resulting from outgrowth of colonic tumors into the intestinal lumen may facilitate translocation of gut microflora into the intestinal lamina propria, thus triggering inflammation and myeloid cell recruitment (Supplementary Figure S4 is available at Carcinogenesis Online). Interestingly, a MyD88-dependent mechanism for enhanced regenerative responses to epithelial injury, through activation of NF-kB has been proposed (13). We therefore backcrossed APCMin+/C176 mice with mice carrying a myeloid-specific deletion in IκB kinase β. Our data revealed a significant drop in the tumor load of SPF APCMin+/C176 mice lacking IκB kinase β in myeloid cells (Supplementary Figure S5 is available at Carcinogenesis Online), suggesting that a MyD88-dependent activation of NF-kB in myeloid cells, by signals from...
the gut microflora and injured epithelium, can contribute to accelerated colonic tumor growth in APCmin/+ mice.

**Colonic tumors display an inflammatory profile**

Subsequent histological analyses of intestinal tumors from APCmin/+ mice revealed that colonic tumors had compromised epithelial-lining integrity, as shown by the discontinuous epithelial lining (marked by arrows in Figure 2A; Supplementary Figure S2 is available at *Carcinogenesis* Online). Moreover, when we assessed mucosal barrier function via FITC-dextran assay, intestinal permeability in 18- to 25-week-old APCmin/+ mice was significantly increased ($P < 0.01$, Wilcoxon rank-sum test) as compared with age-matched B6 mice (Figure 2A, right plot). To assess the inflammatory status of intestinal tumors, we analyzed tumor and surrounding normal tissues for recruitment of myeloid cells as well as lymphocytes by immunohistochemistry. We found significantly elevated numbers of CD11b+ and GR1+ cells in colonic tumors than small intestinal tumors, but no significant differences between them in counts of infiltrating T and B lymphocytes (Supplementary Figures S3 and S4 are available at *Carcinogenesis* Online). NK cells were rarely detected in both tumor and mucosal tissues.

Complementary cytokine expression profiling (quantitative real-time--PCR) revealed significantly higher ($P < 0.05$, analysis of variance) messenger RNA (mRNA) levels of tumor necrosis factor $\alpha$, IL-1$\beta$ and IL-23 (p19) in colonic tumors of APCmin/+ mice, relative to small intestinal tumors as well as normal intestinal mucosa (Figure 2B; Supplementary Figure S6C is available at *Carcinogenesis* Online). Moreover, increased amounts of the biologically active form of IL-1$\beta$ were detected in colonic tumors, distinct from other tissues (Supplementary Figure S6A is available at *Carcinogenesis* Online).

In contrast, expression analysis of T lymphocyte-polarizing cytokines, IL-10 and IL-12A, showed no significant differences between colonic tumors and small intestinal tumors as well as normal mucosa (Supplementary Figure S6B is available at *Carcinogenesis* Online). These findings demonstrate a high inflammatory status in colonic tumors of APCmin/+ mice, marked by an enrichment of myeloid cells and increased production of inflammatory cytokines.

Chronic inflammatory circuits are intimately connected with cellular stress-signaling regulators such as the AP-1 and STAT TCFs. To assess whether chronic inflammatory signaling pathways were activated in APCmin/+ tumors, we looked for the potential activation of c-Jun and STAT3. Protein analysis of tumors from APCmin/+ mice, via densitometry analysis of western blots, revealed significantly higher levels of phosphorylated c-Jun in colonic tumors (Figure 2C). As AP-1 TCFs have been reported to interact with STAT3 and enhance its transactivation potential (14), we further probed whether EPO can modulate STAT3 phosphorylation in APC min/+ mice. For this purpose, we took advantage of EPO, a hormone known to stimulate proliferation of both myeloid and erythroid cells (17); 8- to 10-week-old APCmin/+ mice (without anemia) were injected with EPO or with PBS as a control (Supplementary Figure S9A is available at *Carcinogenesis* Online). After 21 days, animals were killed and analyzed for tumor load. When we scored for tumor load in the colon, a significant increase ($P < 0.05$, Wilcoxon rank-sum test) was observed in the EPO-treated group compared with the PBS-treated group (Figure 4). In contrast, no increase in the tumor load of small intestine was observed (Figure 4). Interestingly, when EPO was administered to GF APCmin/+ mice under similar conditions, no acceleration of tumors in the colon was observed (one of eight mice developed colonic tumors). Thus, exposure of APCmin/+ mice to EPO accelerates tumor growth in the colon in a microflora-dependent manner, without affecting tumors of the small intestine. The EPO-treated group also showed an increase in infiltrating CD11b+ myeloid cells in colonic tumor regions (Supplementary Figure S9B is available at *Carcinogenesis* Online) and increased numbers of mature (c-Kit+) CD11b+ cells in the splenic compartment of APCmin/+ mice (Supplementary Figure S9C is available at *Carcinogenesis* Online).

**EPO induces STAT3 phosphorylation in CD11b+ myeloid cells of APCmin/+ mice**

Having observed activated STAT3 in CD11b+ cells infiltrating colonic tumors from APCmin/+ mice and in view of earlier work depicting an EPO-induced STAT3 phosphorylation in erythroid cells (18), we further probed whether EPO can modulate STAT3 phosphorylation levels in colonic tumors of APCmin/+ mice. Colonic tumors isolated from APCmin/+ mice were cultured ex vivo and exposed to EPO, in the presence or absence of LPS. After 2 h, extracts were prepared and subjected to western blot analysis of phosphorylated STAT3. Intriguingly, ex vivo cultured colonic tumors showed increased p-STAT3 (Tyr-705) following EPO exposure (Figure 5A, lane 2). In contrast, this induction was absent in small intestinal tumors (Figure 5B, lane 5). Having observed elevated levels of activated STAT3 in CD11b+ myeloid cells infiltrating colonic tumors, we next examined whether EPO could influence STAT3 activation in these cells. Anemic APCmin/+ mice display a systemic activation of the immune system, including high numbers of CD11b+ cells in the spleen (Figure 3B). Due to limitations in obtaining sufficient numbers of CD11b+ cells from the tumor directly, we purified CD11b+ cells from the spleen and exposed them to EPO ex vivo. As shown in Figure 5Bii, we detected an increase in STAT3-Tyr705 phosphorylation in these cells following 1 h of EPO exposure. Relative RNA analysis of splenic CD11b+ cells that were stimulated with EPO for a day further showed an increased expression of two known STAT3 target genes, Bcl-3 and IL-23 (Figure 5Bii) (19,20). These findings implicate the potential role of CD11b+ myeloid cells and activation of the JAK/STAT3 signaling pathway, mediated via anemia-driven signals, in the acceleration of colon tumorogenesis observed in EPO-treated APCmin/+ mice.

To validate the role of STAT3 activation in colon tumorigenesis, we treated fecal occult blood-positive APCmin/+ mice with curcumin, a known STAT3 inhibitor (21). As curcumin has been demonstrated recently to decrease intestinal inflammation and tumor load in APCmin/+ mice (22), we sought to establish if this effect in tumorigenesis is linked to STAT3 activation. Remarkably, we find that after 5 days of curcumin feeding, there was a significant reduction in
Permeability was assessed via FITC-dextran assay (right plot). Concentration colon (right column) at higher magnification. Intestinal gut barrier represents cross-section of the adenomas in small intestine (left column) and were fixed, sectioned and stained with hematoxylin and eosin. Picture 4

Colonic but not small intestinal tumors display signs of inflammation. (Fig. 2.) Gut samples from 18- to 25-week-old SPF APC Min/þ mice were collected from anemic, colon-tumor bearing APC Min/þ mice and exposed to LPS, EPO or LPS and for 24 h ex vivo. Conditioned media were collected and incubated with CT26 cells. As a control, CT26 cells were directly exposed to EPO or LPS. We found that conditioned medium from LPS-stimulated macrophages induced c-Jun phosphorylation in the epithelial cells (Fig. 6A). Similarly, when primary colonic epithelial cells from GF B6 mice were exposed to LPS, an increase in c-Jun phosphorylation was observed (Fig. 6C).

As c-Jun/JNK signaling has previously been reported to promote cell growth (23), we thus checked whether the increased c-Jun phosphorylation affected epithelial cell growth. To examine this, we measured the proliferation of CT26 cells following exposure to conditioned media from LPS, EPO or LPS and LPS stimulated myeloid cells, in the presence and absence of a c-Jun kinase inhibitor. As shown in Figure 6B, conditioned medium from LPS-stimulated CD11b+ cells accelerated CT26 cell proliferation, with an additive effect observed when cells were incubated with conditioned medium from LPS- and EPO-stimulated macrophages. This proliferative effect was diminished in the presence of JNK inhibitor II (JNKi), indicating that the promotion of cell growth was dependent on c-Jun phosphorylation (Figure 6B). As a negative control and to eliminate the possibility that the growth inhibition observed with JNKi was due to cytotoxic effects of the reagent, we measured cell viability via Trypan blue cell counting and did not find a significant reduction in cell viability with the use of JNKi (Supplementary Figure S10 is available at Carcinogenesis Online).

Our data thus demonstrate that LPS-induced c-Jun phosphorylation and conditioned media from EPO stimulated myeloid cells can accelerate epithelial cell growth.
Gut microbiota accelerate tumor growth

Discussion

Here we identify two pathways, the c-Jun/JNK and STAT3 signaling pathways, which are triggered by microflora-originating signals and anemia, respectively, that act synergistically to enhance colonic tumor growth in APCMin/+ mice. Our data suggests that microflora invasion into the lamina propria, following tumor-mediated disruption of the intestinal epithelial lining, may be more critical than previously perceived in initiating a ‘vicious cycle’ of anemia and inflammation leading to enhanced tumor growth through myeloid STAT3-dependent signals.
Gut microbiota has previously been suggested to be associated with regenerative epithelial cell growth as well as with tumor growth (4, 24–26). This is further supported by the importance of a MyD88-dependent activation of NF-κB in myeloid cells for tumorigenesis in APCMin/+ mice (12). Herein, we provide two lines of evidence that reinforce the critical role of gut microflora and myeloid-dependent inflammation in accelerating colonic tumor growth, namely that (i) GF APCMin/+ mice are virtually devoid of colonic tumors (Figure 1) and (ii) macrophage-specific deletion of IκB kinase β abrogates colonic tumors in SPF APCMin/+ mice (Supplementary Figure S5 is available at Carcinogenesis Online).

The analysis of GF APCMin/+ mice has previously been investigated by Dove et al. (27). Although our tumor counts for GF APCMin/+ mice are similar to their study, we find that contrary to their conclusions whereby no significant difference in total intestinal tumor load between GF mice and conventional controls was found and thus independence of tumorigenesis from microbial status, our SPF APCMin/+ mice developed significantly more intestinal tumors. These distinct observations highlight the potential impact of different animal housing conditions and environment on microflora composition and diversity in the APCMin/+ mice and thus tumor load. This is an interesting proposition that needs further investigation that is not within the scope of our present study.

In our characterization of the intestinal tumors of SPF APCMin/+ mice, we observe a distinct inflammatory status in colonic tumors, relative to small intestinal tumors and normal mucosa tissues. Notably, we detect elevated mRNA levels of IL-23, a pro-inflammatory cytokine which has been reported to be an important factor in tumorigenesis and is induced by enterotoxins from Bacteroides fragilis (26), in our APCMin/+ colonic tumors. Moreover, our ex vivo macrophage stimulation assays show an induction of IL-23 mRNA expression following exposure to LPS and EPO, which correlates with increased STAT3 phosphorylation (Figure S5Bii). These findings thus lead us to speculate that the innate immune MyD88-dependent NF-κB signaling pathway (triggered by gut microbiota components), as well as anemia-mediated signals, can induce IL-23 in myeloid cells through activation of STAT3.

A major inflammation-related culprit of pathogenesis in APCMin/+ mice with advanced intestinal and particularly, colonic tumors is intestinal bleeding, which induces anemia and concomitant erythropoiesis (7). EPO is a hormone that is known to stimulate erythropoiesis and activate myeloid cells (17) and in an attempt to mimic the anemic signaling pathway in non-anemic APCMin/+ mice, we treated them with EPO. Our findings show that administration of EPO in vivo accelerates colonic tumor growth in non-anemic APCMin/+ mice (Figure 4). In contrast, the lack of EPO effect on the tumor load of GF APCMin/+ mice suggests that anemia-driven factors interact with signals from the gut microbiome to accelerate tumor growth. In our ex vivo culture assays using APCMin/+ colonic tumors and purified CD11b+ myeloid cells, an increased phosphorylation of STAT3 with EPO exposure in both colonic tumors and myeloid cells was detected (Figure 5A and B). Additional analysis of these cells revealed an increased expression of two STAT3 responsive genes, Bcl-3 and IL-23 (Figure S5Bii). Interestingly, Bcl-3 and IL-23 have been linked to apoptotic resistance and inflammation, respectively, trademarks of tumor promotion (3, 28). Notably, oral administration of APCMin/+ mice with curcumin, a chemical known to reduce intestinal tumorigenesis in these mice, decreased STAT3 phosphorylation in colonic tumors, thus linking STAT3 activation with colonic tumor growth. Hence, while our results are in line with previous work on myeloid cells and tumor progression (29, 30), they also highlight the role of anemia in promoting tumor growth via the JAK/STAT3 signaling pathway.

Earlier work by several groups have identified elevated levels of tyrosine-phosphorylated STAT3 as a negative prognostic factor for various cancers including CRC (10), propelling STAT3 as a major molecular bridge between chronic inflammation and tumorigenesis. However, while STAT3 has been shown to have pro-proliferative effects in the initial stages of tumor growth, its expression in intestinal epithelial cells has been suggested in a recent study to inhibit tumor progression at later stages (31). In another recent study, the genetic ablation of STAT3 in macrophages (and also partially in other hematopoietic cells) was linked to the development of colonic tumors (32). However, the tumor incidence reported was extremely low and the genetic ablation affected various hematopoietic cell lineages, which may cause defects in hematopoietic differentiation that are cancer promoting. Thus, the correlation of STAT3 activation with respect to the prognosis of cancers needs to be interpreted with caution. Intriguingly, the presence of high levels of nuclear p-STAT3 (Tyr-705) that we detect in the myeloid cells associated with APCMin/+ colonic tumors (Figure 2E; Supplementary Figure S7 is available at Carcinogenesis Online), as well as the EPO effects that we observe, suggest the ability of STAT3 to cooperate with NF-κB in myeloid cells to promote colonic tumor growth in these mice. Our findings are also in line with previous reports demonstrating alternative roles for EPO besides erythropoiesis, such as regeneration of myocardia in ischemic tissue and EPO-induced STAT3 activation in myeloid cells (33).

There appears to be a discrepancy in EPO effect on tumor cell lines and in vivo cancer models whereby EPO did not exert proliferative effects in vitro but promotes tumor growth and...
angio genesis in mouse tumor models (34,35). This is probably due to EPO exerting its tumor-promoting effects on bystander cells in the tumor microenvironment, as suggested by our data. While EPO initially was used as a vehicle to mimic anemia-induced signaling pathways in APCMin/+ mice, our data disclose a potentially harmful effect of EPO. This raises some concerns about the use of EPO under anemic conditions in patients with high tumor load. Indeed, the Breast Cancer Erythropoietin Trial (BEST) (36) and the Amgen 20010103 clinical trial (Amgen 103) (37), both reported that EPO treatment was associated with a higher mortality rate. Our data may in part explain some of these unwanted effects.

While tumorigenesis in APCMin/+ mice is known to depend on an active Wnt/β-catenin pathway, additional tumor-promoting signals have been reported (25,38,39). One such accessory signal is the phosphorylation and activation of c-Jun a member of the AP-1 transcription complex (9). Here we show that c-Jun phosphorylation in epithelial cells can be induced by LPS alone or in combination with factors secreted by CD11b+ myeloid cells and stimulate cell proliferation in a JNK-dependent manner (Figure 6A–C). Thus, our results support an AP-1 driven tumor-promoting mechanism, acting in addition and distinct from the Wnt/β-catenin pathway (40).

Major health problems such as cardiovascular diseases (41), Crohn’s disease (42) and various cancers (43–45) have recently been reported to have host-microbe connections. Yet we are only beginning to understand the molecular mechanisms underlying the link between microbes, chronic inflammation and disease development. Our data should therefore be viewed as an initial attempt to mechanistically explain how gut microbiota can fuel a vicious cycle of epithelial lining disruption that accelerates inflammation and anemia and ultimately tumor growth (Figure 6D). Further studies utilizing STAT3 and c-Jun mediated signaling pathways in myeloid and tumor cells, respectively, in other inflammation driven tumor models outside the intestinal tract is therefore highly warranted.

Supplementary material

Supplementary Figures 1–11 can be found at http://carcin.oxfordjournals.org/

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References


