IL–22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine

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Chronic mucosal inflammation and tissue damage predisposes patients to the development of colorectal cancer1. This association could be explained by the hypothesis that the same factors and pathways important for wound healing also promote tumorigenesis. A sensor of tissue damage should induce these factors to promote tissue repair and regulate their action to prevent development of cancer. Interleukin 22 (IL-22), a cytokine of the IL-10 superfamily, has an important role in colonic epithelial cell repair, and its levels are increased in the blood and intestine of inflammatory bowel disease patients4,5. This cytokine can be neutralized by the soluble IL-22 receptor, known as the IL-22 binding protein (IL-22BP, also known as Il22ra2); however, the significance of endogenous IL-22BP in vivo and the pathways that regulate this receptor are unknown4,5. Here we describe that IL-22BP has a crucial role in controlling tumorigenesis and epithelial cell proliferation in the colon. IL-22BP is highly expressed by dendritic cells in the colon in steady-state conditions. Sensing of intestinal tissue damage via the NLRP3 or NLRP6 inflammasomes led to an IL-18-dependent downregulation of IL-22BP, thereby increasing the ratio of IL-22/IL-22BP. IL-22, which is induced during intestinal tissue damage, exerts protective properties during the peak of damage, but promoted tumour development if uncontrolled during the recovery phase. Thus, the IL-22–IL-22BP axis critically regulates intestinal tissue repair and tumorigenesis in the colon.

IL-22 is produced by innate lymphoid cells, T helpers 17 cells, and T helpers 12 cells, particularly at mucosal surfaces7–9. The membrane-bound IL-22 receptor 1 (IL-22R1, also known as Il22ra1) is absent on immune cells, but expressed within tissues, such as the epithelial cells of the gastrointestinal tract and skin1. IL-22 has an important function in the promotion of antimicrobial immunity via induction of antimicrobial peptides, and in tissue repair via induction of epithelial cell proliferation and survival2,10–12. However, IL-22 can also promote pathological inflammatory responses in the skin10 or intestine11 in mouse models, and its concentration is increased in a variety of human diseases including psoriasis, rheumatoid arthritis, infections and inflammatory bowel disease12. In line with the pleiotropic role(s) of IL-22, it is known that this cytokine signals via STAT3, which is important for wound healing, but also for tumour development1. However, the role of IL-22 during tumour development needs to be clarified, because both inhibitory and promoting effects have been reported (for review see ref. 4).

IL-22BP is a soluble IL-22 receptor, which lacks a transmembrane and intracellular domain. IL-22BP specifically binds to IL-22 but not to other IL-10 family members, and prevents the binding of IL-22 to membrane-bound IL-22R112–15. The binding of IL-22 to IL-22BP is of 20- to 1,000-fold higher affinity compared to its binding to the membrane-bound IL-22R112–15. It is also known that IL-22BP expression is downregulated in the intestine during tissue damage. However, the cellular source of IL-22BP is unclear. Moreover, the mechanism regulating IL-22BP expression and the significance of endogenous IL-22BP in vivo are unknown4,5.

IL-22BP is highly expressed in the colon (Supplementary Fig. 1) and IL-22 has been suggested to have a role in tumour development. We therefore generated Il22bp2/2 mice (Il22bp is also known as Il22ra2) (Supplementary Fig. 2) and used a colitis-associated colon cancer model, which resembles the pathology of human colitis-associated neoplasia16,17. We then examined the role of IL-22BP during tumorigenesis in the colon. Interestingly, tumour development was strongly accelerated and the number and size of the tumours were increased in Il22bp2/2 mice compared to wild-type control mice (Fig. 1a–c). Tumour morphology was similar between Il22bp2/2 and wild-type control mice (Fig. 1c). Thus, IL-22BP deficiency leads to accelerated and increased tumorigenesis in a colitis-associated colon cancer model.

Inflammation is one of the major drivers of tumour development in the colitis-associated colon cancer model16,18. IL-22 has been reported to have both protective and pathogenic properties during colitis4,5,19. However, wild-type and Il22bp2/2 mice showed no difference in colitis disease severity in the acute dextran sodium sulphate (DSS)-induced colitis model (Supplementary Fig. 4a–g). In line with a previous report1, we found that Il22bp was downregulated during acute DSS-colitis, and was not detectable on day 10 of the experiment (Supplementary Fig. 4g), which could explain the lack of phenotype in Il22bp2/2 mice. We then examined a model of chronic DSS-induced colitis in which DSS is administered for 5 days followed by 16 days of a water regimen for four separate cycles, similar to the colitis-associated colon cancer model. No difference in disease severity was observed between wild type and Il22bp2/2 in this chronic colitis model (Supplementary Fig. 5).

IL-22 is well known to promote epithelial cell proliferation3,4. Therefore another hypothesis for the increased experimental colon tumorigenesis in Il22bp2/2 mice is that the effect of IL-22BP is to inhibit IL-22-induced epithelial cell proliferation. However, the significance of endogenous IL-22BP for the control of IL-22 is currently unclear4,5. Furthermore, both IL-22 and IL-22BP expression are modulated during intestinal tissue damage4,5. We examined the expression of IL-22 and IL-22BP during one cycle of DSS administration for 5 days followed by 16 days of water. Mice treated with DSS lost weight and developed histological signs of colitis (Fig. 2a). The peak of disease occurred between day 7 and day 8 (Fig. 2a). Il22bp messenger RNA was expressed under steady-state conditions. The lowest expression of Il22bp correlated with the peak of disease. However, Il22bp was expressed once again during the recovery phase (Fig. 2a, b). Consistent with previous studies4,9, we found increased Il22 mRNA levels in the colon with maximal expression between day 7 and day 8 (Fig. 2b). Accordingly

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IL-22 serum levels were increased with the same pattern (data not shown). We next validated the inverse expression pattern of IL-22 and IL-22BP in another model of intestinal damage. Following wounding of the colon using an endoscopic biopsy forceps (Fig. 2c), Il22bp was expressed in the initial biopsy (Fig. 2d). However, 2 days later Il22bp expression was reduced in a re-biopsy close to the wound, but not in a control biopsy taken at a distance of 0.5 cm from the initial biopsy (Fig. 2d). Thus IL-22BP is downregulated upon damage of the epithelial barrier in the colon, but induced once again during the recovery, and IL-22 shows an inverse expression pattern to IL-22BP.

On the basis of these results we tested if IL-22BP has a significant effect on epithelial cell proliferation during DSS-induced intestinal tissue damage. There was no difference in epithelial cell proliferation between wild-type and Il22bp−/− mice on day 0 or 7 of the experiment. However, whereas in wild-type mice epithelial cell proliferation on day 14 was comparable to steady-state conditions, Il22bp−/− mice continued to demonstrate elevated epithelial cell proliferation (Fig. 2e and Supplementary Fig. 6). At this time point both Il22 and Il22bp were expressed in the colon (Fig. 2b). In line with these data, tumour cells of Il22bp−/− mice proliferated more compared to wild type (Fig. 1d). Thus endogenous IL-22BP is required to terminate the IL-22-induced regenerative program.

We generated Il22bp−/− Il22−/− double KO (dKO) mice to establish that the effect of IL-22BP is dependent on the presence of IL-22. Il22bp−/− Il22−/− mice showed a similar tumour number and score compared to Il22−/− mice, which was lower than the score observed in Il22−/− mice. These data confirm that the effect of IL-22BP is dependent on the presence of IL-22 (Supplementary Fig. 7). Unexpectedly Il22−/− mice developed a higher tumour load compared to wild-type mice (Supplementary Fig. 7), which seems to be in contrast to the increased tumour load in Il22bp−/− mice. It is known, however, that Il22−/− mice exhibit an increased disease severity in the DSS-colitis model. We therefore proposed the hypothesis that IL-22 has a dual function during colitis-associated colon cancer: Deficiency of IL-22 might lead to delayed colonic repair and increased intestinal inflammation, thereby promoting tumour development. However, the increased availability of IL-22 in Il22bp−/− mice during the recovery phase caused prolonged epithelial proliferation, thereby also promoting the development of intestinal tumours. In support of this hypothesis, we confirmed that Il22−/− mice have increased disease and inflammation with marked IL-6 production after DSS administration (Supplementary Fig. 8). To further validate our hypothesis we performed two sets of experiments. First we showed, by the administration of neutralizing IL-22 antibody, that IL-22 is protective during the peak of disease, but detrimental during the recovery phase (Supplementary Fig. 9). Second we used a model of spontaneous tumorigenesis, in which a genetic mutation and not inflammation induces tumour development in the colon. We crossed Il22−/− and Il22bp−/− with Apcmin/+ mice. Min (multiple intestinal neoplasia) mice carry a dominant mutation in the adenomatous polyposis coli (Apc) gene and develop multiple adenomas throughout their intestinal tract, mainly in the small intestine. This mouse model resembles the human disease known as familial adenomatous polyposis, which is also caused by mutations in the Apc gene. Humans carrying this mutation develop polyps, mainly in the colon, and malignant transformation into colon cancer occurs, if untreated, in almost 100% of the cases. Il22bp−/− Apcmin/+ mice developed an increased number and size of tumours, whereas Il22−/− Apcmin/+ developed fewer tumours in the colon compared to Apcmin/+ mice. In contrast to the human disease, in the mouse model most tumours are seen in the small intestine. The tumour number and load was also significantly lower in the small intestine of Il22−/− Apcmin/+ compared to wild-type mice, although Il22bp−/− Apcmin/+ showed similar tumour development in the small intestine compared to wild-type mice (Fig. 3). This lack of effect of the Il22bp genotype in the small intestine is in line with the expression of Il22bp, which is high in the colon and low in the small intestine (Supplementary Fig. 1). Taken together, these data support our hypothesis that the increased tumour burden in Il22−/− mice in the colitis-associated colon cancer model is due to the increased susceptibility to DSS-induced colitis. In summary, IL-22 and IL-22BP are regulated during intestinal tissue damage. IL-22 has both protective and detrimental effects during intestinal tissue damage and therefore needs to be controlled via IL-22BP.

The cellular source of IL-22 and the mechanism regulating IL-22 expression have been studied. In contrast, the mechanism regulating IL-22BP expression is unknown. IL-22BP expression has been reported in different haematopoietic cells and also in intestinal epithelial cells. However, these studies contradict each other. Interestingly, we observed Il22bp expression in haematopoietic cells and preparations of epithelial cells isolated from the colon (Fig. 4a). One caveat, however, was that the purity of the epithelial cells was about 98% and the Il22bp expression was about 50 times lower than in the haematopoietic cells. We used bone marrow chimaeras to show that the vast majority of IL-22BP expression in the colon was due to the haematopoietic compartment both in steady-state condition (Fig. 4b) and in the colitis-associated colon cancer model (Supplementary Fig. 10). We next used a stepwise approach to identify the haematopoietic source of IL-22BP in the colon. Il22bp was expressed in TCR-β− cells, but...
not in TCR-β+ cells (data not shown). Within the TCR-β− cells MHCII+CD11c+ cells were the main source of Il22bp (Fig. 4c and Supplementary Fig. 10).

Next we aimed to identify the trigger and the pathway regulating IL-22BP expression. As shown in Fig. 2 we found that Il22bp is downregulated at day two after DSS administration before the development of histological signs of disease. However, it is known that DSS induces the loss of tight junctions within 2 days, and that the epithelial barrier is therefore permeable, allowing penetration of bacteria and bacterial products.

Interestingly, depletion of the bacterial flora by administration of antibiotics in the DSS-colitis model impaired the downregulation of IL-22BP (data not shown, Fig. 4e). We therefore tested whether the downregulation of IL-22BP is dependent on MYD88 and/or TRIF signalling. To that end we used the endoscopic wounding model as described in Fig. 2. Remarkably, Il22bp was not downregulated in Myd88−/−Trif−/−dKO mice after wounding of the colon. Moreover, expression appeared to be elevated in the wounded dKO mice compared to the unwounded tissue of these mice. As expected, Cd11c (also known as Itgax) was also upregulated in the wounded compared to the unwounded tissue. We therefore normalized Il22bp expression to Cd11c, which showed that the Il22bp expression remained stable in Myd88−/−Trif−/−dKO mice, but was drastically reduced in wild-type mice after wounding (Fig. 4d). Finally, we could demonstrate that the downregulation of Il22bp was independent of the TLR2, TLR4, TLR5 or IL-1 pathways, but dependent on IL-18 (Fig. 4e).

Inflammasomes are cytoplasmic multiprotein complexes that function as sensors of endogenous or exogenous stress. Inflammasomes typically assemble with the adaptor protein ASC (apoptosis-associated speck-like protein) into a multiprotein complex that leads to caspase 1 activation and subsequent cleavage of pro-IL-18 (or pro-IL-1)23. Accordingly, the downregulation of IL-22BP is dependent on the IL-18 (or IL-1) pathway.

Figure 2 | Inverse expression of Il22bp and Il22 during chemical and mechanical intestinal tissue damage. a. Weight loss during DSS-colitis (2.5% for 5 days; mean ± s.e.m.; n = 11), and histology (bars, 500 μm). b. Il22 and Il22bp mRNA (normalized to Hprt) in total colon (mean ± s.e.m. of triplicates). c, d. Il22bp and Il22 mRNA after mechanical wounding (mean ± s.e.m.; n = 4).

Figure 3 | IL-22BP controls tumorigenesis in Apcmin/+ mice. Il22bp−/− and Il22−/− mice were crossed with Apcmin/+ mice. Six-months-old mice were analysed. Tumour number and size were measured using a dissecting microscope (size 1, <2 mm; size 2, 2–5 mm; size 3, >5 mm). Each dot represents one mouse, lines indicate mean ± s.e.m. All mice displayed Apcmin/+ . No tumours were observed in Apcmin/+ regardless of the Il22 and Il22bp genotype.

c. Number of 5-bromo-2′-deoxyuridine (BrdU)-positive cells per crypt during DSS-colitis and BrdU immunohistochemistry on day 14 (bars, 200 μm). Each dot represents one animal. Lines indicate mean ± s.e.m. Results are representative of at least three experiments.
we found that the downregulation of Il22bp in inflammasome-deficient mice. We therefore co-housed wild-type mice with CD11c+ cells; mean of triplicates. Expression of Il22bp normalized to Hprt in WT and Myd88−/− mice (n = 3). Il22bp expression (re-biopsy relative to initial biopsy; Abs, antibiotic-treated WT mice). Il22bp expression in cultured human-monoctye-derived dendritic cells (n = 8). 10 ng ml−1 IL-18, variable time; g, variable IL-18 concentration. Results are representative of two independent experiments (a-d, f, g), cumulative from seven experiments (e). Mean ± s.e.m.

activated by host-derived factors which are released upon tissue damage, such as ATP, uric acid and hyaluronan, and also by microbial ligands19. Using the antibiotic treatment that we validated previously20, we found that the downregulation of Il22bp was partially dependent on the intestinal microbiota (Fig. 4e). We recently reported that Nlrp6−/− mice have an altered microflora24. We therefore co-housed wild-type mice with Nlrp6−/− mice, conditions under which the dysbiotic flora are transferred to co-housed recipients. But the downregulation of Il22bp upon wounding of the wild-type intestine was not impaired (fold reduction of Il22bp: wild type 0.0123 ± 0.002, wild type (co-housed) 0.0118 ± 0.0003, Nlrp6−/− (co-housed) 0.405 ± 0.152). These data show that, although the downregulation of Il22bp is partially dependent on the microflora (Fig. 4e), it is not affected by the transmissible flora of inflammasome-deficient mice.

It is known that IL-18 is upregulated very early after DSS-induced intestinal tissue damage25.26. We therefore tested the role of IL-18 in the modulation of IL-22BP in this model. We observed that IL-18 is crucial for the complete downregulation of Il22bp also upon DSS-induced tissue damage (Supplementary Fig. 11). Whereas loss of IL-18 strongly reduced the level of downregulation of Il22bp, there was residual downregulation of Il22bp even in the absence of IL-18, indicating that other factors may also contribute to the regulation of Il22bp. We next aimed to further support and extent our murine in vivo model to human biology. We first validated that human dendritic cells express IL-22BP, and then we established, in vitro, that IL-18 is able to downregulate IL-22BP (also known as IL22RA2) in human (Fig. 4f, g) and mouse dendritic cells (data not shown) in a time- and dose-dependent manner. Taken together, these data show that IL-18 downregulates Il22bp expression by CD11c+ cells upon intestinal wounding.

Recent publications have shown that IL18−/− mice have increased inflammation and tumour development in the colitis-associated colon cancer model26.27. This is in line with our finding that IL-18 downregulates IL-22BP, and that IL18−/− mice have increased inflammation and tumorigenesis in the colitis-associated colon cancer model. However, our data do not exclude other important functions of IL-18 besides the regulation of IL-22BP.

Chronic mucosal inflammation and tissue damage as seen in inflammatory bowel disease predisposes patients to the development of colorectal cancer, one of the most frequent fatal cancers in the world. This association could be explained by the hypothesis that the same factors and pathways that are important for wound healing, also promote tumorigenesis. Our data indicate that IL-22 is such a factor. Several studies have linked the microbial status with colon tumorigenesis28.30. These data are in line with our results that sensing of microbial ligands and intestinal tissue damage by the NLIRP3 and NLRP6 inflammasomes regulates IL-22BP expression by CD11c+ cells via caspase-1-mediated IL-18 activation. This regulation of IL-22BP is crucial to control the effects of IL-22 during intestinal tissue damage and tumorigenesis (Supplementary Fig. 12). Therefore we propose a link between the microflora, the epithelium and the immune system regulating the balance between tissue regeneration and tumour development in the intestine. The regulation of IL-22BP via the inflammasome provides an unexpected mechanism, controlling IL-22 and thereby the development of colon cancer.

METHODS SUMMARY

Tumour induction. Co-housed mice were injected intraperitoneally with AOM (azoxymethane) (Sigma) at a dose of 7.5 mg kg−1 body weight. After 5 days, mice were fed 2–2.5% DSS (MP Biomedicals, molecular mass 36,000–50,000 Da) in the drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated twice.

Endoscopic procedures. Colonoscopy was performed in a blinded fashion for colitis and tumour monitoring using the Coloview system (Karl Storz). Colitis scoring was based on granularity of mucosal surface, stool consistency, vascular pattern, translucency of the colon and fibrin visible (0–3 points for each). Tumour sizes were graded from 1 to 5. The total tumour score per mouse was calculated as sum of all tumour sizes. In some experiments the colon was wounded using the endoscope and a biopsy forceps. A re-biopsy was taken 2 days after the wounding event to the initial biopsy. A control biopsy was taken at a distance of about 0.5 cm.

Statistical analysis. For comparison of groups, the non-parametric two-sided Mann–Whitney test or analysis of variance (ANOVA) and post-hoc analysis was applied. The significance level was set to 0.05.

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

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

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**Author Contributions** R.A.F., S.H. and N.G. designed the experiments, analysed the data and wrote the manuscript. L.A.Z. performed colitis-associated colon cancer experiments with Il22<sup>−/−</sup> single-KO mice, and provided Il22<sup>+/−</sup> mice. F.J.H. assisted during the mouse endoscopy. L.B. performed immuno histochemistry. B.H. provided mice for colitis-associated cancer experiment. W.O. made key suggestions for experiments and edited the manuscript. A.J.M., D.M.V. and G.D.Y. generated Il22<sup>−/−</sup> mice and are employees of Regeneron Pharmaceuticals Inc. C.J.B. performed the histopathological analyses. W.O. provided IL-22 antibody and is an employee of Genentech Inc. W.Z., J.H., C.A. and M.H. did the experiments using human material. S.H. and N.G. performed all other experiments.

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METHODS
Animals. Generation of Il22bp−/− mice: the gene encoding Il22bp (Il22ra2) was ablated in embryonic stem cells using the Velocigene method31. Briefly, a bacterial artificial chromosome (BAC) containing the Il22ra2 gene (clone 305Q4 from Incyte Genomics mouse "BAC embryonic stem cell release 2" library) was isolated and bacterial homologous recombination (BHR) was performed to replace 9.4 kb of the Il22ra2 gene with a cassette containing a transmembrane (TM)-lacZ reporter gene and a floxed neomycin resistance selectable marker gene. The resulting mutant allele (designated VG437) encodes a fusion protein containing 7 amino acids of the mature form of Il-22BP, fused to a transmembrane segment, fused to Escherichia coli beta-galactosidase under transcriptional control of the Il22ra2 gene. BHR resulted in a large targeting vector (RACvec) in which the reporter/cassette cassette was flanked with homology arms of 155 and 45 kb. The RACvec was electroporated into VGF1 (ref. 31) embryonic stem cells and G418-resistant colonies were screened for targeting using a loss of native allele (LONA) assay with two quantitative PCR probe/primer sets within the deleted region: 437TU (primers 437TUF: GGGACCTCAGCTTCCGTGC and 437TUR: CTAAACGATGTTGCTGCCAGC and probe 437TU P: TCGCAAAAAGCTCAGATTGCTCAATG) and 437TD (primers 437TDF: CCAGCCCATGTTGCAGAAAG and 437TDR: TAGGGCTCAGACCATTCTACAT and probe 437TD P: TCGCAACGATCCTCCTTGTCGGGC) and appropriate reference probes as described31. Correctly targeted embryonic stem cells were microinjected into C57BL6 blastocysts to produce chimaeras that were subsequently bred to generate knockout mice. Il22bp−/− mice were backcrossed for 12 generations on C57BL6 background. For all experiments age- and sex-matched KO mice and co-housed in house breed C57BL6 wild-type animals between 8 and 14 weeks of age were used. Casp1−/− were generated in our laboratory34. Apem1−/−, Il18−/−, Il1r−/−, Myd88−/−, Trif−/−, Tlr4−/− and Trs−/− mice were obtained from The Jackson Laboratory. Nlrp3−/−, Nlrp6−/−, Asc−/−35 and Il22−/− mice are described elsewhere. For antibiotic treatment, mice were given either a combination of ciprofloxacin (0.2 g l−1) and metronidazole (1 g l−1) or a combination of vancomycin (1 g l−1), ampicillin (1 g l−1), kanamycin (1 g l−1), and metronidazole (1 g l−1) for 4 weeks in the drinking water35.

Mice were cared for in accordance with institutional animal care and use committee-approved protocols at the Yale University School of Medicine animal facility.

Tumour induction. Mice were injected intraperitoneally with AOM (Sigma) at a dose of 7.5 mg kg−1 body weight. After 5 days, mice were fed 2.5% DSS (MP biomedicals, molecular mass 36,000–50,000 Da) in the drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated twice31. Mice were killed on day 80 of the experiment.

DSS colitis. For acute DSS colitis induction mice were administered 2–2.5% DSS in the drinking water for 7 days, followed by 3 days of regular water. For chronic DSS colitis induction mice were fed 2.5% DSS for 5 days followed by 16 days of regular water. This cycle was repeated three times. According to the animal protocol, mice were killed if they lost more than 20% of their initial body mass.

Endoscopic procedures. Colonoscopy was performed in a blinded fashion for colitis and tumour monitoring using the Coloview system (Carl Storz) as previously described3. Briefly, colitis scoring was based on granularity of mucosal surface, stool consistency, vascular pattern, translucency of the colon and fibrin visible (0–3 points for each). Tumour sizes were graded from 1 to 5. Tumours observed during endoscopy were counted to obtain the total number of tumours per animal. The total tumour score per mouse was calculated as sum of all tumour sizes. The colon was wounded using the endoscopy and a biopsy forceps as described previously31,32. A re-biopsy was taken 2 days after the wounding either close to the initial biopsy or at a distance of about 0.5 cm.

Histopathology procedures. Colons were evaluated and were assigned scores by investigators blinded to experimental manipulation. Each section was evaluated by a semiquantitative criterion-based method (score 0–5) essentially as described before31. For immunohistochemistry paraffin-embedded sections were stained with anti-BrdU (Sigma) or anti-Kit (Thermo (Lab Vision)). DAKO EnVision System was used for detection. All sections were counterstained with haematoxylin. TUNEL (TdT-mediated dUTP nick end labelling) staining was performed using an ApoAlert DNA fragmentation assay kit according to the manufacturer's instruction. BrdU was injected 4 h before the animals were killed.

Isolation of epithelial cells and haematopoietic cells from the intestine. and haematopoietic cells were isolated from the freshly obtained colon. After removal of the Payer's patches and the adventitial fat, the colon was cut longitudinally and washed with PBS. For disruption of the epithelial cells the colon was incubated in HBSS/EDTA at 37 °C. The supernatant was collected and further separated in CD45-positive (intraepithelial lymphocytes) and -negative cells using MACS (magnetic-activated cell sorting). The remaining colon was digested using collagenase/DNase incubation at 37 °C. CD45-positive cells (lamma propria lymphocytes) were purified using MACS. In a second step CD45-positive IEL and LPL cells were further purified using a MoFlo. The purity of CD45-positive cells was >95%, epithelial cells were >98% CD45 negative.

Colon explant culture. One centimeter of the distal colon was removed, washed with PBS, scaled and cultured for 3 days in X-Vivo medium containing penicillin, streptomycin and tetracycline. IL-6 was measured using CBA (cytometric bead array).

RNA analysis. Total RNA was extracted from colon tissue, tumours, colon biopsies or cells using TRIzol reagent, followed by RNA clean up using the RNeasy Kit (Qiagen) or the Dynabeads mRNA Kit (Applied Biosystems). The high capacity cDNA synthesis kit (Applied Biosystems) was used for synthesis of cDNA. Real-time PCR analysis using TaqMan Fast Universal PCR Mater Mix and TaqMan Gene Expression Assays (Applied Biosystems) was performed on 7500 Fast Real-time PCR system machine (Applied Biosystems).

Human subjects. Informed consent was obtained from healthy participants per protocol approved by the institutional review board at Yale University.

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