LETTER

Adenoma–linked barrier defects and microbial products drive IL-23/IL-17–mediated tumour growth

Sergei I. Grivennikov1,2a, Keping Wang1,2a, Daniel Mucaj3,4, C. Andrew Stewart5, Bernd Schnabl6, Dominik Jauch1, Koji Taniguchi1,2, Guann-Yi Yu1, Christoph H. Oestreicher6,8, Kenneth E. Hung9, Christian Datz10, Ying Feng11, Eric R. Fearon11, Mohamed Oukka12, Lino Tessarollo11, Vincenzo Coppola14, Felix Yarovinsky15, Hilde Cheroutre3, Lars Eckmann6, Giorgio Trinchieri5 & Michael Karin1

Approximately 2% of colorectal cancer is linked to pre-existing inflammation known as colitis-associated cancer, but most develops in patients without underlying inflammatory bowel disease. Colorectal cancer often follows a genetic pathway whereby loss of the adenomatous polyposis coli (APC) tumour suppressor and activation of β-catenin are followed by mutations in K-Ras, PIK3CA and TP53, as the tumour emerges and progresses1–5. Curiously, however, ‘inflammatory signature’ genes characteristic of colitis-associated cancer are also upregulated in colorectal cancer1–4. Further, like most solid tumours, colorectal cancer exhibits immune/inflammatory infiltrates, referred to as ‘tumour-elicited inflammation’. Although infiltrating CD4+ T cells and CD8+ T-cytotoxic T cells constitute a positive prognostic sign in colorectal cancer2,5,6, myeloid cells and T-helper interleukin (IL)-17-producing cells are also detected in colorectal cancer6–8. Tumour infiltrating CD11b–CD11c– myeloid cells and T-helper interleukin (IL)-17-producing cells (Th17) cells promote tumorigenesis5,6, and a ‘Th17 expression signature’ in stage I/II colorectal cancer is associated with a drastic decrease in disease-free survival5. Despite its pathogenic importance, the mechanisms responsible for the appearance of tumour-elicited inflammation are poorly understood. Many epithelial cancers develop proximally to microbial communities, which are physically separated from immune cells by an epithelial barrier9. We investigated mechanisms responsible for tumour-elicited inflammation in a mouse model of colorectal tumorigenesis, which, like human colorectal cancer, exhibits upregulation of IL-23 and IL-17. Here we show that IL-23 signalling promotes tumour growth and progression, and development of a tumoral IL-17 response. IL-23 is mainly produced by tumour-associated myeloid cells that are likely to be activated by microbial products, which penetrate the tumours but not adjacent tissue. Both early and late colorectal neoplasms exhibit defective expression of several barrier proteins. We propose that barrier deterioration induced by colorectal-cancer-initiating genetic lesions results in adenoma invasion by microbial products that trigger tumour-elicited inflammation, which in turn drives tumour growth.

Specimens of human colorectal cancer (CRC) show increased expression of IL-23p19 and IL-17A messenger RNA (mRNAs) (Fig. 1a). IL-23p19 is the specific subunit of IL-23, a positive regulator of Th17 and other IL-17-producing cells10, previously found to promote skin carcinogenesis11. To address the role of these cytokines in CRC-related tumour-elicited inflammation and tumour growth, we used ApcFwt mice that harboured a Cdx2-Cre transgene (CPC-APC mice) in which colorectal tumorigenesis was driven by Apc allelic loss12. Unlike ApcBlin mice, CPC-APC mice develop tumours primarily in the distal colon, providing a relevant model of human CRC13. Mouse colon tumours also exhibited marked upregulation of IL-23 and IL-17A mRNA and IL-23 protein relative to matched non-tumour colon (Fig. 1b, c). IL-23 induction was specific, as tumoural expression of other IL-12/23 family members was not substantially elevated and IL-23 itself was already seen in early tumours (Supplementary Fig. 1a, b). IL-23 expression did not further increase in more advanced mouse CRC caused by Apc loss and forced K-Ras or B-Raf activation (Supplementary Fig. 1c). Analysis of I23–/– mice harbouring green fluorescent protein (GFP) gene in the I23p19 locus, staining with IL-23p19 antibody and flow cytometry showed that IL-23p19 is particularly expressed in tumour-infiltrating cells such as CD11b+ and F4/80+ myeloid cells (Supplementary Fig. 1d–f). Both IL-23 subunits, p19 and p40, need to be simultaneously expressed to generate a functional cytokine. Analysis by quantitative PCR with reverse transcription (RT–qPCR) and fluorescence-activated cell sorting (FACS) of tumoural cell populations showed that CD11b+ cells expressed substantial amounts of both p19 and p40 (Fig. 1d). Th17- and other IL-17-producing cells were detected in mesenteric lymph nodes and tumours of CPC-APC mice and demonstrated dependence on IL-23 signalling (Fig. 1e). Thus, expression of IL-23 and its downstream target IL-17A is increased during spontaneous colorectal tumorigenesis.

Colorectal tumour multiplicity and growth were diminished upon ablation of IL-23 or IL-23R in CPC-APC mice (Fig. 1f, g and Supplementary Fig. 2a). Although intra-tumoural apoptosis was unaffected, cancer cell proliferation was reduced in I23–/–/CPC-APC mice (Supplementary Fig. 2b, c). Epithelial STAT3 phosphorylation was decreased without IL-23, but genetically driven nuclear β-catenin accumulation was unaffected (Fig. 1g and Supplementary Fig. 2d). IL-23 signalling is important for intra-tumoural production of downstream cytokines, which are either direct (IL-6, IL-22) or indirect (IL-17A) STAT3 activators (Fig. 1h). Pro-tumorigenic IL-23 signalling was particularly confined to the haematoipoietic compartment, as CPC-APC chimaeras harbouring I23–/– or I23p19–/– bone marrow also exhibited reduced tumour load (Supplementary Fig. 3a–d) and diminished expression of IL-23-dependent cytokines (Supplementary Fig. 3e). To examine the role of IL-17 signalling in CRC tumorigenesis,
Figure 1 | IL-23 controls CRC inflammation and tumorigenesis. a, b, RT-qPCR for IL-23p19 and IL-17A mRNAs from colorectal tumours (T) and matching normal (N) colon of (a) human CRC patients (n = 7, P = 0.037 for IL-23p19) or (b) CPC-APC mice (n = 8, P = 5 × 10^{-4}, 3.6 × 10^{-4}, respectively). c, IL-23 protein was measured by ELISA in supernatants of cultured tumour populations: CD11b+ (n = 5, P = 0.04). d, RT-qPCR analysis of sorted haematopoietic myeloid cells (CD45+ TCRβ+ CD11b+) from tumours of CPC-APC mice (n = 4; pooled); populations: CD11b+ = Gr1+ F4/80+; Gr1+ = F4/80+; Gr1high = F4/80+; F4/80+ = Gr1high F4/80+ and T cells were deceased upon TLR/MyD88 inactivation in the haematopoietic compartment (Fig. 2a). Intratumoural expression of IL-6 and IL-17A was also dependent on MyD88 in bone-marrow-derived cells (Fig. 2b). Transplantation with Myd88Δ/Δ or TLR2,4,9Δ/Δ bone marrow reduced colorectal tumour growth (Fig. 2c and Supplementary Fig. 5). By contrast, deletion of IL-1R in bone marrow had no effect on tumorigenesis or IL-23 production by TAM (Supplementary Fig. 6a, b). Short-term depletion of intestinal microflora with a combination of broad-spectrum antibiotics, which reduced microbial counts by over 99.9%, also inhibited IL-23 expression by TAM, resulting in reduced tumoural IL-17A and decreased STAT3 activation in cancer cells (Fig. 2d–f). Prolonged depletion of commensal microflora by antibiotic treatment.

we crossed CPC-APC mice with Il7raΔ/Δ mice, which do not respond to either IL-17A or IL-17F. Both tumour multiplicity and growth were reduced in the absence of IL-17RA (Supplementary Fig. 4a–c).

We examined the cause of IL-23 upregulation in CRC. The commensal microflora regulates basal colonic IL-23 expression in naive mice. Microbial products are sensed by Toll-like receptors (TLRs), which rely on the adaptors MyD88 and TRIF. Similar to its effect on small intestinal polyps in Apcmin mice, whole body MyD88 ablation reduced tumour multiplicity and growth in CPC-APC mice (data not shown). We transplanted Myd88Δ/Δ, Tlr2,4,9Δ/Δ triple knockout or control bone marrow into lethally irradiated CPC-APC mice. Expression of IL-23p19 mRNA by sorted tumoural myeloid cells (CD45+ TCRβ+) was decreased upon TLR/MyD88 inactivation in the haematopoietic compartment (Fig. 2a). Intratumoural expression of IL-6 and IL-17A was also dependent on MyD88 in bone-marrow-derived cells (Fig. 2b). Transplantation with Myd88Δ/Δ or Tlr2,4,9Δ/Δ bone marrow reduced colorectal tumour growth (Fig. 2c and Supplementary Fig. 5). By contrast, deletion of IL-1R in bone marrow had no effect on tumorigenesis or IL-23 production by TAM (Supplementary Fig. 6a, b). Short-term depletion of intestinal microflora with a combination of broad-spectrum antibiotics, which reduced microbial counts by over 99.9%, also inhibited IL-23 expression by TAM, resulting in reduced tumoural IL-17A and decreased STAT3 activation in cancer cells (Fig. 2d–f). Prolonged depletion of commensal microflora by antibiotic treatment.
**Figure 2 | TLR-MyD88 signalling and commensal microflora promote cytokine expression and tumorigenesis.** a–c, CPC-APC mice were transplanted with Myd88+/+, Tlr2,4,9−/− or control bone marrow (BM) and analysed 4 months later by RT–qPCR for cytokine mRNAs in sorted tumour myeloid cells (Live/Dead− TCRβ+ CD11b+) , representative of two independent experiments, each including four pooled mice (a) or in tumours and normal tissues (n = 5, P = 0.004, 0.032) (b). c, Tumour number, size and load in mice transplanted with indicated bone marrow (n = 5, P = 0.14, 0.048, 0.046, respectively). BMT, bone marrow transfer. d–f, CPC-APC mice were treated with a cocktail of antibiotics (Abx) for 3 weeks. d, Myeloid cells were sorted from tumours and analysed by RT–qPCR, representative of two independent experiments, each including four pooled mice. e, IL-17A mRNA expression in normal and tumour tissues from the control and Abx-treated CPC-APC mice (n = 9, P = 0.003). f, Colon sections from mice were stained with phospho-STAT3 antibody, and intensity of staining was quantified (n = 8, P = 0.049). g, WT (Il23r−/−) or Il23r−/− CPC-APC mice were treated with antibiotics for 3.5 months and tumour size and load were determined (n = 5, P = 0.027 and 0.043). Data represent averages ± s.e.m. *P < 0.05. Scale bars, 50 μm.
and IL-17A in adenoma tissue (Supplementary Fig. 9a). Early CRC lesions, such as aberrant crypt foci, are marked by enhanced expression and nuclear translocation of β-catenin, and such areas were devoid of WGA and JAM-A staining (Fig. 4e). To demonstrate further that barrier loss is an early and direct consequence of tumour emergence, we studied rapid colonic transmigration in Cdx2ERT-Cre × ApcMin mice using tamoxifen to induce bi-allelic APC inactivation. APC loss resulted in patches of proliferating transformed cells with enlarged nuclei, which coincided with loss of mucin and induction of IL-23 (Fig. 4f and Supplementary Fig. 9b).

Nearly all solid malignancies contain inflammatory infiltrates, influencing tumour promotion, progression and metastasis9,6. The contribution of inflammation to CRC pathogenesis is emphasized by the protective effect of non-steroidal anti-inflammatory drugs, such as aspirin19, but sources of tumour-elicited inflammation in CRC were heretofore unknown. We now suggest that early CRC-inducing genetic events may cause local loss of barrier function and entry of microbial products into the tumour microenvironment. This results in activation of IL-23-producing myeloid cells, which regulate expression of downstream tumour-promoting cytokines, including IL-17 and IL-6. A similar mechanism may apply to other cancers that develop in epithelia that are exposed to commensal and pathogenic bacteria. Deregression of tight junctions and cell–cell contacts was described in advanced human adenocarcinomas, but suggested to cause detachment of carcinoma cells from their neighbours, increased motility and metastasis20. The importance of the mucus layer is emphasized by whole body Muc2 deficiency, which causes colonic inflammation17,18. Prolonged treatment with dextran sulphate sodium, which promotes development of colitis-associated cancer in wild type (WT) or ApcMin mice, also causes erosion of the intestinal epithelial barrier and microbial-dependent inflammation21,22, and so do the barrier-disrupting pathogens Citrobacter rodentium23 and enterotoxic Bacteroides fragilis24.
affected by distinct microflora components. Segmented filamentous bacteria persisting in proximity to epithelial cells initiate T\textsubscript{H}17 responses in the small intestine\textsuperscript{29}. In tumours lacking protective barrier, other microbial species and products may invade the tumour and influence the ‘IL-23/T\textsubscript{H}17’ signature.

Loss of APC and activation of β-catenin induces a proliferative state and blocks the differentiation of certain IEC lineages, including mucin-producing goblet cells\textsuperscript{30}. Epithelial barrier loss may therefore be a consequence of β-catenin activation and/or of APC loss, which apart from β-catenin can also control cytoskeleton dynamics. Hence, early barrier loss and activation of IL-23/IL-17-driven tumour-elicited inflammation act additively and sequentially to genetically controlled events that govern CRC development and progression (Supplementary Fig. 10). We propose that screening of early tumours for IL-17 expression and adjuvant treatment of patients showing a ‘T\textsubscript{H}17 signature’ with IL-23 or IL-17 antagonists should reduce mortality due to CRC.

**METHODS SUMMARY**

Mice were backcrossed to the C57BL/6 background for at least seven (CPC-APC) or ten (others) generations and maintained in filter-topped cages. CPC-APC mice were surgically clipped and FITC-dextran or Alexa488-LPS were venously injected with 10\textsuperscript{7} nucleated bone marrow cells from various donors to generate radiation chimaeras. Endotoxin was measured in portal vein blood/plasma by a Limulus assay. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349 (2008).


**Full Methods** and any associated references are available in the online version of the paper.

Received 8 February; accepted 31 July 2012.

**Published online 3 October 2012.**


**Supplementary Information** is available in the online version of the paper.

**Acknowledgements** We thank eBioscience, GeneTex, Santa Cruz, BioLegend and Cell Signaling for antibodies; Genentech and Amgen for il23 \textsuperscript{+} and il17a \textsuperscript{−}, mice, respectively, and S. Reid and E. Southon for the help in generating Il23 \textsuperscript{−} mice. This work was supported by Crohn's and Colitis Foundation of America (Career Development Award number 2693), NIH/National Institute of Diabetes and Digestive and Kidney Diseases (K99-DK088589) and a University of California, San Diego, Digestive Disease Research Development Center Pilot Grant (DK08506) to S.G.; Croucher Foundation and China Postdoctoral Science Foundation (20110409019) to K.W.; Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation to K.T.; SPAR Austria to C.D.; NIH (R01CA082253) to E.R.F.; and NIH (A043477; DK035108) and American Association for Cancer Research (07-60-21-KARI) grants to M.K., who is an American Cancer Society Research Professor. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

**Author Contributions** S.G. and M.K. conceived the project. S.I.G., K.W., D.M., B.S., D.J., K.T., M.K., J.C., Y.F. and K.E.H. performed the experiments. S.I.G., K.W., D.M., D.J., H.C., L.E. and M.K. analysed data. C.A.S., V.C., L.T. and G.T. generated Il23 \textsuperscript{−} mice. M.O. and F.Y. provided Il23 \textsuperscript{−} mice and Tg2,4.9+/- bone marrow, respectively, and Y.F. and E.R.F. provided CPC-APC mice and tissues from Cdx2 \textsuperscript{−}APC mice. C.A.S., E.R.F., H.C. and G.T. provided conceptual advice. C.D. collected and provided human specimens. S.I.G., K.W. and M.K. wrote the manuscript, with all authors contributing to the writing and providing advice.

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METHODS

Human samples. Paraffin-embedded human ulcerative colitis and CRC specimens were provided by Oberdorf Hospital, Paracelsus Medical University Salzburg, Austria. Patients with ulcerative colitis or sporadic CRC underwent colonoscopy as a part of the diagnostic workup at the Department of Internal Medicine, Oberdorf Hospital (Salzburg, Austria). Diagnosis of ulcerative colitis was based on appropriate clinical, endoscopic, histopathological and radiological findings that satisfied the internationally accepted Lennard-Jones criteria. Additionally, colonoscopic findings were classified as tubular adenoma, size less than 6 mm (early lesion), advanced adenoma (that is, villous or tubulovillous features), size at least 1 cm or high-grade dysplasia or carcinoma after a combined analysis of macroscopic and histological results. Biopsies from colonic tissue with macroscopically normal appearance were taken in patients who underwent colonoscopy for CRC screening. For histological examination, specimens were fixed in 4% buffered formalin and embedded into paraffin. When available, a small tissue portion of the biopsy specimen was separated and preserved for RNA and protein extraction in RNAlater (Ambion’s RNAlater solution, Applera, Brunn am Gebirge, Austria). Biopsies from colonic tissue with macroscopically normal appearance were taken in patients who underwent colonoscopy for CRC screening. Written informed consent was obtained from all study participants to use one biopsy specimen for scientific purposes. The study was approved by the local ethics commission of the University of Salzburg, Austria (Ethikommission des Landes Salzburg, approval number 415-E/1262/2-2010). Fresh human CRC and normal colon tissue were obtained from Cooperative Human Tissue Network of Vanderbilt University Medical Center.

Generation of Il23r–/– and Il23r+/+ mice. The targeted region includes exons 2 (3,708–3,810) and 3 (8,085–8,381) of Il23r that encode the portion of the signal peptide and the amino (N)-terminal Ig-like domain (Supplementary Fig. 11a). The targeting vector was generated by recombining using a phage Red as described by Liu et al. Homology arms for the retrieval vector were amplified by PCR on C57BL/6 genomic DNA. Homology arms were cloned into pLMJ235 (pBSKS+ targeting vector. First, LoxP-Neo-LoxP was inserted at position 3,524, followed by bacterial rRNA was analysed by RT–qPCR. Mice were kept on antibiotics for the indicated time and killed for tumour and tissue analysis.

Portal blood vein drainage and endotoxin measurement. Mice were anaesthetized with isoflurane and their portal vein exposed to insert a 28-gauge needle and draw 100–200 μl of blood, which was collected into EDTA coated tubes (BD Biosciences). Plasma was isolated by centrifugation and endotoxin amounts were determined by microplate Limulus Amebocyte Lysate (LAL) colorimetric bioassay (Lonza) according to the manufacturer’s recommendations.

Bone marrow transplantation. Six- to eight-week-old littermate recipient mice were irradiated twice during one day to achieve a lethal dose (2 × 600 rad) and intravenously injected with single-cell suspension containing 107 donor bone marrow cells. Every cage of recipient mice contained receiving both gene-deficient and wild-type bone marrow to compare mice living in the same cage. For 2 weeks after irradiation, mice were placed on sulphanemthoxazole and trimethoprim antibiotics in drinking water following transfer to the cages from biological containment. Mice were intravenously injected with single-cell suspension containing 107 donor bone marrow cells to the distal colon, which resulted in solitary colonic tumours, as described. Subsequent tumour formation was monitored by serial optical colonoscopy. Tumours were collected after more than 75% occlusion of the colonic lumen was observed and banked in RNA later for subsequent analysis.

Colonics loops and injections of FITC-dextran and Alexa488-LPS. After anaesthesia, a midline laparotomy incision was made. A 2–3 cm long segment of the colon with or without tumours inside was created with two vascular haemoclips without disrupting the mesenteric vascular arcades. The length of intestine between the two clips was injected with 50 μl of 100 mg ml−1 FITC-dextran solution or 50 μl of Alexa488-LPS at 70 μg per mouse. At the indicated time points, mice were killed and fluorescence was measured in the plasma with a fluorimeter, or colon fragments were embedded into OCT compound, and frozen sections prepared and analysed for Alexa488-LPS tissue distribution by fluorescent microscopy.

Antibiotic treatment. Mice were given the following antibiotics in their drinking water: 100 μg ml−1 neomycin, 50 μg ml−1 vancomycin, 50 μg ml−1 imipenem, 100 μg ml−1 metronidazole, 50 μg ml−1 streptomycin and 100 U ml−1 penicillin. Fresh antibiotics were supplied every week. For long-term antibiotic treatment after 2 months of initial treatment, drinking water was further supplemented with streptomycin (1 mg ml−1), gentamycin (170 μg ml−1), ciprofloxacin (125 μg ml−1) and bacitracin 1 mg ml−1 as previously described. Control mice were placed on bottled water. Faeces were collected and cultured on 5% sheep blood agar plates (Fisher Scientific) under aerobic or anaerobic conditions at 37 °C to determine the extent of colon sterilization, which exceeded 99.9%. Alternatively, DNA was isolated from stools of mice using QIAamp DNA Stool mini kit (Qiagen) and content of 16S bacterial rRNA was analysed by RT–qPCR. Mice were kept on antibiotics for the indicated time and killed for tumour and tissue analysis.

Antibodies and stains. Fluorescent-labelled antibodies for flow cytometry were from eBioscience. Immuno blot analysis and immunohistochemistry were performed with antibodies to Ki-67, claudin7, claudin 5, claudin 4, claudin 3, JAM-B, mucin2, GEP, IL-23p19 (Genetex), JAM-A (Santa Cruz), active caspase 3, phospho-STAT3 (Cell Signaling), F4/80 and E-cadherin (BD Biosciences). Plasma was isolated by centrifugation and endotoxin amounts were determined by microplate Limulus Amebocyte Lysate (LAL) colorimetric bioassay (Lonza) according to the manufacturer’s recommendations.

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**Bacteria fluorescent in situ hybridization.** Paraffin embedded slides were deparaffinized and hybridized to universal eubacterial or control probes labelled with Alexa594. Hybridization was performed in 48 °C oven for 2 h followed by washing and counter-stained with DAPI. The sequences of the probes are listed below: Eubacteria: GCTGCCTCCCGTAGGAGT; control: CGACGGAGGGCATCCTCA.

**Flow cytometry and cell sorting.** Isolated cells were stained with labelled antibodies in PBS with 2% FCS and analysed on a BD LSRII or on an Accuri C6 flow cytometer. Dead cells were excluded on the basis of staining with Live/Dead Fixable Aqua dye (Invitrogen). For intracellular cytokine staining cell were restimulated in the presence of Brefeldin A as indicated, fixed and permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) according to the manufacturer’s recommendations and stained with labelled antibody for the cytokine of interest. For cell sorting, a BD FACS Aria II cell sorter was used. Data were analysed using FlowJo software (Treestar).

**ELISA.** Pieces (20–40 mg) of normal or tumour tissue were cultured in 0.5 ml RPMI medium supplemented with antibiotics and 2% FCS at 37 °C for 24 h. Supernatants were collected, centrifuged and concentration of IL-23 protein was determined using ELISA Quantikine kit (R&D Systems) according to the manufacturer’s recommendations.

**RT–qPCR analysis.** Total RNA was extracted using RNeasy Plus kit to eliminate genomic DNA contamination (Qiagen). RNA was reverse transcribed using a lScript kit (Biorad). RT–qPCR was performed using EvaGreen PCR mix (Biorad) on a Biorad CFX96 machine. Expression data were normalized to L32 mRNA expression. The data are presented in arbitrary units and were calculated as $2^{\Delta\Delta Ct}$ (gene of interest). Primer sequences are listed in Supplementary Table 1 and generally were obtained from the NIH qPrimerDepot for mouse (http://mouseprimerdepot.nci.nih.gov) and human (http://primerdepot.nci.nih.gov). Whenever possible, primers were intron-spanning, such that amplification was only feasible on complementary DNA.

**Statistical analysis.** Data are presented as averages ± s.e.m. and were analysed by built-in $t$-test using Microsoft Excel software. $P$ values less than 0.05 were considered significant.