AUS DEM LEHRSTUHL FÜR CHIRURGIE
PROF. DR. H. J. SCHLITT

DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Interleukin 21 controls tumor growth and tumor immunosurveillance in colitis-associated tumorigenesis in mice

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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Fakultät für Medizin
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Für meine Familie und Freunde

Inhaltsverzeichnis

3 Adenoma - linked barrier defects and microbial products drive IL-23/IL-17 - mediated tumor growth

S.Grivennikov & K.Wang, D. Jauch et al., Nature, 2012.

Abbildungsverzeichnis

Abkürzungsverzeichnis

APC	adenomatous polyposis coli
β-catenin	catherin-associated protein beta
k-ras	Kirsten rat sarcoma viral oncogene homolog
IβL	Interleukin
Th	T-Helfer-Zelle
TNF-α	Tumor-Nekrosefaktor-alpha
IFN-γ	Interferon-gamma
CD	Cluster of differentiation
NK-Zellen	Natürliche Killer-Zellen
IL-21$^{-/-}$	homozygote Interleukin-21 Defizienz
AOM	Azoxymethan
i.p.	intraperitoneal
DSS	dextran sodium sulfate
MLN	Mesenteriale Lymphknoten
LPMC	lamina propria mononuclear cells
H&E	Hämatoxylin-Eosin Färbung
Alcian blue-PAS	Periodic acid-Schiff reaction Färbung
Ki-67	Kiel 67
E-Cadherin	epitheliales Cadherin
TUNEL-Assay	**T**dT-mediated d**U**TP-X **n**ick **e**nd **l**abeling
DNA	Desoxyribonukleinsäure
MACS	magnetic bead sorting
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
CTL-Assay	cytotoxic T-lymphocyte assay
CT-26 Zellen	murine Kolonkarzinomzelllinie
NKT Zellen	Natürliche Killer T-Zellen
GM1	Gangliosid Monosialogangliosid 1

1 Zusammenfassung

1.1 Einleitung

Darmkrebs stellte in Deutschland mit 62.430 Neuerkrankungen im Jahr 2010 die zweithäufigste Tumorerkrankung dar [1]. Grundsätzlich kann man drei verschiedene Entstehungsmechanismen unterscheiden. Die Mehrzahl der Malignome, mit bis zu 95% treten sporadisch auf. Die weiteren Fälle entstehen hereditär oder aufgrund von chronisch entzündlichen Darmerkrankungen, wie zum Beispiel der Kolitis ulzerosa. Patienten mit einer Kolitis ulzerosa haben ein deutlich erhöhtes Risiko im Laufe ihres Lebens ein Kolorektales Karzinom zu entwickeln. Das kumulative Risiko ein Karzinom nach 10 Erkrankungsjahren zu entwickeln beträgt 2% und steigt nach 20 Jahren auf 8% und nach 30 Jahren auf 18% an [2]. Ob das Risiko aufgrund besserer Therapien, frühzeitiger Diagnosestellung und kurativer Operationsmethoden reduziert wird kann derzeit noch nicht sicher beurteilt werden [3].

Zur Etablierung einer Präventionsstrategie ist somit die Aufklärung des Pathomechanismus chronisch entzündlicher Darmerkrankungen unumgänglich. Während die sporadischen und die hereditären Neuerkrankungen molekulargentisch weitgehend beschrieben sind, bleiben die genauen immunologischen Mechanismen der entzündungsassoziierten Darmkrebsentstehung noch ungeklärt.

Die sporadische Darmkrebsentstehung folgt oftmals einer Sequenz genetischer Veränderungen, basierend auf dem Verlust des Tumorsuppressorgens APC und folgender β-Catenin Aktivierung und k-ras Mutation. Zugrunde liegt hierbei die Adenom-Karzinom-Sequenz mit einer Zunahme von molekulargenetischen Veränderungen und Zellproliferation. Auch ohne existente Entzündung werden in diesen Tumoren entzündliche Infiltrate gefunden. Die Aktivierung des Immunsystem erfolgt hierbei jedoch im Unterschied zur Kolitis-assoziierten Tumorentstehung intrinsisch durch, vom Tumor exprimierte Antigene [4].

Beiden, der sporadischen und der entzündungsvermittelten Tumorentstehung, ist ein spezielles immunologisches Tumormilieu gemein. Charakterisiert ist dies einerseits durch zytotoxische, tumorbekämpfende T-Zellen, anderseits gleichzeitig

durch Tumorproliferation begünstigende T-Helfer-Zellen. Proinflammatorische Zytokine werden von tumorinfiltrierenden T-Helfer-Zellen produziert. Hierzu zählen vor allem IL-1, IL-6, IL-17, IL-23, IFN-γ und TNF-α. Interleukin-21 ist dabei von besonderer Bedeutung, da dieses Zytokin maßgeblich an der Polarisierung von zwei Subtypen der T-Helfer-Zellen beteiligt ist, der Th-1 und Th-17 Zelle. Die Sekretion dieses Zytokins erfolgt durch NK-Zellen und Th-17 Zellen.

Ferner spielt auch die Tumor-Immunosurveillance - eine immunologisch gesteuerte Tumorbekämpfung - eine tragende Rolle in der Regulation des Wachstums entzündsassoziierter Malignome. Vermittelt wird dies durch zytotoxische CD8 T-Zellen und NK-Zellen. Die Produktion von Perforinen und Granzyme sorgen hierbei für Apoptose der Tumorzellen und regulieren das Tumorwachstum.
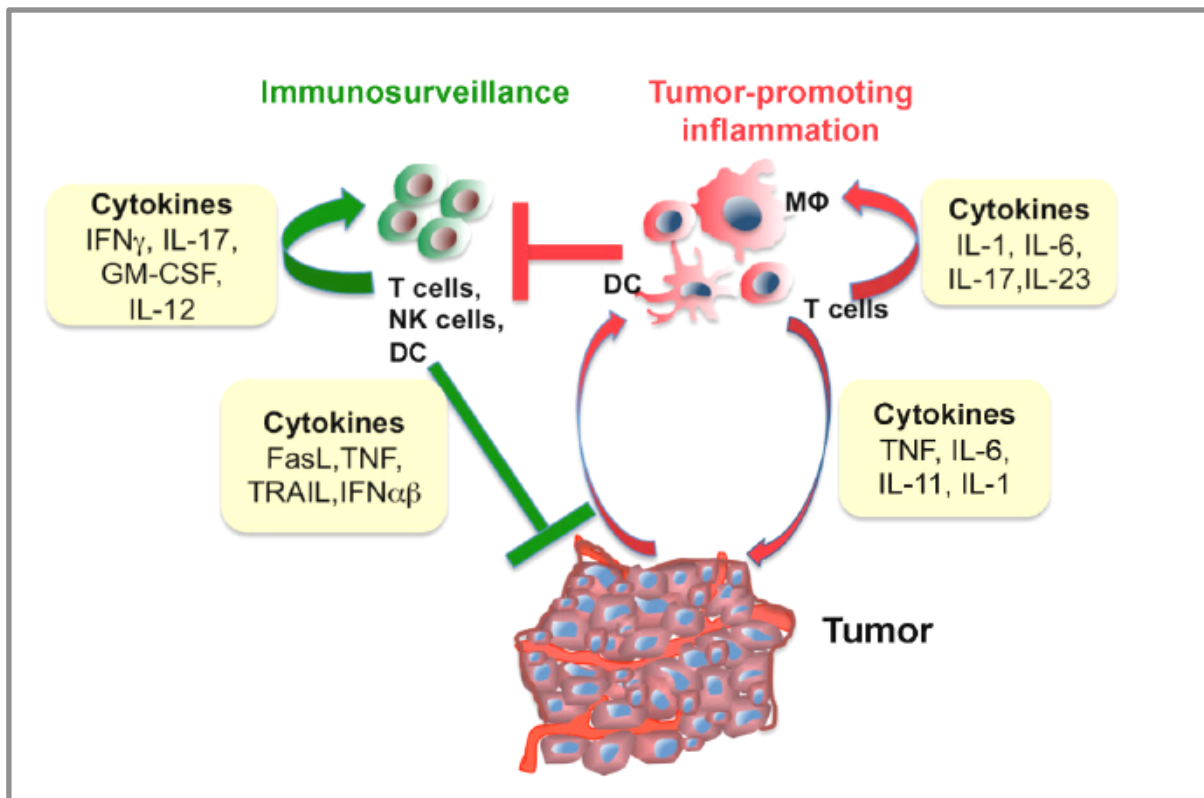

Abb. 1: Immunosurveillance, Tumor-promoting inflammation, modifiziert nach [5].

Ziel dieser Arbeit ist es, herauszufinden inwieweit Interleukin-21 Einfluss auf die entzündungsassoziierte Tumorentstehung im Kolon nimmt und im Rahmen dieser das Zytokinmilieu beeinflusst.

1.2 Methoden

IL-21$^{-/-}$ (B6;129S5) und wild-typ Kontrolltiere wurden pathogen frei gehalten. Für die Tierversuche wurden alle Tiere genetisch bezüglich der IL-21-Defizienz analysiert und im Alter von 2-4 Wochen für die Versuche ausgewählt. Für die Tierversuche wurden je Gruppe 10 Tiere untersucht.

Die chronische Kolitis wurde unter Verabreichung von abwechselnd 7 Tagen 1,5% DSS Trinklösung und 7 Tagen Trinkwasser für insgesamt 3 Zyklen induziert. Zur Einleitung der Tumorentstehung wurden 10mg/kg Körpergewicht AOM initial an Tag 1 i.p. injektziert. Gewichtskontrollen wurden wöchentlich durchgeführt. Die Auswertung erfolgte am Endpunkt nach 42 bzw. 70 Tagen.
Analysiert wurden die Versuchstiere hinsichtlich:

- Tumoranzahl und -größe
- Histologie und Immunhistochemie der Tumore und entzündeter Areale
- Zytokinproduktion von MLN und LPMC
- T-Zell Zytotoxizität

Die Tumoranzahl wurde makroskopisch nach Beenden der Versuchstiere bestimmt und deren Größe anschließend histologisch, mithilfe der Mirax Viewer Software, Carl Zeiss AG, verifiziert.

Für die histologische Beurteilung wurden Teilstücke des Kolons, tumortragende und entzündlich veränderte, in Formalin fixiert und anschließend durch das Institut für Pathologie der Universität Regensburg in Paraffin gebettet. Die Entzündungsreaktion wurde anhand eines *histology scores* [6] von H&E gefärbten Gewebeschnitten verblindet quantifiziert.
Immunhistochemische Gewebefärbungen erfolgten nach Anleitung der Antikörperhersteller. Alcian blue-PAS Färbungen zeigen die Verteilung von Schleim-produzierenden Becherzellen im Epithel an, Ki-67 gibt Aufschluss über die Proliferation der Epithelzellen, β-Catenin und E-Cadherin positive Signale beschreiben die Degradation der Zellwand-Barrierefunktion, Foxp3 färbt regulatorische T-Zellen an, anti-INF-γ und anti-CD-8 Antikörper reagieren jeweils mit Zytokin produzierenden bzw. Zelloberflächenstrukturen exprimierenden Zellen. Der TUNEL-Assay färbt frühapoptotische Zellen bzw. deren DNA-Strangbrüche mithilfe von Fluoreszin-gebundenem Uracil an.

CD4 und CD11b positive Zellen wurden mit Hilfe der MACS-Technik aus MLN oder LPMC isoliert und für 48 Stunden mit anti-CD3 und anti-CD28 Antikörpern kultiviert. Die Bestimmung der Zytokinkonzentration erfolgte mittels ELISA Messung des kultivierten Überstands.

Zur Bestimmung der CD103-, Granzyme B- und Perforin-Expression wurden LPMC isoliert und mit entsprechenden fluoreszenz-markierten Antikörpern markiert. Die darauffolgende FACS-Analyse der markierten Zellen gibt dann Aufschluss über die jeweils prozentual positiv gefärbten Zellen der gesamten Zellpopulation.

Erkenntnis über die zytotoxische Aktivität CD8$^+$ Zellen lieferte der CTL assay. Hierbei wurden CD8$^+$ LPMC isoliert und mit CT-26 Zellen, E-Cadherin-transfiziert oder nativ, kokultiviert. Die zytotoxische Aktivität wurde nach 24 Stunden mit einem Luminescent Cell Viability Assay bestimmt.

Die statische Auswertung erfolgte mit Hilfe der Software GraphPad Prism, Werte von $p<0.05$ wurden als signifikant bezeichnet.

1.3 Ergebnisse

1.3.1 Chronische Kolitis

Vorversuche konnten zeigen, dass IL-21 in chronischen und akuten Kolitiden in Mäusen, auch ohne Tumorwachstum deutlich hochreguliert ist. Gleichermaßen zeigen sich erhöhte IL-21 Level in Patienten mit ulzerativer Kolitis oder Morbus Crohn [7]. Zudem scheint es einen genetischen Zusammenhang zwischen Veränderungen im IL-2/IL-21 Lokus und der Entwicklung von chronisch-entzündlichen Darmerkrankungen zu geben [8].

In Abwesenheit von IL-21 konnte in einer chronischen DSS-Kolitis kaum eine Entzündungsreaktion nachgewiesen werden.
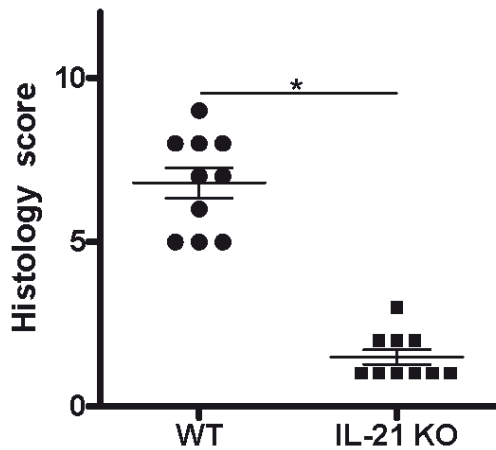
Abb. 2: Histology score bei einer chronischen DSS-Kolitis.

Wohingegen wild-typ Mäuse eine schwere Kolitis, mit Verlust der epithelialen Struktur entwickelten und ein deutlich erhöhtes proinflammatorischer Zytokinlevel (IL-17A , IFN-γ) aufwiesen. Ähnliche Resultate konnten im Rahmen der Transfer-Kolitis gezeigt werden. Zudem zeigten IL-21$^{-/-}$ Mäuse eine signifikant reduzierte Proliferation von Epithelzellen, intraepithelialen und submukösen Lymphozyten.

Abb. 3: Zytokinexpression von CD4^{+} T-Zellen aus MLN bei chronischer DSS-Kolitis.

1.3.2 Kolitis-induzierte Tumorentstehung

Im Tiermodell der Kolitis-assoziierten Tumorentstehung konnten wir in IL-21$^{-/-}$ durchschnittlich nur 1 Tumor pro Maus evalu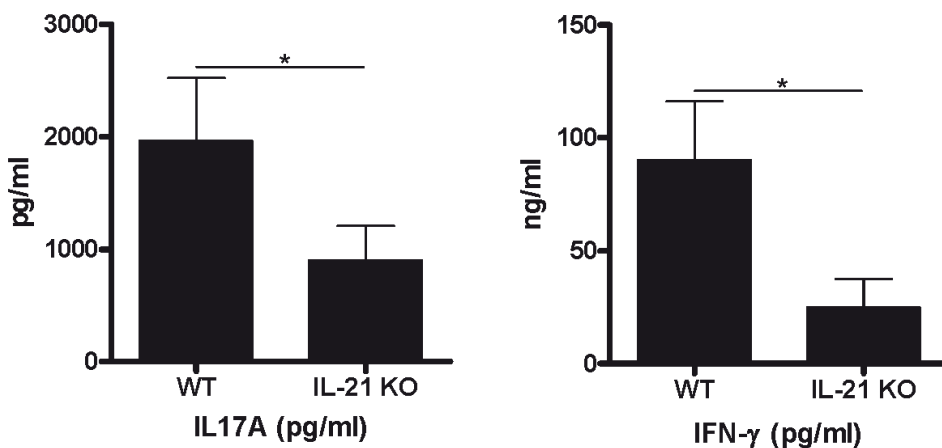ieren, wohingegen wild-typ Mäuse im Durchschnitt 8 Tumoren aufwiesen. Die Tumore waren zudem in der Vergleichsgruppe größer und wiesen auch eine signifikant erhöhte Proliferationsrate innerhalb des Tumorgewebes auf. Auch im Langzeitversuch über 10 Wochen entwickelten IL-21 defiziente Mäuse nicht mehr Tumoren wie bereits nach 6 Wochen, bei den wild-typ Mäusen verdoppelte sich jedoch nahezu die Tumoranzahl.
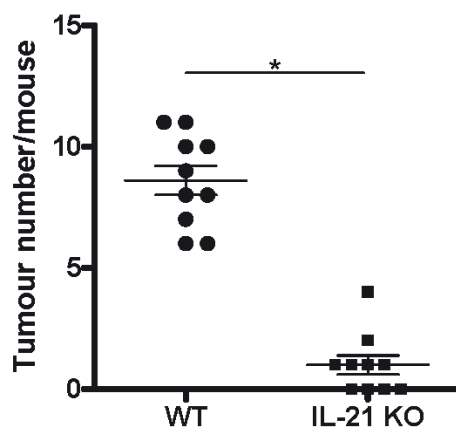
Abb. 4: Tumoranzahl an Tag 42 bei Kolitis-induzierter Tumorgenese.
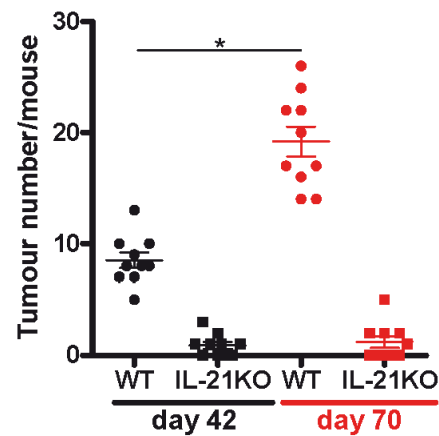
Abb. 5: Tumoranzahl an Tag 70 bei Kolitis-induzierter Tumorgenese.

Die Bestimmung des Zytokinmilieus in entzündeten und tumorösen Anteilen der Kolonschleimhaut zeigt, dass in beiden Arealen die IL-17A Produktion in IL-21$^{-/-}$ Mäusen signifikant reduziert ist. Hinsichtlich der IFN-γ Produktion ließ sich jedoch das Gegenteil nachweisen, in Abwesenheit von IL-21 ist die Produktion von IFN-γ gegenüber der wild-typ Mäuse deutlich gesteigert. Andere proinflammatorische und antiapoptotisch wirkende Zytokine wie IL-6 und IL-22 zeigten keine unterschiedlichen Expressionsmuster in beiden Versuchsgruppen.

Abb. 6: Zytokinexpression an Tag 42 von CD4$^+$ LPMC mittels ELISA bei Kolitis-induzierter Tumorentstehung.

1.3.2.1 Funktion von IFN-Y bei der Kolitis-induzierten Tumorentstehung

Der Schweregrad der chronischen Entzündungsreaktion zeigte trotz des unterschiedlichen Zytokinmilieus und der veränderten Tumorproliferation keine Unterschiede. Nur in der frühen Phase der Kolitis-induzierten Tumorentstehung ließen sich Unterschiede im Schweregrad der Entzündung im Kolonepithel 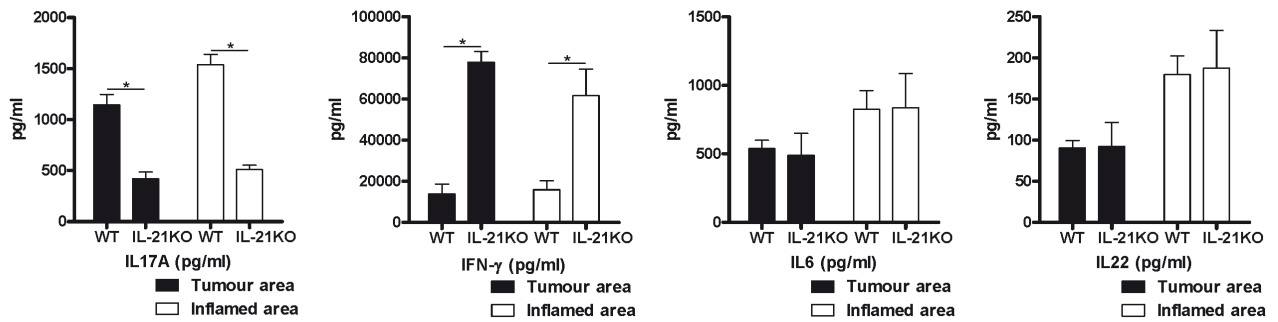feststellen. Begleitend dazu zeigte sich auch, dass IL-21$^{-/-}$ Mäuse erst im Verlauf der 3. Woche eine überschießende IFN-γ zeigten.

Inwieweit antigenpräsentierende Zellen und deren Zytokine Einfluss auf die Entzündungsreaktion und das Tumorwachstum nehmen wurde durch die Zytokinexpression CD11b positiver Zellen hinsichtlich IL-12 und IL-23 bestimmt. Es war dabei zu beobachten, dass IL-23 in wild-typ Mäusen deutlich hochreguliert ist, wohingegen IL-12p70 in IL-21$^{-/-}$ Mäusen eine höhere Expression aufwies. Somit sind auch antigenpräsentierende Zellen, zumindest teilweise an der Immunantwort der Kolitis-assoziierten Tumorentstehung beteiligt.

Die Rolle von IFN-γ in der chronischen Kolitis mit Tumorwachstum wurde durch weitere Analysen und Experimente bestimmt. Hierfür wurde die chronische DSS-Kolitis mit initialer AOM-Injektion durch die Gabe von anti-INF-γ Antikörpern ab Tag 14 ergänzt. Der Schweregrad der Kolitis in wild-typ Mäusen blieb davon unbeeinflusst in IL-21$^{-/-}$ Mäusen führte es jedoch zu einer signifikanten Reduktion

der Kolitis. Hinsichtlich der Tumoranzahl beider Versuchsgruppen zeigten wild-typ Mäuse keine veränderte Tumoranzahl. In IL-21 defizienten Mäusen stieg die Anzahl der Tumore unter Blockierung von IFN-γ signifikant an.

Somit kann man folgern, dass IFN-γ zumindest in IL-21$^{-/-}$ Mäusen, sowohl die Entzündungsreaktion, als auch die Entzündungs-vermittelte Tumorentstehung reguliert.
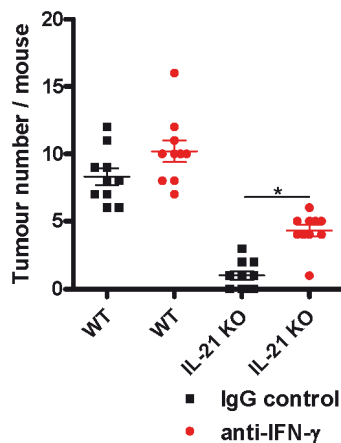
Abb. 7: Tumoranzahl nach IFN-γ Blockierung an Tag 42.

1.3.2.2 CD8$^+$ T-Zell vermittelte Tumor-Immunosurveillance

CD8$^+$ zytotoxische T-Zellen besitzen die Möglichkeit, auch unter Einfluss von IFN-γ das Tumorwachstum zu begrenzen. Unter Administration von anti-CD8 Antikörpern konnte in beiden Versuchsgruppen eine Reduktion der Tumorgröße und der Tumoranzahl erreicht werden. Die Kolitis blieb von der Antikörpertherapie unbeeinflusst.

Auch können NK- und NKT-Zellen einen zytolytischen Effekt ausüben, dieser konnte in unseren Versuchen unter Gabe von CD1 und asialo-GM1 Antikörpern nicht nachgewiesen werden.

CD8/CD103$^+$ T- Zellen können Tumorwachstum regulieren, indem sie den Liganden E-Cadherin auf Tumorzellen binden. Das Expressionsmuster beider Versuchsgruppen hinsichtlich E-Cadherin in den Tumoren zeigte keine

Unterschiede, so dass ein zytolytischer Angriffspunkt in wild-typ und IL-21$^{-/-}$ Tumoren zur Verfügung steht. FACS - Analysen zeigten jedoch, dass wild-typ Mäuse im Tumormodell weniger CD8/CD103$^+$ T-Zellen besitzen. Neben der quantitativen ist auch die qualitative zytotoxische Kapazität in wild-typ CD8/CD103$^+$ T-Zellen vermindert. Granzyme B wird von IL-21$^{-/-}$ zytotoxischen T-Zellen, verglichen mit wild-typ Zellen, deutlich vermehrt ausgeschüttet. Hinsichtlich Perforin, einem weiteren apoptotisch wirksamen Zytokin konnte nur eine gering erhöhte Produktion von IL-21 defizienten T-Zellen gezeigt werden.
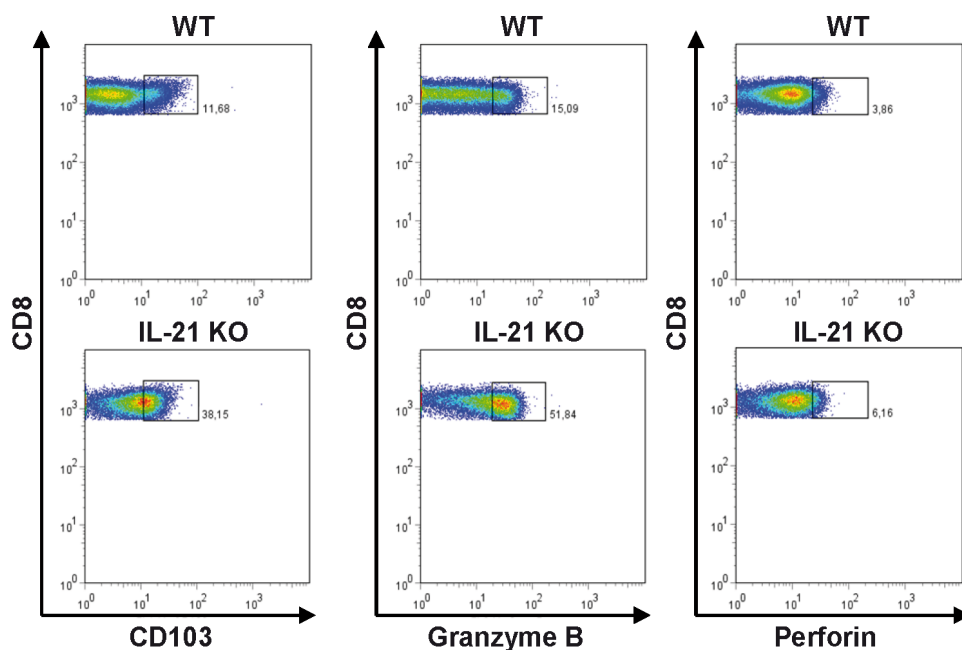
Abb. 8: CD103/CD8, Granzyme B und Perforin Expression,
FACS von LPMC an Tag 28.

Die zytotoxische Kapazität von isolierten CD8$^+$ T-Zellen wurde des Weiteren gegenüber einer allogenen Kolontumorzelllinie getestet. Zusätzlich wurden diese CT-26 Zellen mit einem E-Cadherin (ein CD103 Ligand) exprimierendem Plasmid transfiziert. Wild-typ CD8$^+$ T-Zellen entwickelten nur eine geringe zytolytische Aktivität gegenüber nativen CT-26 Zellen, jedoch eine erhöhte Aktivität gegen E-Cadherin positive Kolontumorzellen. CD8$^+$ T-Zellen aus IL-21 defizienten Mäusen zeigten eine höhere zytotoxische Aktivität sowohl gegenüber nativer als auch gegenüber E-Cadherin transfizierten Kolontumorzellen.
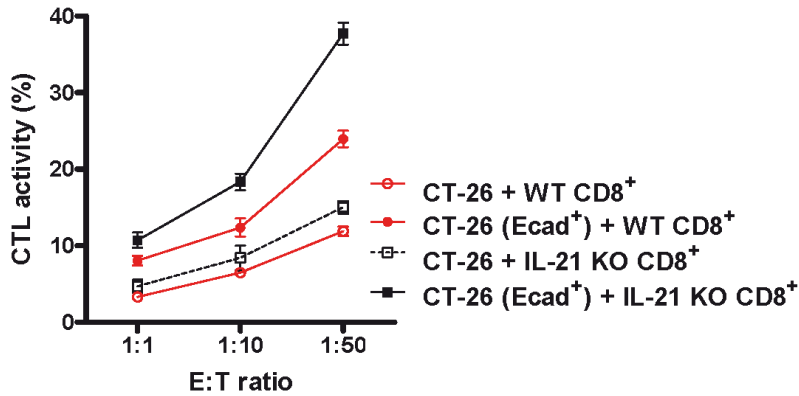
Abb. 9: Zytotoxizität von CD8+ LPMC an Tag 28.

Diese zytotoxisch vermittelte Apoptose konnte auch immunhistochemisch durch einen TUNEL-Assay verifiziert werden. In Tumoren von IL-21$^{-/-}$ Mäusen konnten deutlich mehr apoptotische Zellen nachgewiesen werden.
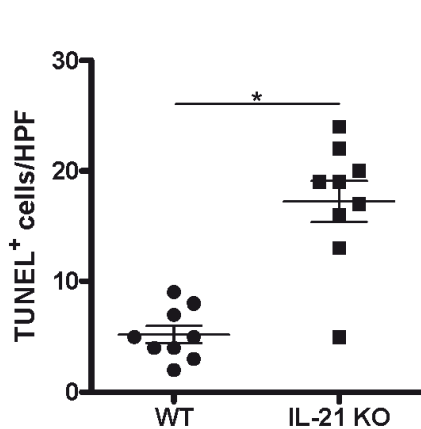
Abb.10: TUNEL-Assay von Tumoren an Tag 42.

Eine Inhibition von IFN-γ führte zu einem Rückgang CD8/CD103+ T-Zellen und verminderte somit die zytotoxische Kapazität dieser Zellpopulation.
Daraus konnten wir feststellen, dass das Vorkommen zytotoxischer CD8/CD103+ T-Zellen in IL-21 defizienten Mäusen abhängig von der IFN-γ Konzentration ist.

14

1.4 Diskussion

Die Ätiologie chronisch entzündlicher Darmerkrankungen, wie Morbus Crohn und Kolitis ulzerosa, ist noch nicht hinreichend aufgeklärt. Eine der schwerwiegenden Langzeitkomplikation der Kolitis ulzerosa ist die Entstehung von kolorektalen Karzinomen.

Immunologisch wird die chronische Entzündungsreaktion durch eine Vielzahl von proinflammatorischen Zytokinen, wie z.B. IL-21 charakterisiert. Wir konnten hier zeigen, dass IL-21 entscheidend an der Polarisierung proinflammatorischer T-Helfer Zellen beteiligt ist, im Besonderen der Th-17 Zelle.

Neben der Tumorproliferation spielt auch die Tumor-Immunosurveillance eine tragende Rolle in der Entwicklung von Entzündungs-assoziierten Tumoren. IL-21 trägt in unserem Tiermodell dazu bei, dass sich das Zytokinmilieu in Tumoren verändert und damit auch Einfluss auf die körpereigene Tumorregulation nimmt.

In der experimentellen, chronischen Kolitis konnte nahezu keine Entzündungsreaktion in Abwesenheit von Interleukin-21 beobachtet werden. Niedrige IL-17 und IFN-γ Konzentrationen bewahrten die Versuchstiere vor der Entwicklung einer chronischen Kolitis.

Im Kolitis-induzierten Tumorwachstum konnte bei beiden Versuchsgruppen eine Entzündungsreaktion beobachtet werden. Gekennzeichnet war diese in wild-typ Tieren erneut durch eine hohe Produktion von IL-17. Hingegen zeigten sich in IL-21 defizienten Mäusen hohe IFN-γ Konzentrationen, welche die Kolitis hervorriefen.

Die mukosale Entzündung beeinflusst das immunologische Tumormilieu nachhaltig und führt in kolorektalen Karzinomen zu einer Infiltration von Th-17 Zellen [9]. In dieser Arbeit war das Kolitis-assoziierte Tumorwachstum in wild-typ Mäusen durch eine hohe IL-17A Produktion und eine verringerte IFN-γ Konzentration gekennzeichnet. Ebenfalls zeigen unsere Ergebnisse, dass das Vorkommen dieser zwei T-Zell Subtypen entscheidend durch IL-21 bestimmt wird.

In Abwesenheit von IL-21 war die Entzündungsreaktion durch hohe IFN-γ und niedrige IL-17A Konzentration gekennzeichnet. Die Reduktion der Tumorentstehung ist dabei in unserer Arbeit nicht auf eine ausbleibende Kolitis zurückzuführen, sondern vielmehr Resultat des veränderten Tumormilieus. *Stolfi et al* kamen dagegen zu dem Ergebnis, dass genau diese ausbleibende Entzündung zu einer Reduktion

der Tumoranzahl in IL-21$^{-/-}$ Mäusen geführt hat [10]. Diese Reduktion der Entzündung konnte in unseren Experimenten jedoch nur in der chronischen Kolitis ohne Tumorwachstum nachgewiesen werden.

Die Administration von AOM bewirkt neben der Initiierung des Tumorwachstums auch die Apoptose der Kolonepithelzellen. Resultat dieser gestörten epithelialen Barrierefunktion ist eine erhöhte bakterielle Translokation in die Kolonschleimhaut [11]. Bedingt durch diese bakterielle Fehlbesiedlung kommt es zu einer gesteigerten intestinalen Entzündungsreaktion und der Infiltration von proinflammatorischen Makrophagen und T-Zellen, welche nachhaltig das Tumorwachstum unterstützen.
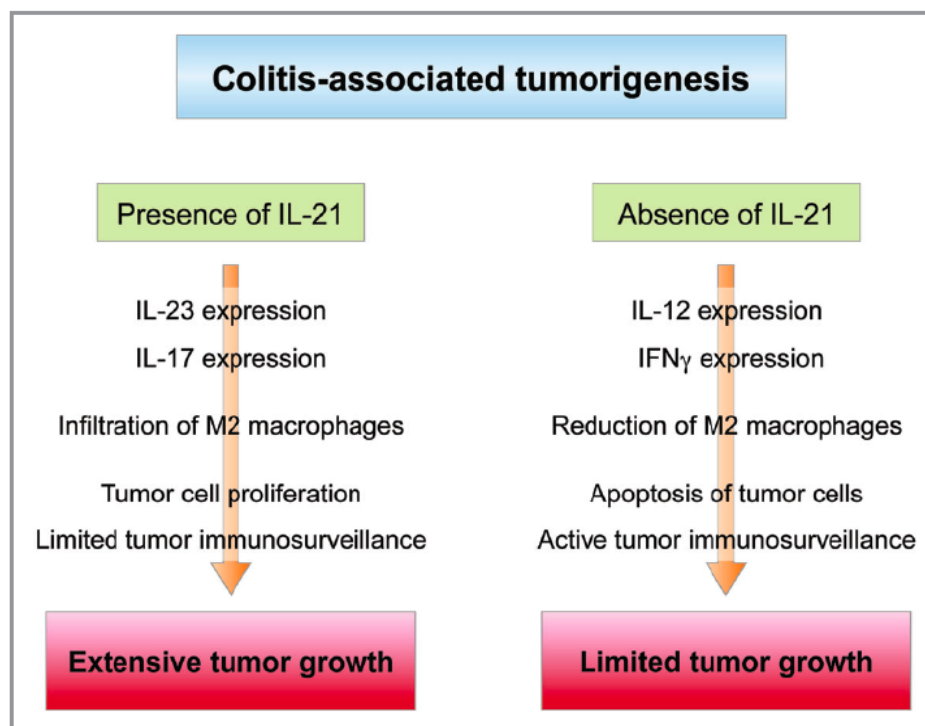
Abb. 11: Schematische Charakteristik des Tumorwachstums in Abwesenheit von IL-21 im Vergleich zu einem IL-21 reichen Tumormilieu [12].

Vielmehr deuten unsere Daten daraufhin, dass die Infiltration von Entzündungszellen ins Tumormilieu eine entscheidende Rolle in der Regulation des Tumorwachstums darstellt. Dieser Effekt beruht in unseren Beobachtungen auf der Tumor-Immunosurveillance durch zytotoxische T-Zellen. CD8^{+} T-Zellen besitzen die Möglichkeit Tumorepithelzellen zur Apoptose zu bringen indem E-Cadherin - ein

CD103 Ligand - gebunden wird. Die zytotoxische Kapazität wurde vorranging durch eine erhöhte Granzyme B Sekretion CD8/CD103^{+} zytotoxischer T-Zellen, in IL-21$^{-/-}$ Mäusen, vermittelt. Eine hohe IFN-γ Konzentration in Abwesenheit von IL-21 im Tumormilieu ermöglicht die Reifung und Infiltration von zytotoxischen CD8^{+} T-Zellen und erhöht deren Vorkommen [13]. Unter Blockade von IFN-γ wird die Tumorregulation teilweise inhibiert und führte zu einem deutlichen Anstieg der Tumoranzahl in IL.21$^{-/-}$ Mäusen. Die erhöhte IFN-γ Produktion sorgt in IL-21 defizienten Mäusen somit sowohl für die Entwicklung einer chronischen Kolitis als auch für eine verbesserte Tumorabwehr.

Der Zusammenhang von IFN-γ und tumorbekämpfender Immunität konnte bereits bei verschiedenen experimentellen Tiermodellen und humanen Tumorentitäten beschrieben werden und bedingt weiteren Untersuchungen [14], [15], [16].

Zusammenfassend konnten wir nachweisen, dass IL-21 die Fähigkeit besitzt das Tumormilieu und die Tumor-immunosurveillance, im experimentellen Tiermodell, nachhaltig zu beeinflussen.

Interleukin 21 controls tumour growth and tumour immunosurveillance in colitis-associated tumorigenesis in mice

Dominik Jauch, Maria Martin, Gabriela Schiechl, Rebecca Kesselring, Hans Jürgen Schlitt, Edward K Geissler, Stefan Fichtner-Feigl

ABSTRACT

Background and aims Colitis-associated tumorigenesis is a balance between proliferation of tumour cells and tumour immunosurveillance. The role of T-helper-cell-derived cytokines in tumour growth is not fully understood. In this study the authors investigated the influence of interleukin (IL) 21 on intestinal tumorigenesis.

Methods Chronic colitis was induced in IL-21$^{-/-}$ and littermate control wild-type mice with three cycles of 1.5% dextran sulphate sodium (DSS) over 7 days followed by 7 days of drinking water. Mice received an azoxymethane injection on day 0 of DSS-colitis to induce tumorigenesis. Immunohistochemistry was performed on inflamed and tumour-bearing areas of colons. Cytokine expression of isolated colonic CD4 T cells was determined by ELISA. Cytotoxic capacity of isolated colonic CD8 T cells targeting tumour cells was evaluated by flow cytometry and quantitative cytotoxicity assay. Apoptosis of tumour cells was determined by TUNEL assay of colonic sections.

Results Increasing expression of IL-21 was observed in chronic colitis, which showed functional importance, since IL-21 deficiency prevented chronic DSS-colitis development. Further, in the absence of IL-21, significantly fewer tumour nodules were detected, despite a similar extent of intestinal inflammation. In wild-type mice, 8.6±1.9 tumour nodules were found compared with 1.0±1.2 in IL-21-deficient mice. In tumour-bearing IL-21-deficient mice, intestinal inflammation was restored and partly dependent on interferon (IFN)-γ, whereas the inflammation in wild-type mice showed high IL-17A concentrations. In these rare tumours in IL-21-deficient mice, tumour cell proliferation (Ki-67) was decreased, while cell apoptosis was increased, compared with wild-type mice. Increased IFNγ expression in tumour-bearing IL-21-deficient mice led to increased tumour immunosurveillance mediated by cytotoxic CD8CD103 T cells targeting E-cadherin$^+$ colonic tumour cells and therefore limited tumour growth.

Conclusion These results indicate that IL-21 orchestrates colitis-associated tumorigenesis, leading to the hypothesis that high IFNγ and low IL-17A expression reduces tumour cell proliferation and increases tumour immunosurveillance.

INTRODUCTION

Long-lasting inflammatory bowel disease is accompanied by an increased risk of colorectal adenocarcinoma development.[1–4] Based on information obtained from a variety of animal studies, it is obvious that the presence of chronic inflammation provides a milieu that favours tumour cell proliferation. For instance, molecules that mediate or regulate proinflammatory innate immune signalling have been shown to influence the local milieu during colitis-associated tumour growth.[5–14] One of the key features of cells of the innate immune system during colonic tumour development is the production of cytokines that induce antigen-driven differentiation of the adaptive immune system.[15] Therefore the role of adaptive immunity in establishing the local cytokine milieu during colitis-

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Significance of this study

What is already known about this subject?
► Chronic inflammation supports tumour growth.
► Tumour immunosurveillance can suppress tumour growth.
► IL-21 can tip the balance between Th1 and Th17 differentiation.
► IL-21-deficient mice are protected from acute intestinal inflammation.

What are the new findings?
► Intestinal tumour growth is reduced in the absence of IL-21 and this reduction is not due to ameliorated colitis.
► IL-21 is necessary to establish a tumour-supportive colonic micro milieu.
► In the absence of IL-21, colonic inflammation is driven by IFNγ which leads to increased tumour immunosurveillance.
► Tumour immunosurveillance is mediated by CD8CD103 T cells targeting E cadherin+ transformed epithelial cells.

How might it impact on clinical practice in the foreseeable future?
► Inflammatory infiltrates can be detected in a broad variety of tumours and their metastases. Understanding the inflammatory infiltrate characterising the tumour microenvironment and its effect on tumour immunosurveillance will help to establish new treatment options in cancer treatment. Targeting IL-21 is a potential target for limiting tumour growth and increasing tumour immunosurveillance in colitis-associated tumour growth.

associated tumour growth is not yet understood. In particular, adaptive Th1 and Th17 cells have the capacity to influence the local cytokine milieu in the colon, polarising the immune response towards intestinal inflammation. IL-21 is a particularly interesting cytokine to study in this respect because it affects the development of naïve T cells into Th1 or Th17 cells.[15] The main sources of IL-21 under autoinflammatory conditions are NK cells and activated CD4 T cells.

In the present study, we aimed to examine the importance of IL-21 in the development of chronic intestinal inflammation as it relates to tumour growth. Further analyses focused on the importance of tumour immunosurveillance mediated by cytotoxic CD8 T cells and its dependency on the local cytokine milieu, which is influenced by the presence of IL-21 during chronic intestinal inflammation with tumour growth. Results from our study suggest that IL-21 is essential for the generation of a tumour-proliferative cytokine milieu and for the control of tumour immunosurveillance, both of which are key aspects of colitis-associated tumour growth.

METHODS

Mice
Specific pathogen-free IL-21$^{-/-}$ (B6;129S5) and littermate control wild-type mice (2—4 months old) were housed in the animal facility at the University of Regensburg. IL-21$^{-/-}$ B6;129S5 mice were initially obtained from the Mutant Mouse Regional Resource Center (University of California Davis). For transfer colitis, IL-21$^{-/-}$ mice and littermate controls were back-crossed on to a C57BL/6 background. Animal use was approved by the laboratory animal care guidelines of the University of Regensburg.

Induction of colitis
Chronic dextran sulphate sodium (DSS)-colitis was induced by three cycles of 1.5% DSS in drinking water for 1 week followed by normal drinking water for 1 week. Colitis-associated tumorigenesis was induced by intraperitoneal injection of a single dose of azoxymethane (AOM) (10 mg/kg; Sigma Aldrich, St Louis, Missouri, USA) on day 0, followed by chronic DSS-colitis.

Cell isolation and cytokine measurement
Lamina propria mononuclear cells were isolated from colonic tissues as previously described.[16 17] CD4 cells were isolated by magnetic bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured for 48 h under stimulation with plate-bound antibody to CD3 (10 μg/ml) and soluble antibody to CD28 (1 μg/ml) (BD Biosciences, San Jose, California, USA). Cytokine concentrations were measured using ELISA kits, according to the manufacturer's instructions (BD Biosciences, San Jose, California, USA).

Immunohistochemistry
Sections were incubated with antibodies to Ki-67 (Dako, Hamburg, Germany), Foxp3 (Abcam, Cambridge, UK), β-catenin (Cell Signaling Technology, Danvers, Massachusetts, USA) and E-cadherin (Cell Signaling Technology). TUNEL assay was performed according to the manufacturer's (Roche, Mannheim, Germany) protocol. For calculation of the extent of inflammation, H&E sections were examined by investigators blinded to the experimental protocol and according to a previously published scoring system.[18]

CTL assays
CTL assays (cytotoxic T-lymphocyte assay) were performed using CD8 cells isolated from DSS-treated, tumour-bearing mice.

Isolated CD8 cells were co-incubated in vitro with either CT-26 cells or E-cadherin-transfected CT-26 cells (5×10^4 cells). Cytolytic activity was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA). Performing a cytotoxicity assay with CD8 T cells obtained from B6;129S5 mice using allogeneic CT-26 cells resulted in a higher baseline level; however, the relative increase in cytotoxicity between treatment groups, compared with baseline levels, remained quite stable.

Statistical analysis
For calculation of differences in histology score and proliferation index, a Kruskal—Wallis test with Dunn's multiple comparison test was used. For calculation of differences in the number of tumour nodules and cytokine concentrations, a two-way analysis of variance test with Bonferroni post-test was used. A value of p<0.05 was considered significant.

RESULTS

IL-21 is upregulated in chronic intestinal inflammation
IL-21 is a key effector cytokine for acute experimental colitis.[19] In our studies, we began to dissect the importance of IL-21 in the development of chronic intestinal inflammation and the related long-term complication of colonic tumour growth by first investigating chronic colitis without tumour growth. We found that increasing amounts of IL-21 are detectable during the course of chronic colitis induced by DSS or 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) in wild-type mice, or by transfer of naïve T cells to Rag1$^{-/-}$ mice (online supplementary figure S1A,B).

IL-21-deficient mice are protected against the development of chronic DSS-colitis
After DSS administration to wild-type mice, we observed severe chronic colitis. However, IL-21-deficient mice were protected from this type of inflammation (figure 1A). Further, on day 42 we found a dramatic loss of colonic crypts and a significant reduction in goblet cell numbers in wild-type mice. In contrast, the morphology and architecture of colonic tissue obtained from IL-21-deficient mice treated with DSS remained mostly intact (figure 1B). Notably, general cellular proliferation measured by Ki-67 staining within intestinal tissue was significantly decreased after DSS treatment in IL-21-deficient mice (online supplementary figure S2).

We further determined the expression levels of IL-17A and IFNγ on day 42 of chronic DSS-colitis induction. As shown in figure 1C, isolated CD4 cells from mesenteric lymph nodes of IL-21-deficient mice produced significantly less IL-17A and IFNγ than wild-type animals. Similar findings were obtained in the cell transfer colitis model (online supplementary figure S3). A potential role for IL-17A and low IFNγ in an 'IL-21-mediated' tumour-promoting effect was then considered.

Indeed, previous reports suggest that IL-21 can modulate the balance between Th17 cells and Foxp3$^+$ Treg.[20] We did not observe any difference in the distribution of Foxp3$^+$ Treg on day 42 after DSS treatment initiation in wild-type versus IL-21-deficient mice (online supplementary figure S4). This observation is in contrast with data obtained from mice with acute DSS-colitis (online supplementary figure S5). Similar changes were obtained via flow cytometric analysis examining for CD4 Foxp3$^+$ Treg isolated cells from the colon of wild-type and IL-21-deficient mice (data not shown).

Tumour growth during chronic DSS-colitis is dependent on the presence of IL-21
We next combined chronic DSS-colitis with an initial intraperitoneal administration of the carcinogenic substance AOM. We

19

Figure 1 Interleukin (IL)-21-deficient mice are protected from chronic dextran sulphate sodium (DSS)-colitis. (A) H&E staining of representative colon sections and histology score on day 42 of chronic DSS-colitis. Data shown are mean values ± SEM and derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (B) Alcian blue/periodic acid Schiff (PAS) staining of representative colon sections on day 42 of chronic DSS-colitis. (C) IL-17A and interferon (IFN)-γ expression on day 42 of chronic DSS-colitis. CD4 cells were extracted from mesenteric lymph nodes and stimulated for 48 h. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *p≤0.05. KO, knock-out; WT, wild-type.
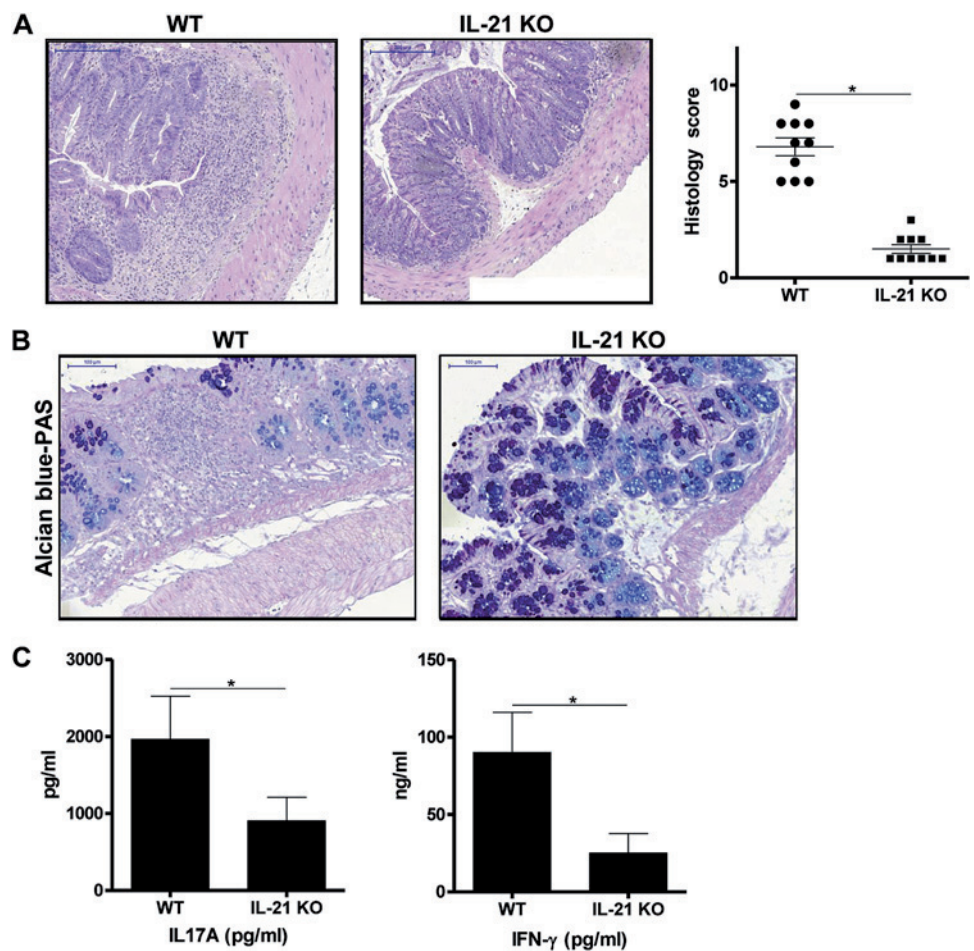
found that wild-type mice displayed an average of eight tumour nodules per colon on day 42, whereas there was only about one in IL-21-deficient mice (figure 2A).

H&E staining revealed that tumour nodules consisted mostly of adenoma tissue, with low- to high-grade intraepithelial neoplasia (figure 2B). Although the morphology of tumours from wild-type and IL-21-deficient mice was similar, the diameter of tumours from IL-21-deficient mice was 30—40% less. Consistent with this observation, Ki-67 staining revealed a lower cell proliferation rate in tumours derived from IL-21-deficient mice (figure 2C). Examination of transformed epithelial cells revealed an intracellular and nuclear expression pattern of β-catenin in tumour cells in wild-type and IL-21-deficient mice, whereas non-tumorous epithelial cells showed weak cell membrane staining of β-catenin (figure 2D). To rule out the possibility that the decreased tumour burden in IL-21-deficient mice on day 42 was because these mice exhibit less severe colitis during the early course of the disease, we observed wild-type and IL-21-deficient mice until day 84 after the initiation of the model. As shown in figure 2E, wild-type mice had developed an average of nine tumour nodules on day 42 and an average of almost 20 tumour nodules on day 84. IL-21-deficient mice showed hardly any tumour burden on either day 42 or day 84, despite the fact that chronic colitis lasted until day 84. This experiment demonstrates that, despite chronic colitis, starting between days 21 and 28, IL-21-deficient mice are largely protected from tumorigenesis.

Tumour growth restores inflammatory signs in IL-21-deficient mice

Initially, we hypothesised that the reason for reduced tumour formation in IL-21-deficient mice would be reduced intestinal inflammation. However, we found that the inflammation was similar in wild-type and IL-21-deficient mice on day 42 of chronic DSS-colitis and AOM-induced tumour growth (figure 3A). It is worth noting that this restoration of intestinal inflammation in IL-21-deficient mice with AOM-induced tumour growth was observed despite the use of a similar approach for inducing the underlying chronic DSS-colitis (as shown in figure 2), with the exception of not administering AOM.

High IFNγ expression in IL-21-deficient mice with intestinal tumorigenesis

To further elucidate the inflammatory milieu associated with intestinal tumour growth with regard to IL-21 deficiency, we determined cytokine expression in inflamed areas of colitis and tumour-bearing areas. Figure 3b shows a significant decrease in IL-17A production on day 42 in tumour-bearing IL-21-deficient mice compared with results in wild-type mice. However, IFNγ expression during chronic DSS-colitis with AOM-induced tumour growth showed the opposite: in the presence of tumours, an increase in IFNγ production was detected in IL-21-deficient mice. These results suggest that the presence of AOM-induced tumours resulted in a change in the local cytokine milieu, and that a high concentration of IFNγ expression in IL-21-deficient mice does not support tumour growth to the extent seen in an IL-17A-dominated micro milieu in wild-type mice.

We further investigated the expression pattern of IL-6 and IL-22, cytokines that are both potentially proproliferative and antiapoptotic for intestinal epithelial cells. There were no changes in IL-6 or IL-22 production on day 42 (figure 3B). In
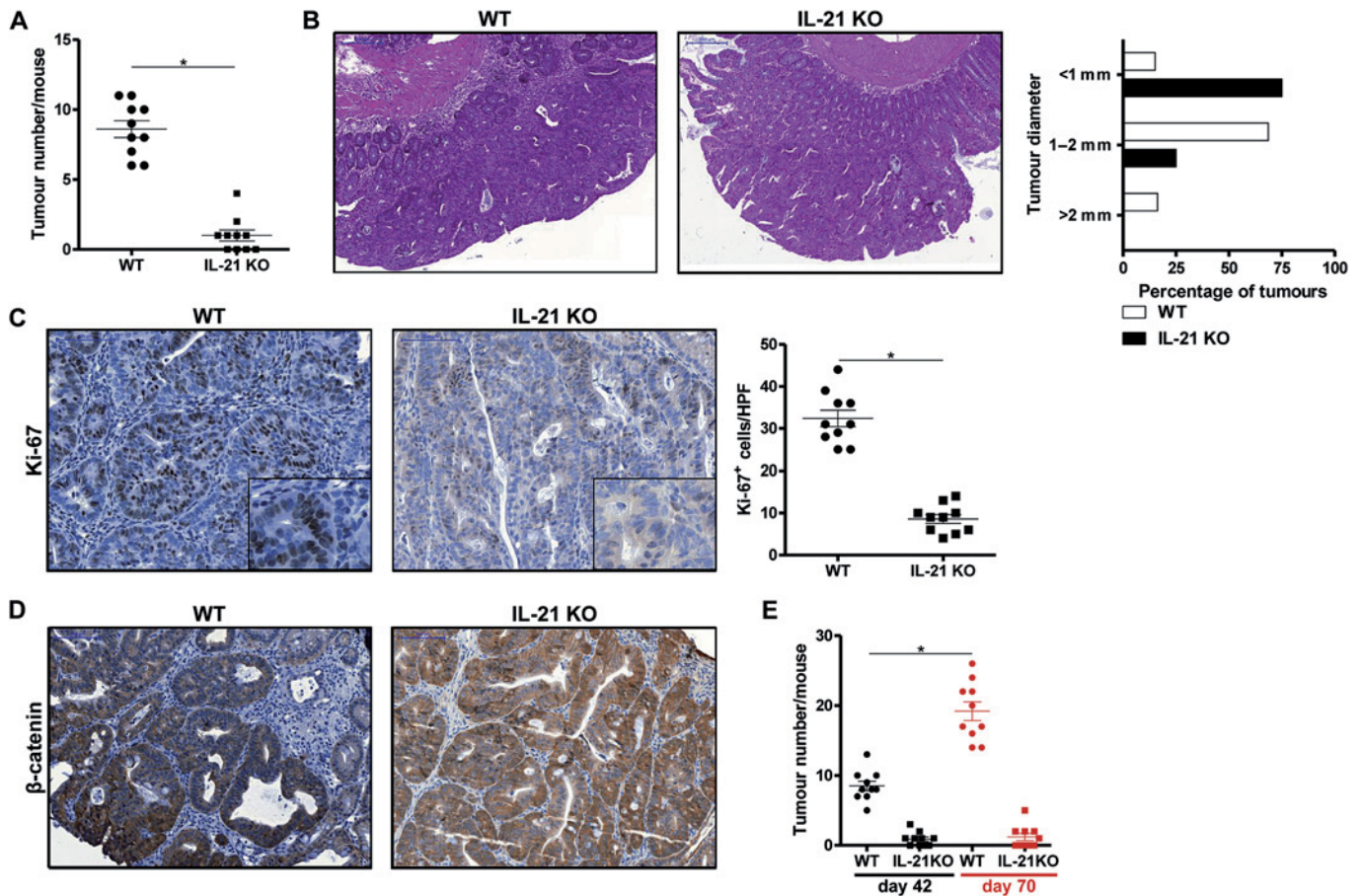
Figure 2 Tumour number is decreased in the absence of interleukin (IL0-21. (A) Number of tumour nodules on day 42 of chronic dextran sulphate sodium (DSS)-colitis with azoxymethane (AOM)-induced tumour growth. Data shown are mean values $\pm$ SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p$\leq$0.05. (B) H&E staining in representative tumour areas of colon sections and tumour size on day 42 of chronic DSS-colitis with AOM-induced tumour growth. Measurements of tumour nodules refer to diameter and were made using a digitally obtained image of the whole tissue section. Evaluation was performed using the Mirax Viewer software (Carl Zeiss AG, Germany). (C) Ki-67 staining of representative tumour areas of colon sections and proliferation score on day 42 of chronic DSS-colitis with AOM-induced tumour growth. Data shown are mean values $\pm$ SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p$\leq$0.05. (D) β-Catenin staining of representative tumour areas of colon sections on day 42 of chronic DSS-colitis with AOM-induced tumour growth. (E) Number of tumour nodules on day 42 and 84 of chronic DSS-colitis with AOM-induced tumour growth. Data shown are mean values $\pm$ SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p$\leq$0.05. HPF, high power field; KO, knock-out; WT, wild-type.

addition, we determined the distribution of follicular helper CD4 T cells in mesenteric lymph nodes on day 35 of chronic DSS-colitis with AOM-induced tumour formation. We found no changes in the number of these cells (as determined by flow cytometry for CD4CD44^{high}PD1^{+}CXCR5^{high} cells) in wild-type or IL-21-deficient mice (online supplementary figure S6). Further, we did not observe any differences in architecture of spleens and mesenteric lymph nodes.

As tumour-bearing IL-21-deficient mice showed an unexpected change in cytokine production on day 42, we determined the expression of IL-17A and IFNγ during the early phase of chronic DSS-colitis with AOM-induced tumour transformation of colonic epithelial cells. IL-17A was only detectable at low concentrations on day 7 and day 21 in IL-21-deficient mice. However, IFNγ showed a significant increase on day 21 in IL-21-deficient mice with tumour growth (figure 3C). These data indicate that the cytokine switch occurs during the transition phase from acute to chronic intestinal inflammation after AOM-induced tumour transformation.

To investigate whether changes in histological architecture of colons from wild-type mice or IL-21-deficient mice during chronic DSS-colitis with AOM-induced tumorigenesis occur

during the transition phase from acute to chronic colitis, we carried out a time course experiment to assess histological scoring on a weekly interval. We found that the onset of increased colitis in IL-21-deficient mice was on day 21 and further increased until day 28 (figure 3D). Therefore the extent of inflammation changes in accordance with the increase in IFNγ production in IL-21-deficient mice during chronic DSS-colitis with AOM-induced tumorigenesis.

An adaptive immune response characterised by the presence of IL-17A or IFNγ is preceded by the production of IL-23 or IL-12, respectively. Therefore we determined the production of these two cytokines by colonic CD11b cells during the transition phase of chronic DSS-colitis with AOM-induced tumorigenesis. We found that IL-23 was upregulated in wild-type mice, whereas IL-12p70 was greatly increased in IL-21-deficient mice (figure 3E). We hypothesised that this change in cytokine production may be based on an epithelial-derived factor that is stimulating antigen-presenting cells to produce either IL-23 or IL-12. We harvested colonic epithelial cells on day 21 from either wild-type or IL-21-deficient mice and incubated naive CD11b cells with lysates of these epithelial cells. Interestingly, we found that lysates obtained from IL-21-deficient mice were able to
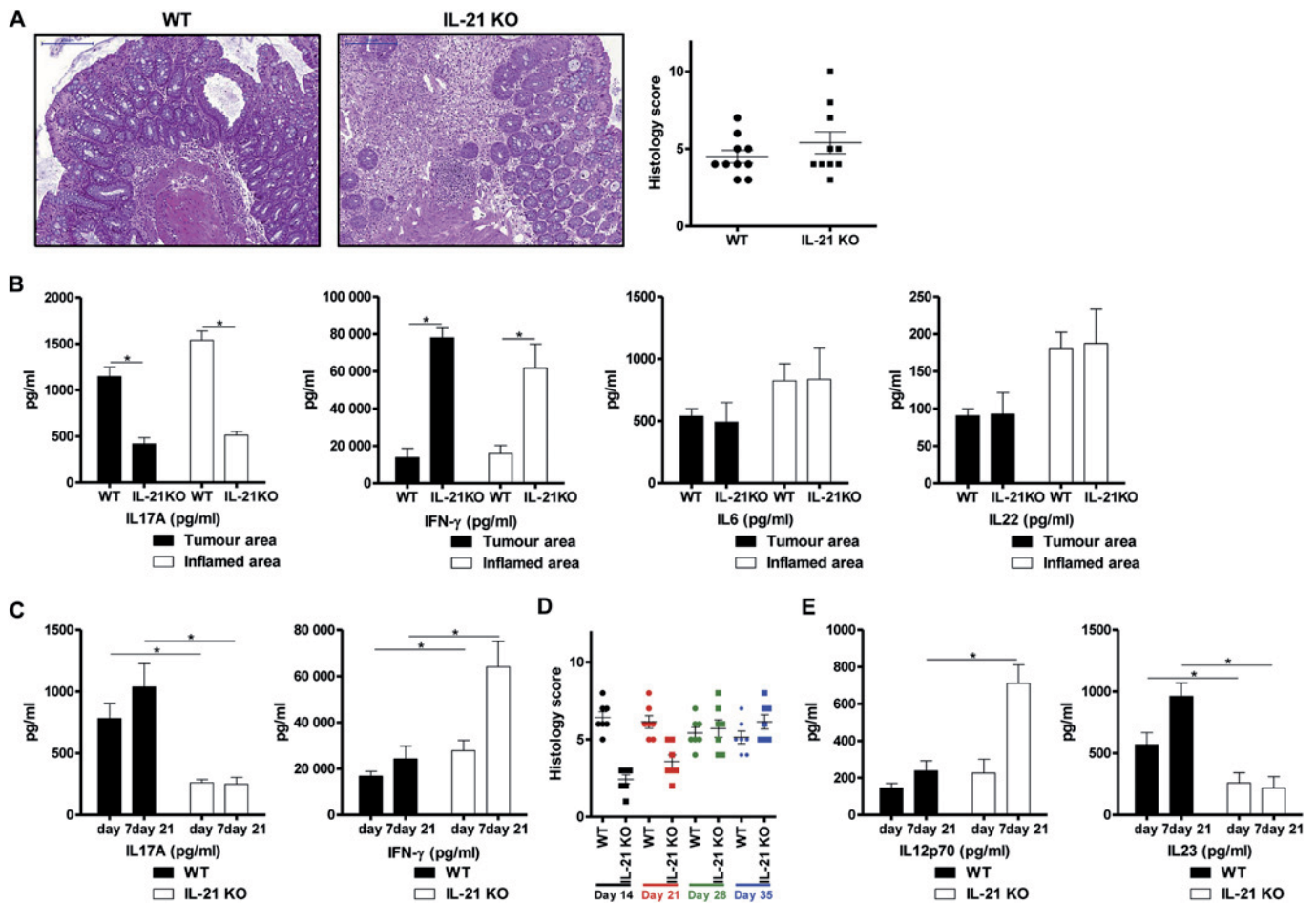
21

Figure 3 Restored inflammation in interleukin (IL)-21-deficient mice with chronic dextran sulphate sodium (DSS)-colitis and tumorigenesis. (A) H&E staining of representative inflammatory areas of colon sections and histology score on day 42 of chronic DSS-colitis with azoxymethane (AOM)-induced tumour growth. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (B) IL-17A, interferon (IFN)-γ, IL-6 and IL-22 expression on day 42 of chronic DSS-colitis with AOM-induced tumour growth. CD4 or CD11b cells were extracted from the lamina propria of inflamed and tumour-bearing areas, and then stimulated for 48 h. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *p≤0.05. (C) IL-17A and IFNγ expression on day 7 and day 21 of chronic DSS-colitis with AOM-induced tumour growth. CD4 cells were extracted from the lamina propria from inflamed and tumour-bearing areas and stimulated for 48 h. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *p≤0.05. (D) Histology score on days 14, 21, 28 and 35 of chronic DSS-colitis with AOM-induced tumour growth. Data shown are mean values ± SEM, derived from at least seven mice per group. Individual points represent one mouse. (E) IL-23 and IL-12p70 expression on day 7 and day 21 of chronic DSS-colitis with AOM-induced tumour growth. CD11b cells were extracted from the lamina propria and stimulated for 48 h. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *p≤0.05. KO, knock-out; WT, wild-type.

induce IL-12p70 production from either wild-type or IL-21-deficient CD11b cells (online supplementary figure S7A). We also generated conditioned media from complete colon tissue, isolated intestinal epithelial cells, lamina propria CD3 T cells, and F4/80[+] macrophages obtained from wild-type and IL-21-deficient mice on day 21 of DSS-colitis with AOM-induced tumorigenesis. Naive CD11b cells were then cultured in conditioned medium, and IL-12p70 production was determined. We found that only conditioned medium from colon tissue or isolated intestinal epithelial cells from IL-21-deficient mice were able to induce an increase in IL-12p70 production (online supplementary figure S7B). These changes in inflammatory response are also reflected in weight curves of wild-type and IL-21-deficient mice during chronic DSS-colitis with or without AOM-induced tumorigenesis (online supplementary figure S8). This set of data strengthens the hypothesis that epithelial-derived factors are at least partly responsible for polarisation of

the adaptive immune response during chronic DSS-colitis with AOM-induced tumorigenesis.

IFNγ induces inflammation and tumour control in IL-21-deficient mice

Further analysis was performed to determine the role of IFNγ during chronic intestinal inflammation and tumour growth. For this purpose, DSS-colitis with initial AOM injection was established and combined with IFNγ antibody administration twice weekly starting on day 14. We found that inhibition of IFNγ did not affect chronic colitis in wild-type mice; however, it significantly reduced colitis in IL-21-deficient mice (figure 4A). Furthermore, antibody interference with IFNγ resulted in an increase in tumour number in IL-21-deficient mice compared with IL-21-deficient mice receiving control IgG (figure 4B). Our results indicate that IFNγ at least partly mediates intestinal inflammation in IL-21-deficient mice with chronic DSS-colitis and AOM-induced tumour growth.

CD8 T cells control tumour growth

Previous work has shown that IFNγ is able to promote an antitumour response, which can be mediated by CD8 cytotoxic T cells. To test for a potential role of CD8 T cells in this model, we administered antibody to CD8 weekly starting on day 14 of chronic DSS-colitis with AOM-induced tumour growth. Although CD8 antibody treatment reduced the number of circulating CD8 T cells by 85% (data not shown), this depletion did not influence the level of inflammation of chronic DSS-colitis with AOM-induced tumour growth (figure 5A). The number of tumour nodules per mouse and the diameter increased significantly with CD8 T cell depletion in wild-type and IL-21-deficient mice (figure 5B and C). To determine if NKT cells or NK cells might be exerting cytotoxic effects in terms of tumour immunosurveillance during chronic DSS-colitis with AOM-induced tumour growth, we depleted these cell types by administering antibody against either CD1 or asialo GM1 (starting on day 14), respectively. As shown in online supplementary figure S9, neither depletion of NKT cells nor depletion of NK cells resulted in a change in tumour burden in wild-type and IL-21-deficient mice.

Cytotoxicity against tumour epithelial cells increases in IL-21-deficient mice

Previous studies show that cytotoxic CD8 T cells specific for tumour cells limit tumour growth (immunosurveillance), as CD8 T cells bearing CD103 ($\alpha_E\beta_7$) can bind E-cadherin on tumour cells.[21–23] Tumour cells from wild-type and IL-21-deficient mice have a similar E-cadherin expression pattern during chronic DSS-colitis with AOM-induced tumour growth (figure 6A). Therefore the potential target cell for cytotoxic CD8 T cells was available in either wild-type or IL-21-deficient mice. However, as shown in figure 6B, wild-type mice with AOM-induced tumour growth had fewer CD8CD103 T cells on day 28 than similarly treated IL-21-deficient mice.

The cytotoxic potential of CD8 T cells was demonstrated by the extent of granzyme B and perforin expression on day 28.[24 25] This evaluation time point was chosen in order to investigate the cytotoxic potential of CD8 T cells at the time of early tumour immunoediting. CD8 T cells from IL-21-deficient mice showed a significant increase in granzyme B and a slight increase in perforin expression. Cytotoxic effects of CD8 T cells isolated from colon tumours against (allogeneic) CT-26 colon tumour cells (not expressing E-cadherin) and CT-26 cells transfected with an E-cadherin-expressing plasmid were tested. CD8 T cells from wild-type mice exhibited only minimal cytotoxicity against untransfected CT-26 cells, although they did exhibit substantial cytotoxicity against transfected E-cadherin-expressing CT-26 cells (figure 6C). CD8 T cells isolated from IL-21-deficient mice showed a relatively enhanced cytotoxic effect against E-cadherin-expressing CT-26 cells. These results are consistent with our finding that tumours from IL-21-deficient, versus wild-type mice, showed a significant increase in apoptotic cells as determined by TUNEL staining (figure 6D). Finally, we examined the dependence of this cytotoxic effect on IFNγ and evaluated the presence of cytotoxic CD8CD103 T cells in the colon after IFNγ antibody treatment. The inhibition of IFNγ resulted in a significant reduction of CD8CD103 T cells in the colon of IL-21-deficient mice (figure 6E). Therefore we could demonstrate that the presence of cytotoxic CD8CD103 T cells in IL-21-deficient mice was dependent on IFNγ. Together, these data show that, while cytotoxic CD8CD103 T cells are potentially capable of exerting immunosurveillance functions against tumour cells, this function is probably controlled by the cytokine milieu in the colon.

Figure 4 Interferon (INF)γ regulates inflammation and tumour growth in the absence of interleukin (IL)-21. (A) H&E staining of representative colon sections of inflamed areas and histology score on day 42 of chronic dextran sulphate sodium (DSS)-colitis with tumour growth after inhibition of IFNγ. Inhibition of IFNγ started on day 14. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (B) Number of tumour nodules on day 42 of chronic DSS-colitis with azoxymethane-induced tumour growth after inhibition of IFNγ. Inhibition of IFNγ was started on day 14. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. KO, knock-out; WT, wild-type.
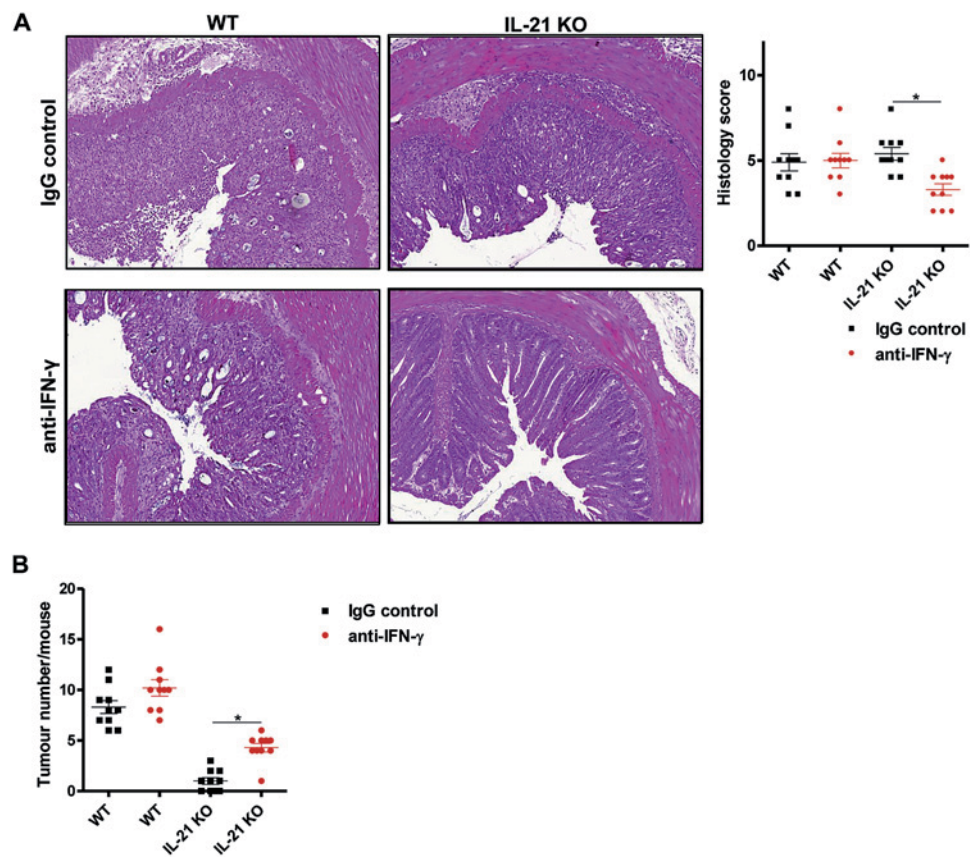
23

Figure 5 CD8 T cells control tumour growth. (A) H&E staining of representative colon sections of inflamed area and histology score on day 42 of chronic dextran sulphate sodium (DSS)-colitis with tumour growth, after deletion of CD8 T cells. CD8 T cell deletion was started on day 14. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (B) Number of tumour nodules on day 42 of chronic DSS-colitis with azoxymethane (AOM)-induced tumour growth, after deletion of CD8 T cells. CD8 T cell deletion was started on day 14. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (C) Diameter of tumour nodules on day 42 of chronic DSS-colitis with AOM-induced tumours after deletion of CD8 T cells. CD8 T cell deletion was started on day 14. Measurements of tumour nodules refer to diameter and were made using a digitally obtained image of the whole tissue section. Evaluation was performed using Mirax Viewer software (Carl Zeiss AG, Germany). KO, knock-out; WT, wild-type.
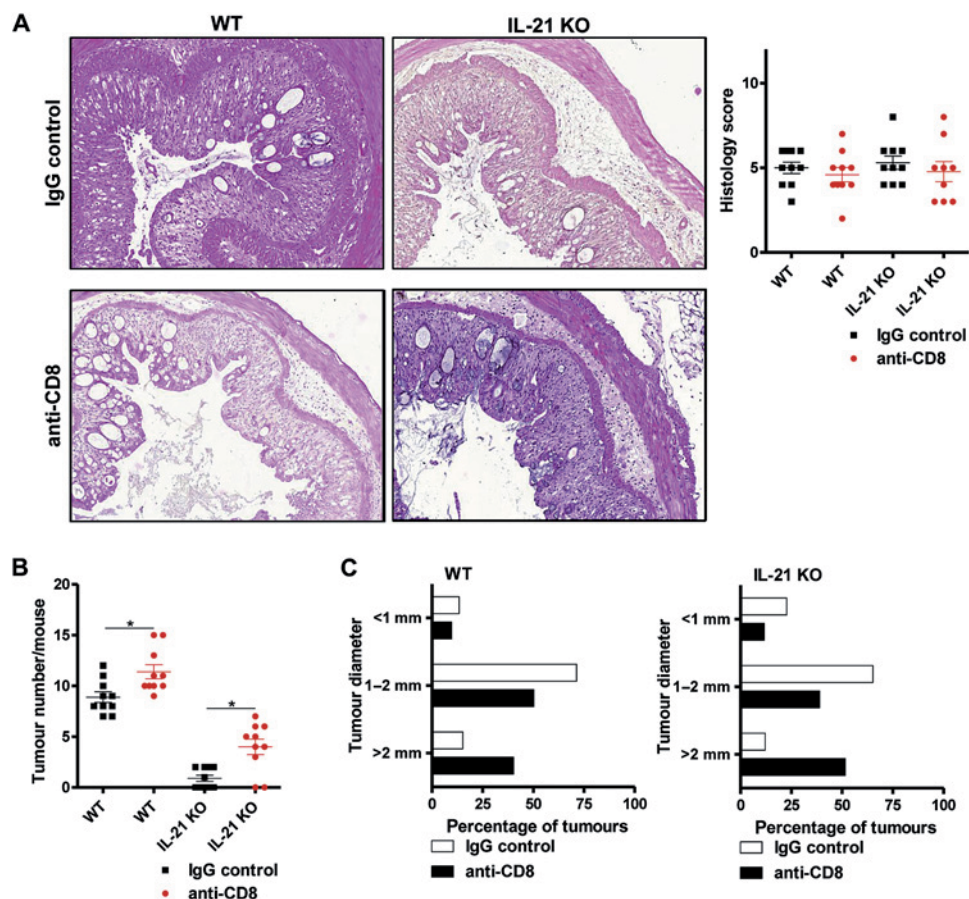
DISCUSSION

The aetiology of inflammatory bowel disease, consisting primarily of Crohn's disease and ulcerative colitis, is not fully understood.[26–28] One long-term consequence of an altered immune homoeostasis at the mucosal surface is development of colorectal cancer. In this article, we demonstrate that IL-21 is an important factor in tumour growth and immunosurveillance during colitis-associated tumorigenesis. In this regard, we show that IL-21 is necessary for the establishment of a tumour-supportive micro milieu in the colon, which is characterised by the presence of the cytokine IL-17A and limited tumour immunosurveillance through reduced concentrations and functional capacity of cytotoxic CD8CD103 T cells.

Although an important effect of IL-21 on the development of chronic intestinal inflammation was expected, the situation changed when tumour growth was accompanied by robust intestinal inflammation even in the absence of IL-21; this inflammation was partly mediated through IFNγ produced by CD4 T cells. It was obvious that the intestinal inflammation and tumour growth were able to interact in both directions. This important link between inflammation and cancer has been known for centuries.[29 30] The inflammation orchestrates the microenvironment around tumours, and the tissue distribution of T cell subsets in human cancers clearly shows that a broad variety of solid tumours are associated with an increase in tumour microenvironmental Th17 cells.[31 32] In our study, tumour growth in wild-type mice was associated with high production of IL-17A by infiltrating CD4 T cells and a reduced concentration of IFNγ, mimicking the cytokine pattern observed in a variety of human solid tumours.[33–36] Importantly, our results indicate that the balance between these two polarised T cell subsets is tipped by IL-21.

It is widely accepted that tumour cell proliferation can be mediated through T cells after stimulation with tumour antigen that is presented by tumour-associated macrophage cells, a cellular interaction known to induce IL-17 expression.[33] Our results indicate that IL-21 has a major role in the generation of a proliferative, IL-17A-based, tumour microenvironment. We have demonstrated that, in the absence of IL-21, the inflammatory response is characterised by high IFNγ concentration, not by high IL-17A concentration. Therefore one can assume that the reduction in tumour number in the absence of IL-21 is not based on the existence of inflammation, but rather on the specific characteristics of the tumour microenvironment. This change in tumour microenvironment is in concordance with findings of previous reports in which IL-21 could inhibit IFNγ expression when IL-21 was present at the time of naïve CD4 T cell priming under Th1 conditions.[37] In addition to our findings in mice, it has been shown that human biopsy specimens obtained from patients with Crohn's disease produce less IFNγ when IL-21 is blocked in an ex vivo culture.[38] This set of data published by Monteleone et al does not necessarily contradict our findings, as we have also found that IFNγ expression is very low in IL-21-deficient mice in the absence of tumours. Furthermore, Sarra et al demonstrated that, in the human gut, IL-21 is mainly produced by CD4 T cells co-expressing IFNγ.[39] Therefore, a likely explanation for reduced IFNγ production by ex vivo culture of biopsy specimens from patients with Crohn's disease is that the inhibition of IL-21 concomitantly blocks the production of IFNγ from the same cell.

It is well accepted that IL-21 plays a pathogenic role in intestinal inflammation. However, this effect has not been seen in a situation when tumour induction was incited by AOM. In our work, we show that epithelial cell lysates and conditioned
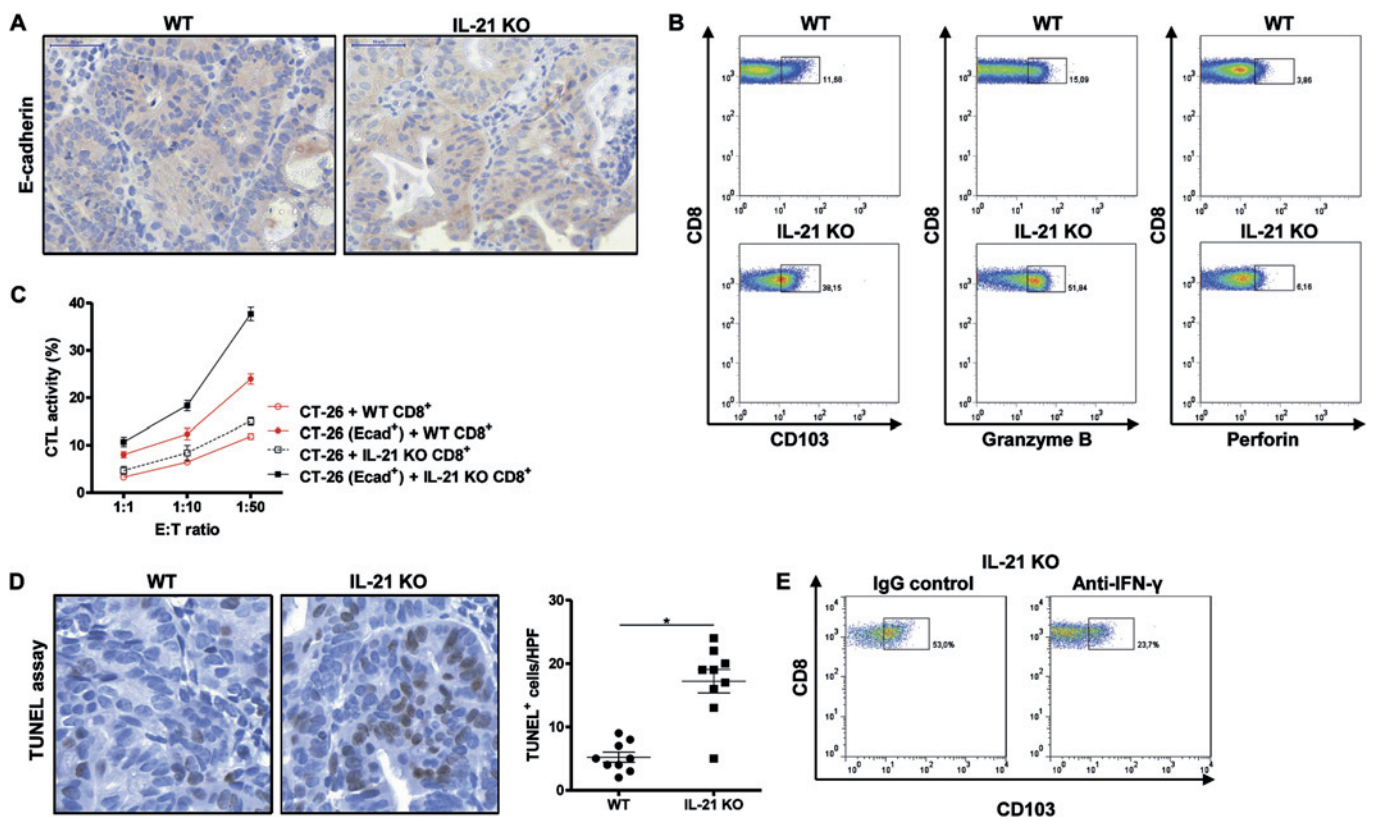
Figure 6 Tumour immunosurveillance increases in the absence of interleukin (IL)-21. (A) E-cadherin staining of representative tumour areas of colon sections on day 42 of chronic dextran sulphate sodium (DSS)-colitis with azoxymethane (AOM)-induced tumour growth. (B) Flow cytometric determination of CD8CD103, CD8granzyme B and CD8perforin cells in the colon on day 28 of chronic DSS-colitis with AOM-induced tumour growth. Cells were extracted from the lamina propria on day 28 and stained with fluorochrome-conjugated antibodies. (C) Cytotoxicity of CD8 T cells against CT-26 cells or E-cadherin+ CT-26 cells. CD8 T cells were isolated from the colon on day 28 of chronic DSS-colitis with AOM-induced tumour growth and co-cultured with CT-26 cells or E-cadherin+ CT-26 target cells. Cytotoxicity was measured after 24 h of cytolytic activity. (D) TUNEL assay of representative tumour areas of colon sections on day 42 of chronic DSS-colitis with AOM-induced tumour growth. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤0.05. (E) Flow cytometric determination of CD8CD103 cells in the colon of IL-21-deficient mice on day 28 of chronic DSS-colitis with AOM-induced tumour growth after inhibition of interferon (IFN)γ. Inhibition of IFNγ was started on day 14. Cells were extracted from the lamina propria on day 28 and stained with fluorochrome-conjugated antibodies. CTL, cytotoxic T-lymphocyte; HPF, high power field; KO, knock-out; WT, wild-type.

media obtained from isolated epithelial cells of IL-21-deficient mice after AOM administration are able to induce a strong Th1 response that is in turn mediating the colitis, albeit with a different cytokine pattern of influence. We have not yet elucidated the actual factor released by intestinal epithelial cells to induce a Th1-polarised colonic microenvironment. One potential candidate for the induction of IL-12 production by CD11b cells with consecutive IFNγ expression is IL-18, which was initially described as IFNγ-inducing factor. IL-18 is upregulated in the intestinal mucosa of patients with inflammatory bowel disease, and blockade of IL-18 reduces the extent of intestinal inflammation induced by DSS.[40] In accordance with the hypothesis that IL-18 might initiate a tumour-suppressive Th1-based microenvironment, recent work from Salcedo et al has shown that IL-18-deficient mice have a much higher tumour burden than wild-type mice during chronic DSS-colitis with AOM-induced tumour formation.[7] However, additional research is necessary to test this hypothesis.

In addition, inflammatory cells in the tumour microenvironment may have an important role in initiating and maintaining protective antitumour immunity by tumour immunoediting. One such effect investigated in our study relates to the immunosurveillance of AOM-induced tumours mediated by cytotoxic cells. After excluding NK cells and NKT cells as possible cyto-

toxic effector cells, we could demonstrate that the capacity of CD8 T cells to be immunosurveillant is inherent in their ability to kill epithelial cell tumour lines bearing E-cadherin (a CD103 ligand) ex vivo. Notably, the presence of CD8CD103 cytotoxic T cells was greatly increased in the absence of IL-21 and dependent on IFNγ. These results are consistent with facilitated binding of antitumour CD8CD103 T cells to E-cadherin-expressing tumour cells.[41] Moreover, under these conditions, killing is presumably mediated by a granzyme B-dependent effect of CD8 cytotoxic T cells, as this potent cytotoxic molecule was highly upregulated in CD8 T cells of IL-21-deficient mice. The reason why a tumour microenvironment with a high expression level of IFNγ promotes an antitumour response probably lies in the capacity of IFNγ to facilitate maturation of CD8 cytotoxic T cells.[42] In fact, IFNγ has been associated with antitumour immunity and its dependence on the adaptive immune system in a variety of different tumour entities.[43 44] Previous in vitro experiments have shown that IL-21 can influence the proliferation of CD8 T cells, whereas the cytotoxic function is not compromised.[45 46] In addition, Hinrichs et al showed that antigen-induced acquisition of effector CD8 T cell phenotype and function is suppressed by the presence of IL-21.[47] These facts are in accordance with data presented in this article, since the number of CD8 T cells remained stable regardless of the presence of IL-21. However, the

25

functional capacity of cytotoxic CD8 T cells was greatly increased in the Th1-dominated environment present in IL-21-deficient mice with intestinal tumour induction.

Owing to the fact that on day 42 of chronic DSS-colitis with AOM-induced tumour growth in IL-21-deficient mice, the tumour cell proliferation index is ∼10%, while the apoptotic index is about 20%, we consider that these tumours may undergo regression to some degree on day 42. Our experience with colitis-associated tumorigenesis models shows that the rate of tumour cell proliferation is highest at the time when tumours start to become visible macroscopically and that tumour cell cytotoxicity becomes apparent slightly later than the first appearance of macroscopic tumour nodules. Therefore this timing difference is probably the explanation for the differences seen between the proliferation index and apoptotic index.

In conclusion, we have demonstrated in this study that the cytokine IL-21 is capable of influencing the development of chronic intestinal inflammation. Importantly, our results show that IL-21 is a key cytokine that tips the balance between a tumour-proliferative and a tumour-suppressive microenvironment, substantially influencing tumour growth.

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Figure S1
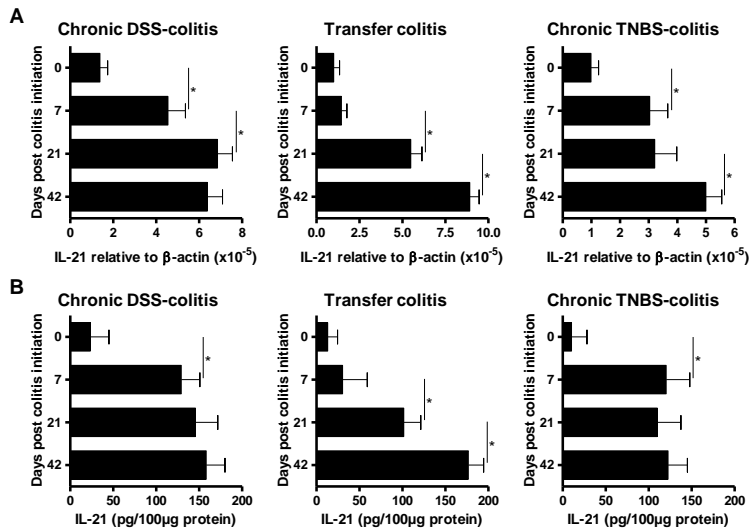
IL-21 is upregulated in chronic intestinal inflammation. (**A**) IL-21 mRNA expression of isolated colonic lamina propria mononuclear cells on indicated time points of chronic DSS-colitis, transfer colitis and chronic TNBS-colitis. IL-21 mRNA expression was determined by quantitative PCR analysis. Data shown are mean values ± SEM from at least five mice per group. *, p ≤0.05. (**B**) IL-21 expression in total colonic protein extracts on indicated time points of chronic DSS-colitis, transfer colitis and chronic TNBS-colitis. Cytokine concentrations were determined by ELISA. Data shown are mean values ± SEM from at least five mice per group. *, p ≤0.05.
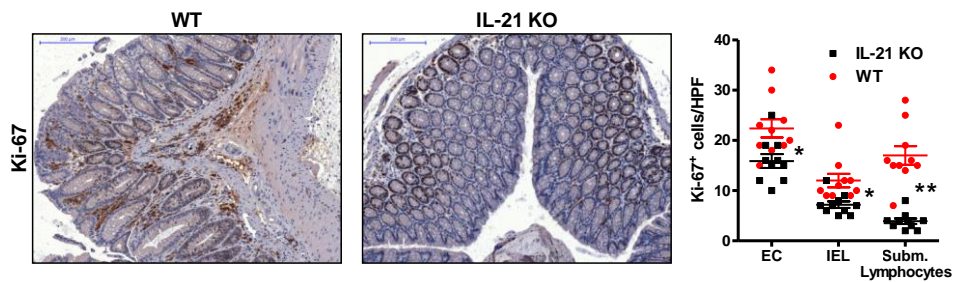
Morphologic characteristics of DSS-colitis. Ki-67 staining of representative colon sections and proliferation score on day 42 of chronic DSS-colitis. Data shown are mean values ± SEM and derived from at least ten mice per group. Individual points represent one mouse. *P≤0.05.

Due to the fact that IL-17A, in particular during an ongoing inflammatory response, is a potent inducer of cell proliferation, we determined the proliferation index on day 42 of chronic DSS-colitis in wild type and IL-21-deficient mice. We distinguished between cell proliferation of colonic epithelial cells, intraepithelial lymphocytes and submucosal lymphocytes by Ki-67 staining. As shown in Figure S2, the proliferation of colonic epithelial cells as well as of intraepithelial lymphocytes was significantly increased in wild type mice compared to IL-21-deficient mice. In addition, there is a highly significant increase of proliferation of lymphocytes in the submucosal area in wildtype when compared to IL-21-deficient mice.

Figure S3
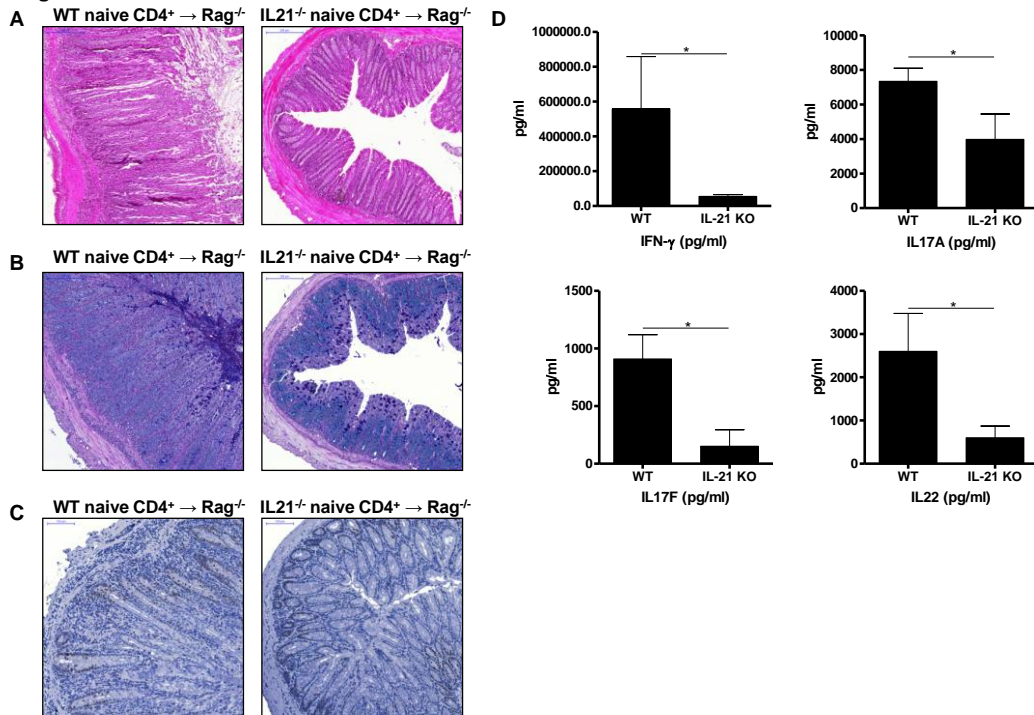
IL-21-deficient naïve T cells hardly induce intestinal inflammation in Rag1^{-/-} mice. (**A**) H&E staining of representative colon sections on day 42 of transfer colitis. (**B**) Alcian blue/PAS staining of representative colon sections on day 42 of transfer colitis. (**C**) Ki-67 staining of representative colon sections on day 42 of transfer colitis. (**D**) IL-17A, IFN-γ, IL-17F, IL-22 expression on day 42 transfer colitis. CD4^{+} cells were extracted from mesenteric lymph nodes and stimulated for 48h. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM and derived from at least five mice per group. *, p ≤0.05.

The transfer of IL-21-deficient naïve T cells resulted in a reduced extent of chronic inflammation, which was accompanied by a significant reduction of IL-17A, IFN-γ, IL-17F, and IL-22 production when compared to Rag1^{-/-} mice that received wildtype naïve T cells.

29

Figure S4
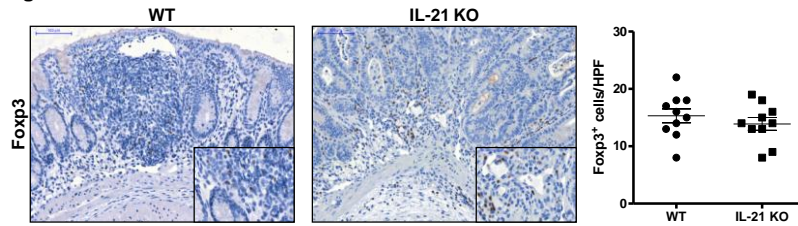
Foxp3$^+$ Treg expression is similar on day 42 of chronic DSS-colitis when comparing wild-type mice and IL-21-deficient mice. Foxp3 staining of representative colon sections on day 42 of chronic DSS-colitis. Data shown are mean values ± SEM and derived from at least ten mice per group. Individual points represent one mouse.

Figure S5
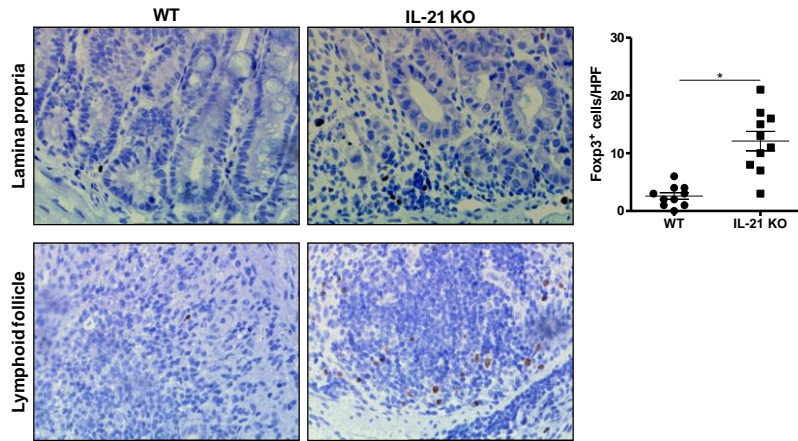
Foxp3+ Treg are upregulated during acute DSS-colitis in IL-21-deficient mice. Foxp3 staining of representative colon sections and colonic lymphoid follicles on day 7 of acute DSS-colitis. Data shown are mean values ± SEM and derived from at least ten mice per group. Individual points represent one mouse. *P≤0.05.

Figure S6
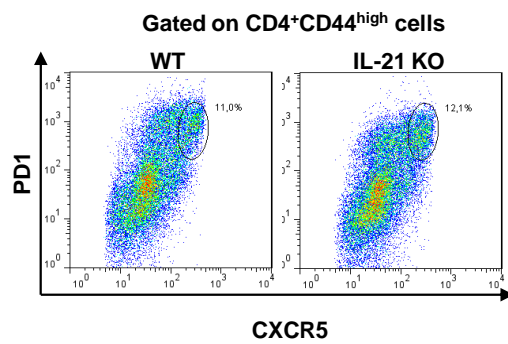
Distribution of follicular helper CD4 T cells in mesenteric lymph nodes on day 35 of chronic DSS-colitis with AOM induced tumour formation. We could not detect changes in the number of TFH cells (as determined by flow cytometry for CD4$^+$CD44^{high}PD1$^+$CXCR5^{high} cells) in wild-type or IL-21-deficient mice.

Figure S7
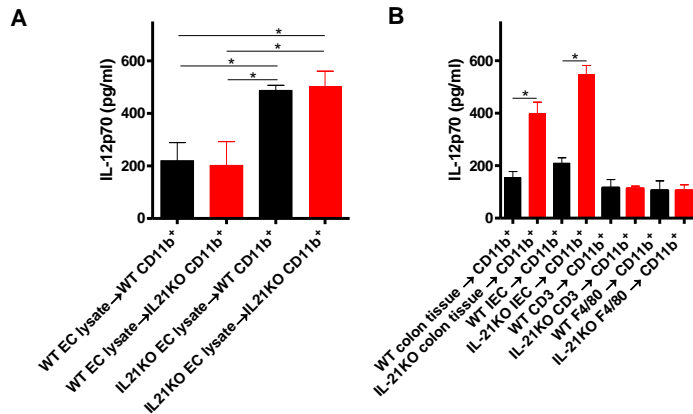
(**A**) IL-12p70 production from isolated splenic naive CD11b^{+} cells of wild-type or IL-21-deficient mice following the incubation with lysates from colonic epithelial cells obtained from wild-type or IL-21-deficient mice on day 21 of chronic DSS-colitis with AOM-induced tumour growth. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *P≤0.05.

(**B**) IL-12p70 production from isolated splenic naive CD11b^{+} cells of wild-type mice following the incubation with conditioned media from complete colon tissue, isolated intestinal epithelial cells, isolated lamina propria CD3^{+} cells and isolated lamina propria F4/80^{+} cells obtained from wild-type or IL-21-deficient mice on day 21 of chronic DSS-colitis with AOM-induced tumour growth. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean values ± SEM, derived from at least five mice per group. *P≤0.05. Conditioned medium was collected from cells incubated in serum-free medium for 24h and filtered through a 0.22-mm filter.

Figure S8
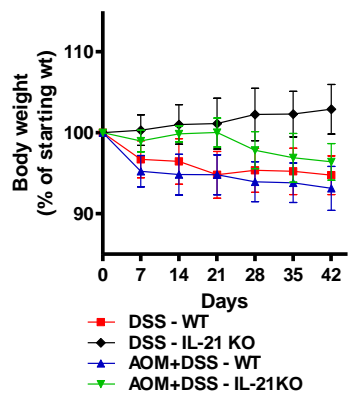
Body weight of mice with chronic DSS-colitis with or without AOM-induced tumour growth. (**A**) Body weight as a percent of starting weight. Data shown are mean values ± SEM and derived from at least six mice per group.

Figure S9
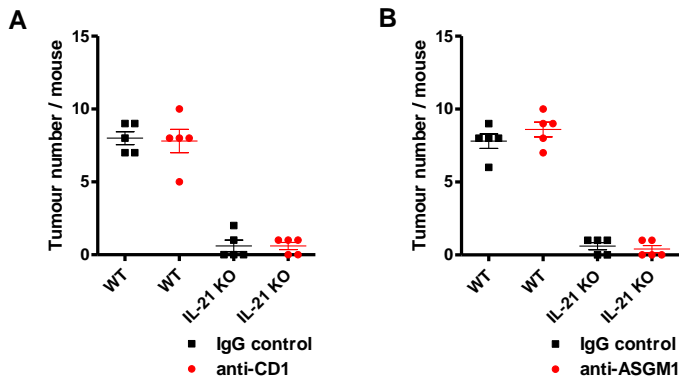
NKT cells or NK cells do not influence tumour growth. Number of tumour nodules on day 42 of chronic DSS-colitis with AOM-induced tumour growth, after deletion of NKT cells (anti-CD1-antibody) or NK cells (anti-asialo GM1- antibody). NKT or NK cell depletion was started on day 14. Data shown are mean values ± SEM, derived from 5 mice per group. Individual points represent one mouse.

Colitis-associated cancer: the dark side of inflammatory bowel disease

Silvio Danese,[1] Alberto Malesci,[1,2] Stefania Vetrano[1]

The intrinsic connection between inflammation and cancer promotion is well established and is especially strong in patients with colorectal cancer (CRC), the second most common cause of cancer-related death in Western countries.[1] [2] The administration of non-steroidal anti-inflammatory drugs in controlled studies reduces the incidence of colon cancer in patients with familial adenoma polyposis, confirming the link between inflammation and colon cancer.[3] Several lines of evidence indicate that chronic inflammation predisposes the tissue to cancer by inducing gene mutation, inhibiting apoptosis or stimulating angiogenesis and cell proliferation. Patients with inflammatory bowel disease (IBD) are at increased risk of developing CRC and, in particular, an association between IBD and the development of colitis-associated cancer (CAC) has been reported. Although the severity and extent of disease and the duration of inflammation seem to be major factors associated with the development of CAC in patients with IBD, the molecular determinants of this link have only recently started to be elucidated. Studies have highlighted the presence of leucocyte infiltration and inflammatory mediators in the tumour microenvironment, indicating that immune cells are major players in tumour promotion.[2] [4]

Preclinical models of CAC are helping to clarify the mechanisms underlying cancer-related inflammation. The direct role of the adaptive immune response in promoting CAC is becoming more evident.[2] Although T cell responses fuel the inflammatory process and orchestrate the microenvironment surrounding tumours by contributing to the proliferation, migration and survival of cancer cells, activation of the adaptive immune

system also plays an important role in inhibiting tumour growth and progression through the recognition and rejection of malignant cells, a process referred to as immunosurveillance or immunoediting.[5] On one hand, the aberrant immune response predisposes tissues to cancer, while on the other hand, the full activation of an effective adaptive immune response is necessary for tumour eradication. Indeed, it is on the battle between the protumour- and antitumour-promoting immune cells that the fate of the tissue depends. Among immune cells, the Th1 helper T subset has long been implicated in the inflammation that underlies IBD; however, in more recent years it has become apparent that Th1 cells are not the primary instigators.[6] A new subset that expresses a distinct set of inflammatory regulators was identified through their expression of the interleukin 17 (IL-17) family cytokines, of which there are five family members (IL-17A—IL-17F) that are termed the Th17 subset. The Th17 subset is implicated as playing a defensive role against extracellular bacteria and also in the pathogenesis of several autoimmune conditions. In a physiological adaptive immune response, the release of IL-17 leads to secretion of cytokines and chemokines that mobilise neutrophils indispensable for pathogen clearance.[7] Human studies have reported that patients suffering from IBD displayed high levels of IL-17-producing T cells in the intestinal mucosa, which also produce interferon γ (IFNγ).[8] Furthermore, elevated levels of IL-17A are detected in the serum of patients with active IBD. Human genetic studies and in vivo studies further support the pathological role of the IL-17 pathway in IBD pathogenesis, but its contribution to colon cancer is still controversial. Tumour-infiltrating Th17 cells have been observed in multiple human cancer types.[9] Th17 cell activation and infiltration in the tumour microenvironment appear to inhibit IL-12 transcription while enhancing IL-23 transcription, thereby shifting the balance

from Th1 to Th17. Consequently, this switch leads to a decreased production of IFNγ, which is the predominant Th1 cytokine that plays an important role for CD4 T cell-mediated antitumour immunity.

Recently, IL-21 has also been identified as an IL-17-inducing cytokine. IL-21 is produced mainly by CD4 T cells, as well as by natural killer T (NKT) cells, and exerts pleiotropic functions on various immune cells.[7]

Enhanced expression of IL-21 is detected in biopsies of patients with IBD and in colitic mice, and seems to trigger an exacerbated inflammatory response, while genetic deletion of IL-21 prevents intestinal colitis.[10]

Although the IL-21 and IL-17 pathway are involved in both the initiation and perpetuation of intestinal inflammation, it remains to be clarified whether these pathways can also be considered as triggers for the development of CAC. In their paper published in this issue of *Gut*, Jauch *et al* demonstrate a key role for IL-21 in supporting intestinal tumourigenesis, as it induces a change in the local cytokine milieu towards Th17 cytokines.[11] In line with previous data, the authors observe that IL-21-deficient mice develop an attenuated chronic intestinal inflammation compared with wild-type mice that is characterised by reduced levels of IL-17A and IFNγ, without any changes to the regulatory T cells. In contrast, when the chronic inflammation is associated with a tumourigenic process, the absence of IL-21 does not prevent the inflammatory process as we may expect, but instead induces a Th1 cytokine shift, reducing the levels of IL-17A.

Although Jauch *et al* do not specify whether the increased levels of IL-17A correlate with a high presence of IL-17 T cells in the mucosa, they demonstrate that IL-21 can tip the balance between Th1 and Th17 differentiation. Additionally, this balance is a determinant for the promotion of intestinal cancer. Indeed, the absence of IL-21 correlates significantly with fewer tumour nodules, which are characterised by a reduced diameter compared with those observed in wild-type mice. Intriguingly, the remarkable reduction in tumour growth is not associated with an ameliorated colitis in IL-21-deficient mice, but with an increased production of IFNγ that achieves tumour immunosurveillance by enhancing the levels of cytotoxic CD8CD103 T cells. Evidence continues to suggest that the unresolved chronic inflammation triggers

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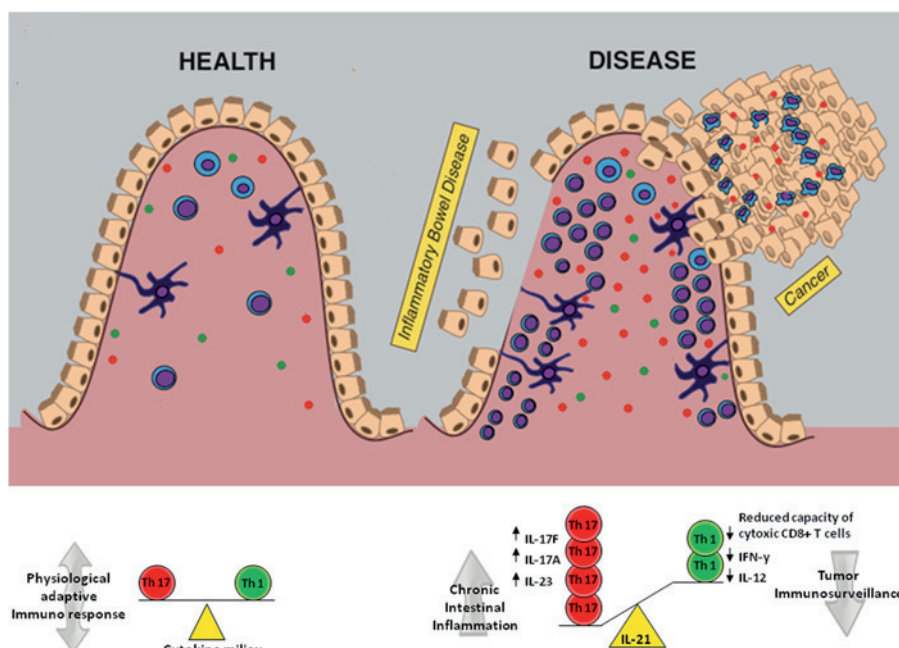
Figure 1 Function of interleukin 21 (IL-21) in a tumour-supportive microenviroment during colitis-associated cancer. In the normal intestine, the physiological inflammation serves to resolve the antigen insult that provokes the infection or damaged tissue. The differentiation of naïve T cells into T helper 1 (Th1) or Th17 is antigen dependent, and is necessary to prevent the spread of the infection. The cytokine milieu influences the development of these lineages. IL-21 cytokine tips the balance towards Th17 differentiation. When the adaptive immune response fails to resolve the inflammation, as happens in patients with inflammatory bowel disease (IBD), the increased production of IL-21 coincides with the transition phase from acute to Th17-mediated chronic intestinal inflammation, characterised by high levels of IL-17A. In addition, IL-21 reduces the production of interferon γ (IFNγ), which exerts antitumour activity enhancing the capacity of cytotoxic CD8 T cells. Thus IL-21 on one hand supports chronic inflammation and on the other hand reduces tumour immunosurveillance and promotes a tumour-supportive microenviroment in the colon.

the initiation and promotion of cancer; studies that describe the kinetics of these events are still lacking. In this work, the authors demonstrated that the switch from a Th1 to a Th17 cell cytokine profile that is induced by IL-21 coincides with the transition phase from acute to chronic intestinal inflammation, and influences the tumour-supportive microenvironment. Thus, these data support the hypothesis that, in the course of acute intestinal inflammation, the environment triggers the differentiation of CD4 T cells to a Th1 immune response. The Th1 cytokines such as IFNγ then induce the production of IL-21 and consequently of IL-23, which leads to Th17-mediated chronic inflammation. This sequence of

events is dependent upon the presence of IL-21. Indeed, the absence of IL-21 reduces the levels of IL-23, and upregulates the production of IL-12, maintaining therefore a Th1 cytokine profile such as IFNγ. The high levels of IFNγ promote an anti-tumour response, enhancing the capacity of cytotoxic CD8 T cells and thereby negatively influencing tumour growth (figure 1). Although this work shows that IL-21 influences the production of epithelial-derived factors involved in the polarisation of the adaptive immune response, it remains unclear whether IL-21 interacts with other cytokines in controlling this switch. Thus, neutralisation of IL-21 could be a target not only for the attenuation of immune inflammatory response, but also

for colon cancer therapy. These findings have relevance to human therapy, but further studies are necessary. In particular, studies in humans will be necessary, as it is well known that animal models of IBD and colon cancer have many limitations, and only partially reflect the complexity of human diseases.

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Interleukin 21 impairs tumor immunosurveillance of colitis-associated colorectal cancer

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The pathogenesis of colitis-associated colorectal cancer is strongly influenced by immune cells, cytokines and other immune mediators present in the inflamed colon. Current research has emerged that T helper cell associated cytokines play a prominent role in tumor growth. In our recent manuscript we have revealed that the Th17 associated cytokine IL-21 prominently influences tumor development and immunosurveillance of colitis-associated colorectal cancer.

Longstanding inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn disease (CD) have an increased risk to develop colorectal cancer.[1] This association of IBD with colorectal cancer was first described by Crohn et al. in 1925.[2] Chronic inflammation promotes carcinogenesis by inducing gene mutations and epigenetic alterations, alteration of the expression of factors involved in carcinogenesis (p53, APC, K-ras, Bcl-2, NFκB, COX-2, DNA mismatch— or base-excision repair proteins), releasing of reactive oxygen and nitrogen species, inhibiting apoptosis or stimulating angiogenesis and cell proliferation. The role of the immune system during the process of cancer development is multifactorial and complex. Whereas some immune cells are able to elicit protumoral effects others diminish tumor progression as part of the antitumoral immune response. However, this extensive network of tumor immune responses is far from completely understood. Among hematopoietic cells which can regulate cancer pathogenesis T cells play a prominent role. Thus, CD4+ T cells as well as CD8+ T cells and regulatory T cells can influence the tumor micromilieu. With this regard T cell subsets especially Th17 cells seem to be a potential target for new immunotherapeutic approaches to treat colitis-associated colorectal cancer as these cells were shown to

have prominent functions in mucosal immunity. Th17 cells are termed according to their secretion of the cytokine IL-17A and are a distinct proinflammatory CD4 effector T-cell lineage.[3] Besides the secretion of IL-17A Th17 cells secrete the cytokines IL-17F, IL-21 and IL-22. The secretion of these Th17 cell-associated cytokines leads to the induction of chemokines, matrix metalloproteinases as well as antimicrobial peptides in the surrounding tissue, leading to inflammation and recruitment of neutrophils and macrophages, but less is known about the function of these cytokines in cancer development.

Among Th17-associated cytokines IL-21 seems to represent an interesting target for immunotherapeutic approaches as IL-21 is able to tip the balance between Th1 and Th17 cells.[4] IL-21 is able to impact both innate and adaptive immune responses due to its ability to act on multiple immune cells expressing the IL-21 receptor like B cells, NK cells, activated T cells, DCs, macrophages as well as fibroblasts and epithelial cells. Upon engagement of its receptor IL-21 signals through JAKs, STAT3 and ultimately Bcl-6, Tcf7, Lef1, Blimp-1 and c-Maf. Therewith IL-21 is able to influence the differentiation, cell fate, proliferation and survival of diverse immune cell subsets. As mentioned above IL-21

promotes the differentiation of Th17 cells whereas it limits the development of Tregs and effector CD8+ T cells. It has been shown that IL-21 is overexpressed in the gut of patients with UC and CD compared with healthy controls[5] but also in tumors of UC-associated cancer.[6] In mouse models of intestinal colitis it was shown that IL-21 exaggerates intestinal acute colitis and that IL-21 is expressed in tumor-infiltrating lymphocytes in a colitis-associated cancer model.[6]

In our recent study our aim was to investigate the function of IL-21 during the development of colitis-associated tumorigenesis and its importance in tumor immunosurveillance.[7] We analyzed the course of chronic colitis in IL-21 deficient mice. Likewise to the acute colitis, we observed a dampened inflammation associated with intact colon architecture and decreased proliferation of intestinal cells in IL-21-deficient mice compared with wild-type (WT) mice due to diminished levels of IFNγ and IL-17. Opposing to previous results in the acute colitis, we found no alterations in Tregs levels in IL-21-deficient mice during chronic colitis.[8] Surprisingly, when we combined chronic colitis with tumorigenesis, IL-21 deficient mice showed a similar extent of inflammation compared with wild-type mice but less tumor burden. The tumors from IL-21 deficient mice were also reduced in

diameter. This effect was due to low tumor cell proliferation and high tumor cell apoptosis in IL-21-deficient mice. The intestinal cytokine milieu of IL-21 deficient mice with colitis-associated colorectal cancer showed a decrease in IL-17, while IFNγ is highly upregulated, which in turn mediated the severe intestinal inflammation. Simultaneously, the Th17 inducing cytokine IL-23 was elevated in WT mice whereas the Th1 inducing cytokine IL-12p70 was upregulated in IL-21-deficient mice. Other protumoral cytokines like IL-6 and IL-22 remain unchanged in IL-21-deficient mice. This cytokine switch from a Th17-dominated cytokine milieu toward a Th1-dominated one happens during the transition phase from acute to chronic intestinal inflammation and is based on epithelial-derived factors that are stimulating antigen-presenting cells to induce this specific adaptive immune responses. In our studies we could verify that the increased IFNγ levels are the reason for an enhanced antitumor response mediated by CD103^{+}CD8^{+} cytotoxic cells specific for tumor cells. These CD103^{+}CD8^{+} T cells were elevated in IL-21-deficient mice in the course of colitis-associated tumorigenesis. Further these cells showed an enhanced cytotoxic potential against E-cadherin[high]-expressing tumor cells. In a
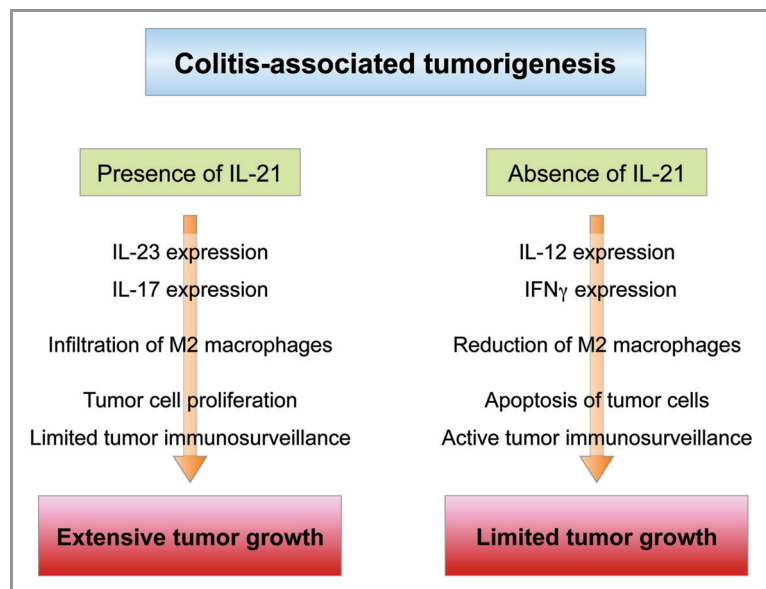

Figure 1. Schematic overview of the characteristics of an IL-21 deficient in comparison to an IL-21 rich tumor milieu.

similar subsequent study Stolfi et al. additionally showed reduced infiltration of alternatively activated macrophages, myeloid derived suppressor cells and reduced phosphorylation of STAT3 and diminished levels of Bcl-X$_L$ in IL-21 deficient mice.[6]

In conclusion, our results clearly elucidate that IL-21 has a prominent function in tumor growth and immunosurveillance

of colitis-associated tumorigenesis. IL-21 controls the balance between Th17 and Th1 cell subsets and therewith is necessary for the homeostasis of a tumor-supportive microenvironment characterized by extensive infiltration of Th17 cells. In addition, IL-21 controls the development of cytotoxic CD103^{+}CD8^{+} T cells whose cytotoxic capacity is also diminished in the presence of IL-21.

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LETTER

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

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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 progresses[1,2]. Curiously, however, 'inflammatory signature' genes characteristic of colitis-associated cancer are also upregulated in colorectal cancer[3,4]. Further, like most solid tumours, colorectal cancer exhibits immune/inflammatory infiltrates[5], referred to as 'tumour-elicited inflammation'[6]. Although infiltrating CD4^{+} T$_H$1 cells and CD8[+] cytotoxic T cells constitute a positive prognostic sign in colorectal cancer[7,8], myeloid cells and T-helper interleukin (IL)-17-producing (T$_H$17) cells promote tumorigenesis[5,6], and a 'T$_H$17 expression signature' in stage I/II colorectal cancer is associated with a drastic decrease in disease-free survival[9]. 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 barrier[10]. 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 tumoural 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 T$_H$17 and other IL-17-producing cells[11], previously found to promote skin carcinogenesis[12]. To address the role of these cytokines in CRC-related tumour-elicited inflammation and tumour growth, we used *Apc*[F/wt] mice that harboured a *Cdx2-Cre* transgene (CPC-APC mice) in which colorectal tumorigenesis was driven by *Apc* allelic loss[13]. Unlike *Apc*[Min] mice, CPC-APC mice develop tumours primarily in the distal colon, providing a relevant model of human CRC[13]. Mouse colon tumours also exhibited marked upregulation of IL-23 and IL-17A mRNA and IL-23 protein relative to matched non-tumour colon (Fig. 1b, c). IL-23 induction was specific, as tumoural expression of other IL-12/23 family members was not substantially elevated and IL-23 itself was already seen in early tumours (Supplementary Fig. 1a, b). IL-23 expression did not further increase in more advanced mouse CRC caused by *Apc* loss and forced *K-Ras* or *B-Raf* activation (Supplementary Fig. 1c). Analysis of *Il23*[−/−] mice harbouring green fluorescent protein (GFP) gene in the *Il23p19* locus, staining with IL-23p19 antibody and flow cytometry showed that IL-23p19 is particularly expressed in tumour-infiltrating cells such as CD11b[+] and F4/80[+] myeloid cells (Supplementary Fig. 1d–f). Both IL-23 subunits, p19 and p40, need to be simultaneously expressed to generate a functional cytokine. Analysis by quantitative PCR with reverse transcription (RT–qPCR) and fluorescence-activated cell sorting (FACS) of tumoural cell populations showed that CD11b[+] cells expressed substantial amounts of both p19 and p40 (Fig. 1d). T$_H$17- 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 *Il23*^{−/−}/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 was 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 haematopoietic compartment, as CPC-APC chimaeras harbouring *Il23*^{−/−} or *Il23r*[gfp/gfp] 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,
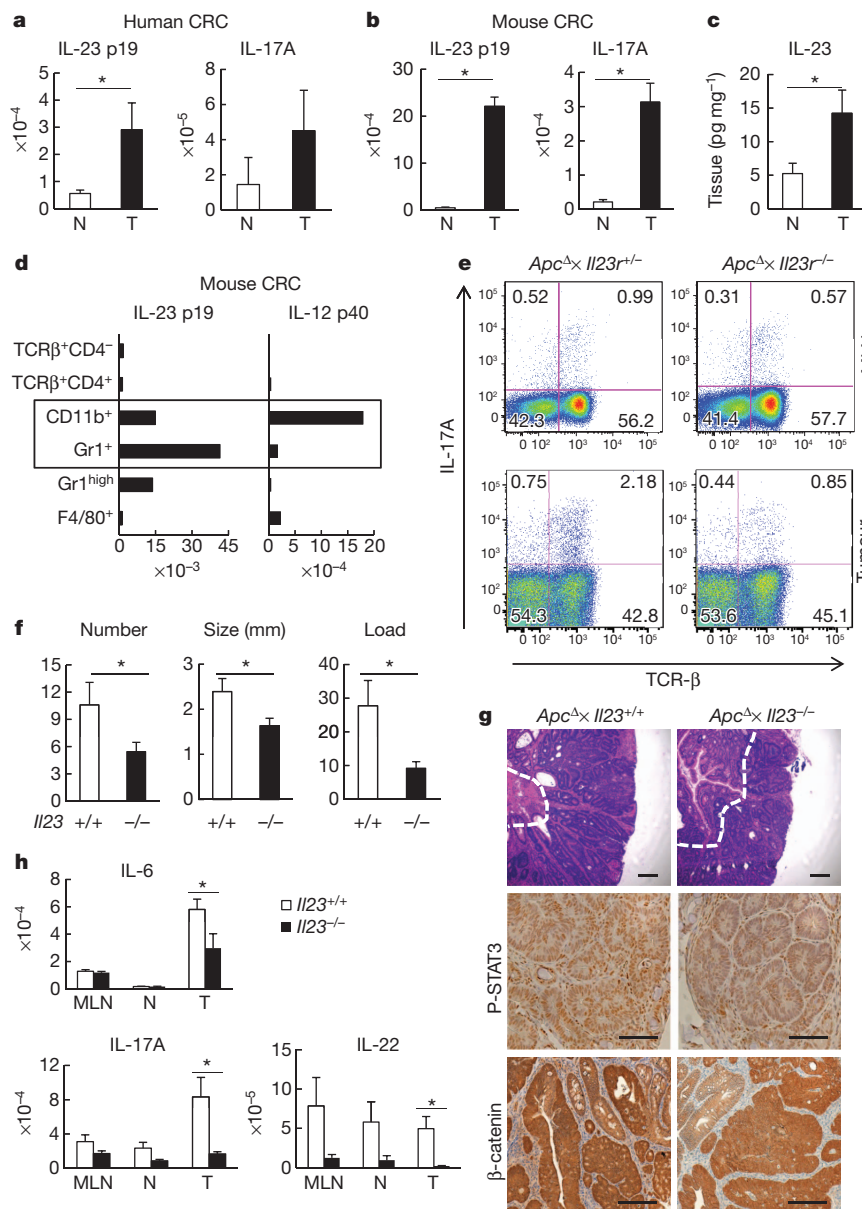
Figure 1 | IL-23 controls CRC inflammation and tumorigenesis. a, b, RT–qPCR for IL-23p19 and IL-17A mRNAs from colorectal tumours (T) and matching normal (N) colons of (**a**) human CRC patients ($n = 7$, $P = 0.037$ for IL-23p19) or (**b**) CPC-APC mice ($n = 8$, $P = 5 \times 10^{-5}$, 3.6×10^{-4}, respectively). **c,** IL-23 protein was measured by ELISA in supernatants of cultured tumours and normal tissues of CPC-APC mice ($n = 5$, $P = 0.04$). **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^{low}; Gr1$^+$ = Gr1$^+$F4/80$^-$; Gr1^{high} = Gr1^{high}F4/80$^-$; F4/80$^+$ = Gr1$^{-/dim}$F4/80$^+$ and T cells (CD45$^+$TCRβ$^+$). **e,** Intracellular cytokine staining of phorbol myristate acetate and ionomycin re-stimulated cells (Live/Dead$^-$CD45$^+$ gate). **f,** Five-month-old $Il23^{-/-}$ and control CPC-APC mice were killed and tumour numbers, size and load were determined ($n = 7$, $P = 0.04, 0.03, 0.01$, respectively). **g,** Tumour sections were stained with haematoxylin and eosin or phospho-STAT3 and β-catenin antibodies. **h,** Cytokine mRNA analysis by RT–qPCR in mesenteric lymph nodes (MLN), normal (N) and tumour (T) tissue of 5-month-old $Il23^{-/-}$ and control CPC-APC mice ($n = 6$, $P = 0.044, 0.007, 0.045$, respectively). Data represent averages ± s.e.m. *$P < 0.05$. Scale bars, 100 μm.

we crossed CPC-APC mice with $Il17ra^{-/-}$ 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[14]. Microbial products are sensed by Toll-like receptors (TLRs), which rely on the adaptors MyD88 and TRIF[15]. Similar to its effect on small intestinal polyps in Apc^{Min} mice[16], whole body MyD88 ablation reduced tumour multiplicity and growth in CPC-APC mice (data not shown). We transplanted $Myd88^{-/-}$, $Tlr2,4,9^{-/-}$ triple knockout or control bone marrow into lethally irradiated CPC-APC mice. Expression of IL-23p19 mRNA by sorted tumoural myeloid cells

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 $Myd88^{-/-}$ or $Tlr2,4,9^{-/-}$ bone marrow reduced colorectal tumour growth (Fig. 2c and Supplementary Fig. 5). By contrast, deletion of IL-18R 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
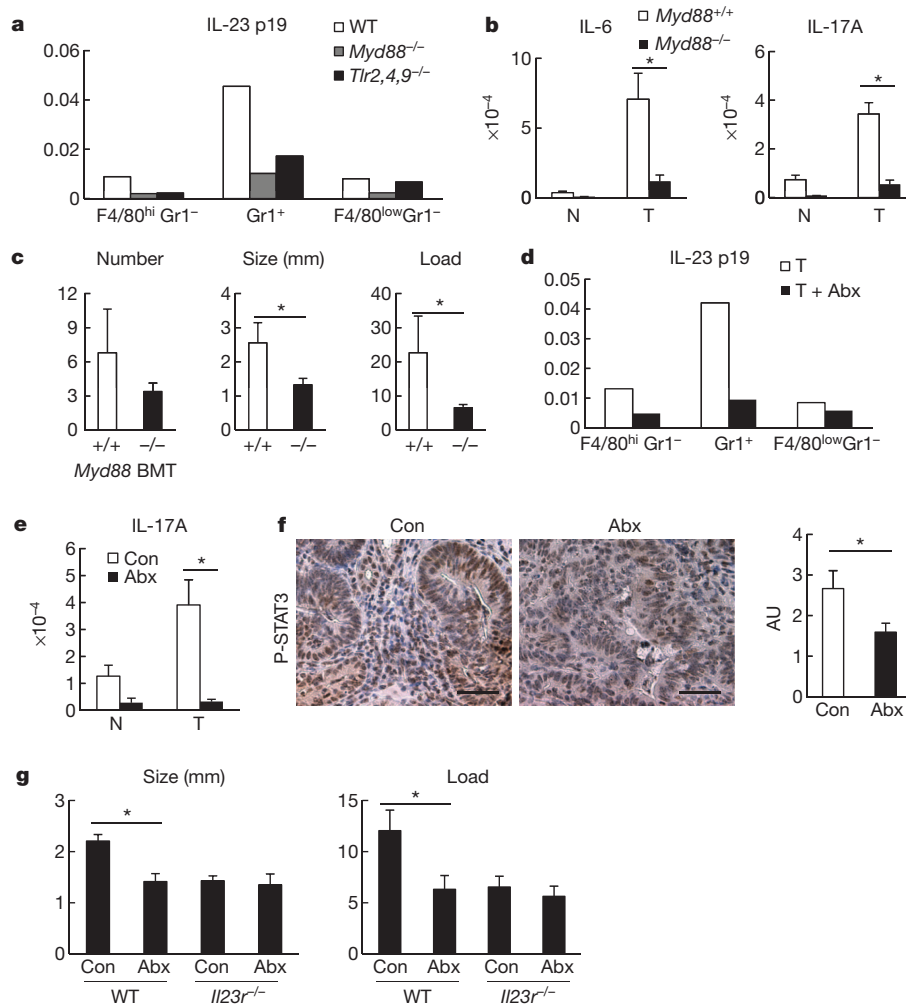
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Figure 2 | TLR-MyD88 signalling and commensal microflora promote cytokine expression and tumorigenesis. a–c, CPC-APC mice were transplanted with *Myd88*$^{-/-}$, *Tlr2,4,9*$^{-/-}$ or control bone marrow (BM) and analysed 4 months later by RT–qPCR 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.

for 3.5 months starting at weaning reduced tumour size and load in control CPC-APC mice but not in *Il23r*$^{-/-}$/CPC-APC counterparts (Fig. 2g), emphasizing the importance of microbial product-driven IL-23 signalling.

By injecting fluorescein isothyocyanate (FITC)-labelled dextran into clamped colonic loops, we found that tumour development in CPC-APC mice was associated with translocation of FITC-dextran into the circulation (Fig. 3a). This suggested that CRC development may result in increased penetration of microbial products or microbes into tumours. Indeed, Alexa488-labelled lipopolysaccharide (LPS) injected into colonic loops of CPC-APC mice translocated into tumours where it particularly co-localized with F4/80$^+$ TAMs, but did not penetrate adjacent normal tissue (Fig. 3b). Tumour development also coincided with elevated endotoxin in portal blood (Fig. 3c). Occasional bacteria were detected by *in situ* hybridization with a eubacterial 16S ribosomal RNA (rRNA) probe within colorectal tumours (Fig. 3d) and proximal to tumour epithelial cells in mouse early lesions that resembled aberrant crypt foci and early human adenomas (Fig. 3e, f).

Mucus from goblet cells prevents bacterial penetration through the colonic epithelial barrier. Correspondingly, *Muc2*$^{-/-}$ mice develop

spontaneous colitis followed by colitis-associated cancer[17,18]. Periodic acid-Schiff staining indicated absence of mucus-producing cells and mucins in tumours but not in adjacent normal tissue (Fig. 4a). Staining with wheat germ agglutinin (WGA) for glycosylated mucus proteins and mucin 2 (Muc2)-specific antibody demonstrated markedly reduced Muc2 production and coating of tumour tissue (Fig. 4a). Muc2 was barely detected in human CRC, but was prominent in adjacent normal tissues (Fig. 4b). Muc2 mRNA was downregulated in human and mouse colorectal tumours relative to healthy tissues (Supplementary Fig. 7a, b). Epithelial barriers also depend on tight and adherent junctions[10]. Human CRC demonstrated notable loss of polarized expression of junctional proteins JAM-A and JAM-B (Fig. 4c). JAM-A/B mRNAs were also reduced in human CRC and JAM-C mRNA was downregulated in mouse tumours (Supplementary Fig. 7a, b) Although claudin 4 mRNA was not downregulated in human or mouse tumours (Supplementary Fig. 7a, b), the protein was no longer distinctly localized to tight junctions in human or mouse tumours, similar to claudins 3, 5 and 7 (Supplementary Fig. 8a, b). Early human adenomas also showed defective mucin expression and organization of tight junctions (Fig. 4d), coinciding with elevated IL-23
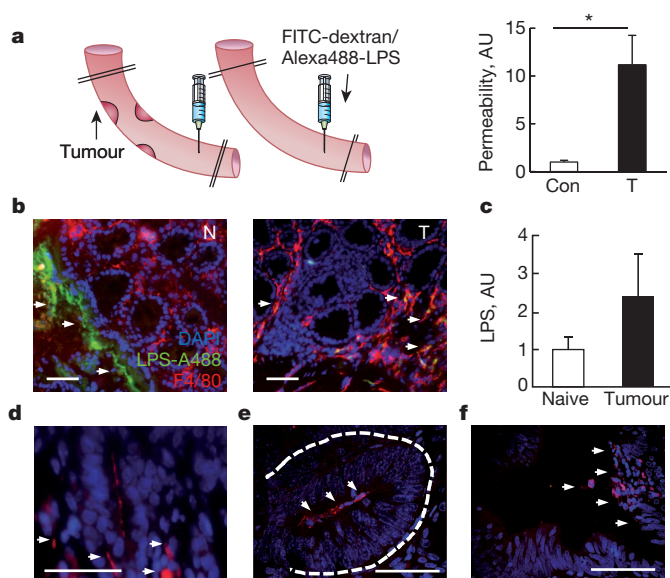
Figure 3 | Colorectal tumours exhibit increased permeability to bacteria and their products. a, Colon segments of CPC-APC and control mice were clipped as indicated and their lumens injected with FITC-dextran or Alexa488-LPS. FITC fluorescence was measured in plasma 1 h later ($n = 7$, $P = 0.017$). **b**, Frozen colon sections prepared 30 min after Alexa488-LPS injection were stained with F4/80 antibody and DAPI and examined by fluorescent microscopy. **c**, Endotoxin was measured in portal blood of naive or tumour-bearing CPC-APC mice by *Limulus* bioassay ($n = 9$, $P = 0.066$). **d–f**, Colon sections from CPC-APC mice containing tumours (**d**) and early lesions (aberrant crypt foci) (**e**), and early human adenomas (**f**) were subjected to fluorescent *in situ* hybridization with eubacterial 16S-rRNA-specific Alexa594-labelled probe and stained with DAPI. Signals are indicated by the arrows. Data represent averages ± s.e.m. *$P < 0.05$. Scale bars, 50 μm.
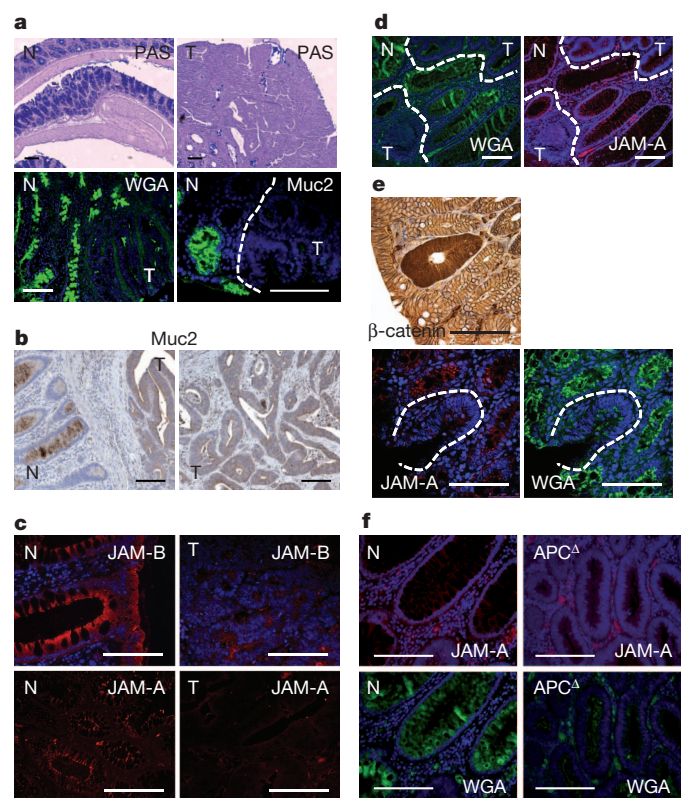

Figure 4 | Colorectal tumours show defective mucin production and aberrant expression of junctional proteins. a, Colon sections from CPC-APC mice were stained with periodic acid-Schiff reagent, Oregon488-WGA or mucin 2 antibody. Normal (N) and tumour (T) areas are depicted. **b, c**, Sections of normal human colon and adjacent CRC specimens were stained with mucin-2-specific antibody (**b**) or JAM-A or JAM-B antibodies (**c**). **d**, Human adenomas were stained with WGA or JAM-A antibodies. Normal and tumour areas are marked. **e**, Sections of CPC-APC colons were stained with β-catenin antibody and analysed by light microscopy or WGA and JAM-A antibodies and analysed by confocal microscopy. **f**, Colon sections of tamoxifen-injected control or $Cdx2^{ERT-Cre} \times Apc^{F/F}$ mice were stained with JAM-A antibody or WGA. Normal (N) and APC-deleted areas are shown. Scale bars, 100 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 transformation in $Cdx2^{ERT-Cre} \times Apc^{F/F}$ 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 metastasis[5,6]. The contribution of inflammation to CRC pathogenesis is emphasized by the protective effect of non-steroidal anti-inflammatory drugs, such as aspirin[19], 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. Deregulation 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 metastasis[20]. The importance of the mucus layer is emphasized by whole body Muc2 deficiency, which causes colonic inflammation[17,18]. Prolonged treatment with dextran sulphate sodium, which promotes development of colitis-associated cancer in wild type (WT) or Apc^{Min} mice, also causes erosion of the intestinal epithelial barrier and microbial-dependent inflammation[21,22], and so do the barrier-disrupting pathogens *Citrobacter rodentium*[23] and enterotoxic *Bacteroides fragilis*[24]. The commensal microflora was suggested to promote adenoma development, as germ-free Apc^{Min} mice exhibit a twofold reduction in small intestinal adenomas[25] and $Tbx21^{-/-};Rag2^{-/-}$ ulcerative colitis (TRUC), and $Il10^{-/-}$ mice fail to develop colitis-associated cancer when rendered germ-free[26,27]. Our work demonstrates that defective expression of barrier proteins is an early event in CRC tumorigenesis, coupled to upregulation of IL-23 and its downstream cytokines. We also found that IL-23 does not act directly on adenoma cells and that its receptor is expressed and signals within the haematopoietic compartment. Probably, IL-23 acts through IL-17 family members, as ablation of IL-17R attenuated colorectal tumorigenesis. IL-23 is instrumental for stabilizing the 'T$_H$17 signature', which includes IL-17A, RORγt and IL-23R, and was linked to extremely poor prognosis in human stage I/II CRC, which otherwise show 75–90% survival[9]. IL-23 itself is not a part of the 'T$_H$17 signature', arguably because its expression is already elevated in early colorectal tumours. The 'IL-23/T$_H$17' and 'T$_H$1-cytotoxic' signatures are not mutually exclusive in human CRC[9]. Although in genetically uniform mice in barrier facilities IL-23 invariably leads to IL-17 induction, the genetic heterogeneity of human populations and their exposure to a broader spectrum of microbes may explain why certain individuals mount a stronger T$_H$17 response than others. T$_H$17 development is promoted by several cytokines, including IL-21, IL-6 and IL-1, all of which can be induced by microbial components[28]. It remains to be investigated which of these cytokines contributes to the tumoural T$_H$17 response and how it is

43

affected by distinct microflora components. Segmented filamentious bacteria persisting in proximity to epithelial cells initiate T_H17 responses in the small intestine[29]. In tumours lacking protective barrier, other microbial species and products may invade the tumour and influence the 'IL-23/T_H17' 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[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_H17 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 and studied in filter-topped cages on autoclaved food and water in a barrier facility at the University of California, San Diego, according to institutional and National Institutes of Health (NIH) guidelines. Male and female mice were used in this study, including littermate and cage mate controls. $Il23r^{F/F}$ mice were generated by C.A.S., L.T., V.C. and G.T. (Supplementary Fig. 11). $Cdx2^{ERT-Cre}$ mice were generated and maintained by Y.F. and E.R.F. and will be reported elsewhere. Mice were allowed to develop spontaneous CRC for 4–5 months. At the end of each study, mice were killed and colonic tumours and non-tumour tissue, along with mesenteric lymph nodes, were analysed using flow cytometry, enzyme-linked immunosorbent assay (ELISA), RT–qPCR, immunohistochemistry and fluorescent microscopy. Lethally irradiated (2×600 rad) littermate CPC-APC recipient mice were intravenously injected with 10^7 nucleated bone marrow cells from various donors to generate radiation chimaeras. Endotoxin was measured in portal vein blood/plasma by a *Limulus* assay. For intestinal permeability analysis, tumour-bearing and control mice were surgically clipped and FITC-dextran or Alexa488-LPS were injected into the colonic lumen, followed by fluorescent detection of FITC in serum or Alexa488-LPS in frozen colon sections.

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Author Contributions S.G. and M.K. conceived the project. S.I.G., K.W., D.M., B.S., D.J., K.T., G.Y.Y., C.O., 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 $Il23r^{F/F}$ mice. M.O. and F.Y. provided $Il23r^{gfp/gfp}$ and $Tlr2,4,9^{-/-}$ bone marrow, respectively, and Y.F. and E.R.F. provided CPC-APC mice and tissues from $Cdx2^{ERT-Cre}$-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.

METHODS

Human samples. Paraffin-embedded human ulcerative colitis and CRC specimens were provided by Oberndorf 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, Oberndorf 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[31]. 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[32,33]. 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 committee (Ethikommission des Landes Salzburg, approval number 415-E/1262/2-2010). Frozen human CRC and normal colon tissues were obtained from Cooperative Human Tissue Network of Vanderbilt University Medical Center.

Generation of $Il23r^{F/F}$ and $Il23r^{-/-}$ mice. The targeted region includes exons 2 (3,708–3,810) and 3 (8,085–8,381) of $Il23r$ that encode a portion of the signal peptide and the amino (N)-terminal Ig-like domain (Supplementary Fig. 11a). The targeting vector was generated by recombineering using λ phage *Red* as described by Liu *et al.*[34] Homology arms for the retrieval vector were amplified by PCR on C57BL/6 genomic DNA. Homology arms were cloned into pLMJ235 (pBSKS+ containing a 2.8kb SalI TK fragment of pGKTK) by triple ligation to form the retrieval plasmid. Retrieval of the targeting region (covering 753-12,812; numbering relative to indicated BamHI site) was performed according to Liu *et al.*[34] using C57BL/6 bacterial artificial chromosome RP23-283M8 (BACPAC Resources) as template in DY380 cells. Recombineering was performed to insert cassettes containing *LoxP*-flanked Neo cassettes from pGKEM7Neo-pA into the targeting vector. First, *LoxP-Neo-LoxP* was inserted at position 3,524, followed by induced expression of *Cre* in EL350 cells to excise the *Neo* selection cassette but leaving the upstream *LoxP* site. A second cassette (*LoxP-Frt-Intron-Eng2SA-Neo-LoxP-Frt*) was inserted into position 8,503. After NotI-linearization, the targeting construct was electroporated into Bruce 4 C57BL/6 ES cells, and G418-selected clones were screened for integration by Southern analysis using sequences outside the targeting vector (Supplementary Fig. 11b). Selected clones were injected into C57BL/6-albino blastocysts and transferred to pseudopregnant females. Chimeric offspring were bred with C57BL/6-Albino partners to generate F_1 mice that were screened for integration by Southern blotting (Supplementary Fig. 11c). Resulting mice with the $Il23r^{SA-F-neo}$ allele were crossed with C57BL/6-FLPe mice to facilitate recombination between *Frt* sequences and generate the $Il23r^F$ allele. The null allele ($Il123r^{-/-}$) was generated by crossing $Il23r^{F/F}$ mice with *CPC-Cre* transgenic mice. The F_2 generation exhibits germline deletion of floxed alleles owing to early *CPC-Cre* expression in caudal regions of the body and gonads[13]. Complete ablation of IL-23R was confirmed by RT–qPCR of spleen and tumour-derived cells.

Animal models. C57BL/6 control mice obtained from Charles River Laboratories were bred locally at University of California, San Diego. $Il23^{-/-}$ and $Il17ra^{-/-}$ mice were from Genentech[35] and Amgen[36], respectively. Bone marrow from $Il23r^{gfp/gfp}$ and $Tlr2,4,9^{-/-}$ mice was provided by M. Oukka[37] and F. Yarovinsky[38], respectively. $Apc^{F/F}$ and *Cdx2-Cre* (*CPC-Cre*) mice[13] were provided by E. Fearon, who also generated the $Cdx2-Cre^{ERT}$ mice, which will be described elsewhere. $Myd88^{-/-}$ mice[39] were obtained from the Jackson Laboratory, and $Il18r^{-/-}$ mice[40] were from H. Hoffman. All mice were maintained in filter-topped cages on autoclaved food and water at University of California, San Diego, according to NIH guidelines, and all experiments were performed in accordance with University of California, San Diego, and NIH guidelines and regulations. Most experiments used littermate controls housed in the same cage; in addition, dirty beddings were switched between cages of the same experiment to make sure that the microflora was balanced.

For spontaneous CRC tumorigenesis, male and female CPC-APC mice were allowed to develop colorectal tumours spontaneously for 4–5 months and then killed. Macroscopic tumours were counted and their diameter measured with a calliper. Average tumour size per individual mouse was determined by averaging diameters of all tumours present. Tumour load was determined as the sum of the diameters of all tumours presented in a given mouse, as previously described[41]. Typically, one-half of the distal colon was taken as a tissue sample, tumours and

normal tissue dissected and snap-frozen in liquid nitrogen or used for *ex vivo* cultures or fluorescence-activated cell sorting. The other half was fixed in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol for subsequent paraffin embedding, or alternatively frozen in optimal cutting temperature (OCT) compound for further sectioning and histological analysis.

To induce colorectal tumours with inactivated *Apc* and activated *K-Ras* or *B-Raf*, open laparotomies were performed in mice bearing floxed *Apc* ($Apc^{F/F}$) in combination with either latent $Kras^{G12D}$ (*Apc-Kras*) or $Braf^{V600E}$ (*Apc-Braf*) alleles under isoflurane anaesthesia. A dosage of adenovirus-Cre was administered to the distal colon, which resulted in solitary colonic tumours, as described[42,43]. 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.

Colonic 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[44]. 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.

Portal blood vein drainage and endotoxin measurement. Mice were anaesthetized with isofluorane 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 10^7 donor bone marrow cells. Every cage of recipient mice contained mice 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 sulphamethoxazole and trimethoprime antibiotics in drinking water followed by transfer to the cages from the same room/rack with dirty bedding to restore microflora. Mice were killed and analysed for tumour development 4–5 months after transplantation.

Antibodies and stains. Fluorescent-labelled antibodies for flow cytometry were from eBioscience. Immunoblot analysis and immunohistochemistry were performed with antibodies to Ki-67, claudin7, claudin 5, claudin 4, claudin 3, JAM-B, mucin2, GFP, IL-23p19 (Genetex), JAM-A (Santa Cruz), active caspase 3, phospho-STAT3 (Cell Signaling), F4/80 and E-cadherin (BD Biosciences). Secondary antibodies (host: donkey) for fluorescent microscopy labelled with Alexa 488 or 594 were from Invitrogen. For TdT-mediated dUTP nick end labelling (TUNEL) assay the *In situ* Cell Death Kit (Roche) was used according to the manufacturer's recommendations. Alexa488-LPS and Oregon488-WGA were from Invitrogen.

Immunohistology. Paraffin-embedded slides were de-paraffinized. Antigen unmasking was performed by incubation in 94 °C water bath in 10 mM sodium citrate buffer with 0.1% Tween 20 for 1 h. Slides were incubated with primary antibodies in PBS containing 1% BSA and 10% normal goat or donkey serum, depending on the host of secondary antibodies used. Biotinylated secondary anti-rat or anti-rabbit antibodies (Pharmingen) were added and incubated at room temperature for 1 h. Streptavidin-HRP (Pharmingen) was added and after 40 min the sections were developed with DAB substrate and counterstained with haematoxylin. Alternatively, paraffin-embedded or acetone fixed frozen slides were stained with antibodies followed by secondary donkey antibodies labelled with Alexa488 or Alexa594, counterstained with DAPI and analysed by immunofluorescence. Images were acquired on an upright light/fluorescent Imager A2 microcope (Zeiss) equipped with an AxioCam camera or on a confocal microscope (Leica SPE).

Bacteria fluorescent *in situ* hybridization. Paraffin embedded slides were de-paraffinized 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 by 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 FACSAria 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 IScript 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 $2^{(Ct(rpl32-gene\ of\ interest))}$. 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.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: List of primers used for Q-RT-PCR.

Human Q-RT-PCR

| Claudin 4	5'-TAACTGCTCAACCTGTCCCC-3'	5'-ATAAAGCCAGTCCTGATGCG-3'
Claudin 5	5'-GCAGCCAATCACAGAGCC-3'	5'-AGTGGTGTCACCTGAACTGG-3'
Claudin 7	5'-GCAAAATGTACGACTCGGTG-3'	5'-CACAAACATGGCCAGGAAG-3'
IL-17A	5'-ACCAATCCCAAAAGGTCCTC-3'	5'-CACTTTGCCTCCCAGATCAC-3'
IL-23 p19	5'-AGAAGCTCTGCACACTGGC-3'	5'-CCACACTGGATATGGGGAAC-3'
JAM-A	5'-CCTCTTCATATTGGCGATCC-3'	5'-CCAGTTGGCAAGAAGGTCAC-3'
MUC2	5'-GACACCATCTACCTCACCCG-3'	5'-TGTAGGCATCGCTCTTCTCA-3'

Mouse Q-RT-PCR

| Claudin 4	5'-GGCGTAATGGCAAGAGTAGC-3'	5'-CTCGGAGTGGATGTCCTCAT-3'
EBI3	5'-GAGAAGATGTCCGGGAAGG-3'	5'-TCCTAGCCTTTGTGGCTGAG-3'
IL-6	5'-ACCAGAGGAAATTTTCAATAGGC-3'	5'-TGATGCACTTGCAGAAAACA-3'
IL-12 p35	5'-GAGGACTTGAAGATGTACCAG-3'	5'-CTATCTGTGTGAGGAGGGC-3'
IL-12 p40	5'-GACCCTGCCCATTGAACTGGC-3'	5'-CAACGTTGCATCCTAGGATCG-3'
IL-17A	5'-GCCCTCAGACTACCTCAACC-3'	5'-ACACCCACCAGCATCTTCTC-3'
IL-17F	5'-AATTCCAGAACCGCTCCAGT-3'	5'-TTGATGCAGCCTGAGTGTCT-3'
IL-22	5'-CAGGAGGTGGTACCTTTCCTG-3'	5'-TCTGGTCGTCACCGCTGAT-3'
IL-23 p19	5'-CCAGCGGGACATATGAATCT-3'	5'-AGGCTCCCCTTTGAAGATGT-3'
IL-27 p28	5'-CTGGCAAGGTACAGGCTGA-3'	5'-CAGGTGACAGGAGACCTTGG-3'
JAM-C	5'-CTGCCTGACTTCTTCCTGCT-3'	5'-ATGTACCACTGGGTTTCGGT-3'
MUC2	5'-GTAGTTTCCGTTGGAACAGTG-3'	5'-ATGCCCACCTCCTCAAAGAC-3'

47
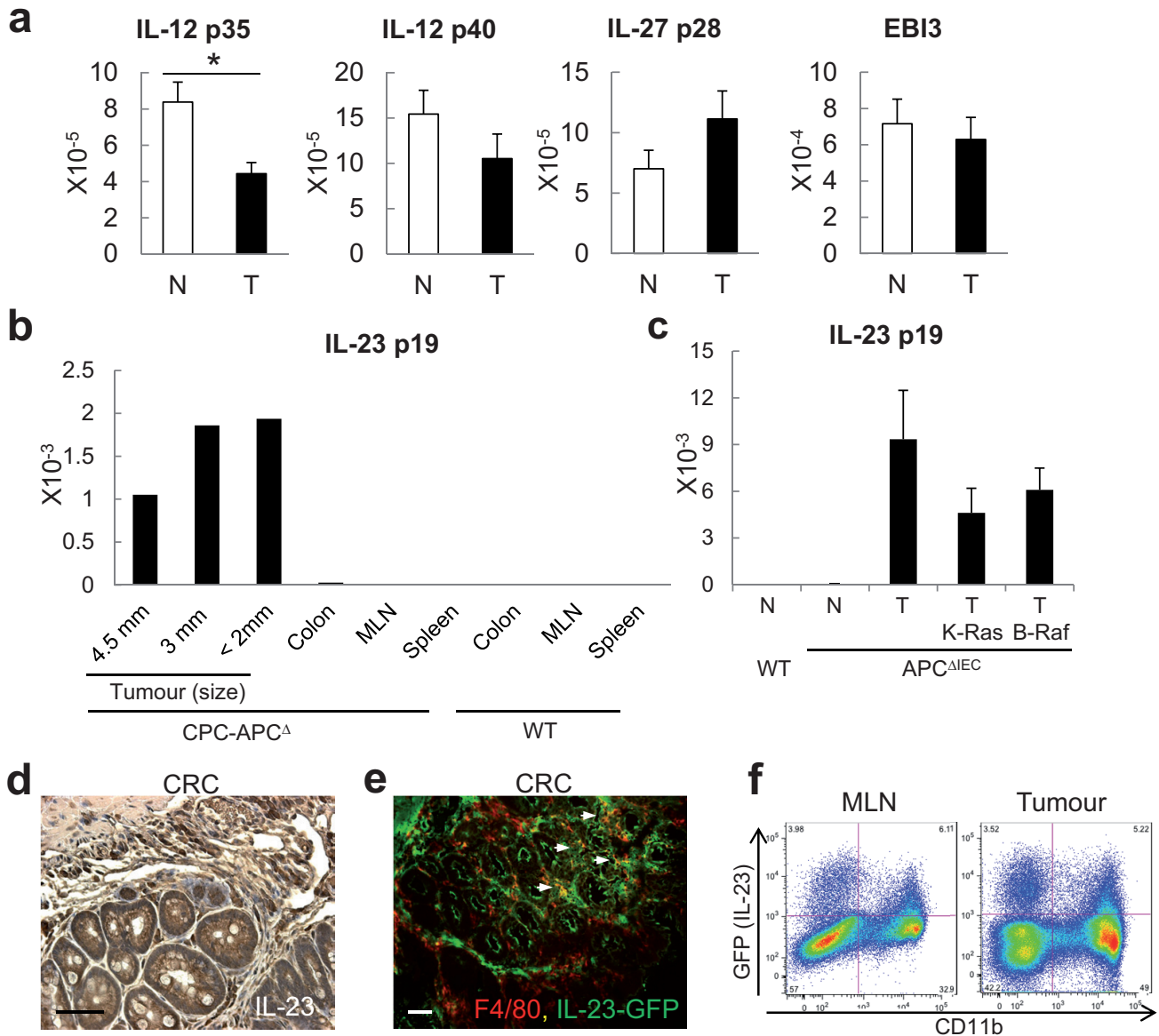
Supplementary Figure 1. Expression of IL-12 family members in normal and tumour tissue of CPC-APC mice.

a-c: RT-qPCR analysis of cytokine mRNA expression in normal colon (N), tumours (T) and mesenteric lymph nodes (MLN) of 5 months old CPC-APC or WT mice **(a, b)** or in tumours of $Apc^{\Delta IEC}$ mice without or in combination with $Kras^{G12D}$ (K-Ras) or $Braf^{V600E}$ (B-Raf) alleles, activated by intra-colonic administration of Adenovirus-Cre into $Apc^{F/F}$, $Apc^{F/F}/K\text{-}ras\text{-}LSL^{G12D}$ or $Apc^{F/F}/Braf^{V600E}$ mice **(c)**. **d,e:** Immunostaining for IL-23 **(d)** or IL-23-driven GFP in CRC, and co-staining of F4/80$^+$ cells **(e)**. Arrows indicate positive cells. **f:** Flow cytometric analysis of single cell suspensions isolated from mesenteric lymph nodes (MLN) or colorectal tumours of $Il23^{-/-}$ CPC-APC mice stained with anti-CD11b antibody. Live/Dead$^-$CD45$^+$ are shown. Data represent averages ± s.e.m., n=6 for **a** and **b**, n=3 for **c**. p=0.006 for IL-12p35 in **a**. Differences between the different tumour models in **c** are not significant. * p<0.05. Scale bars, 50 μm.
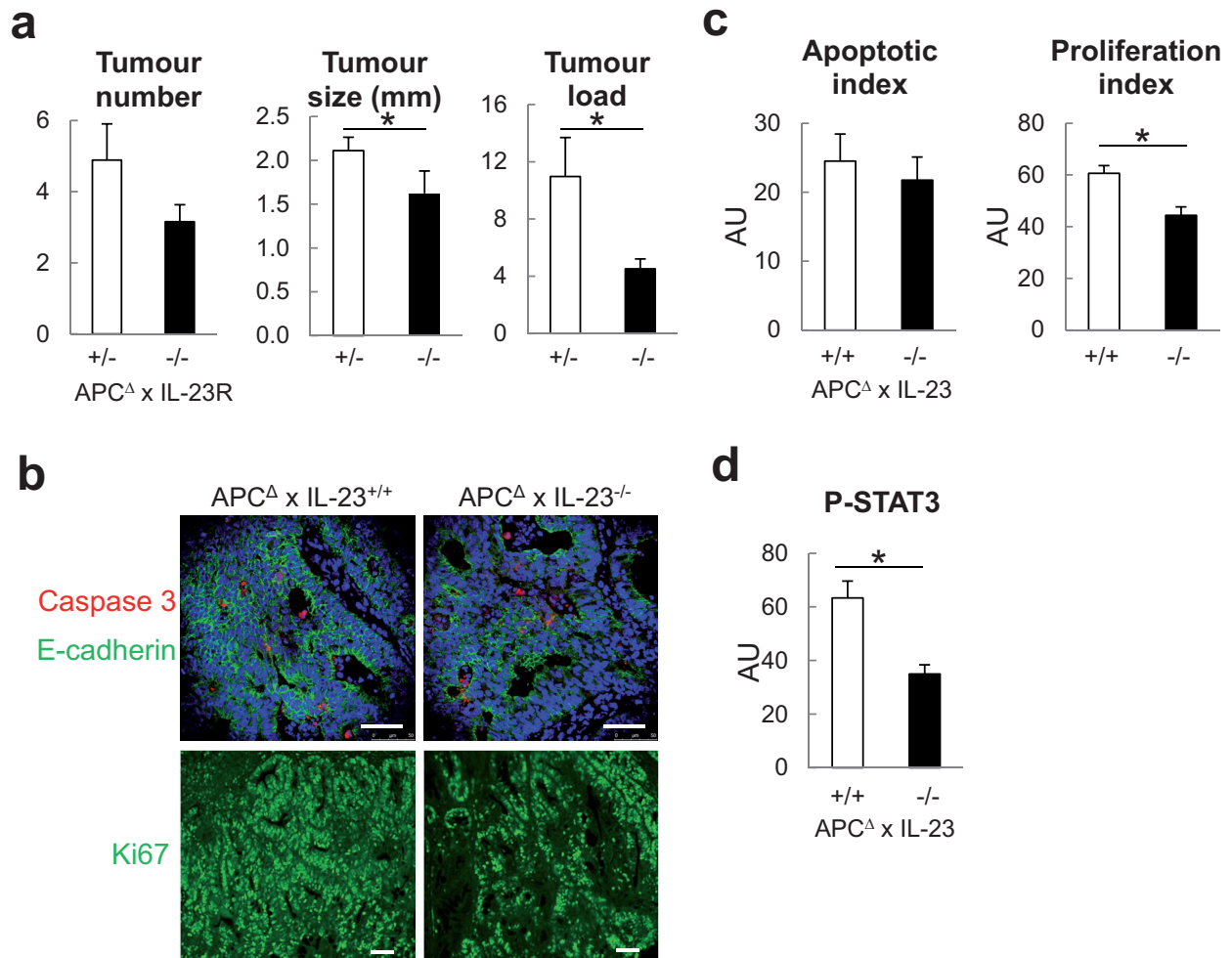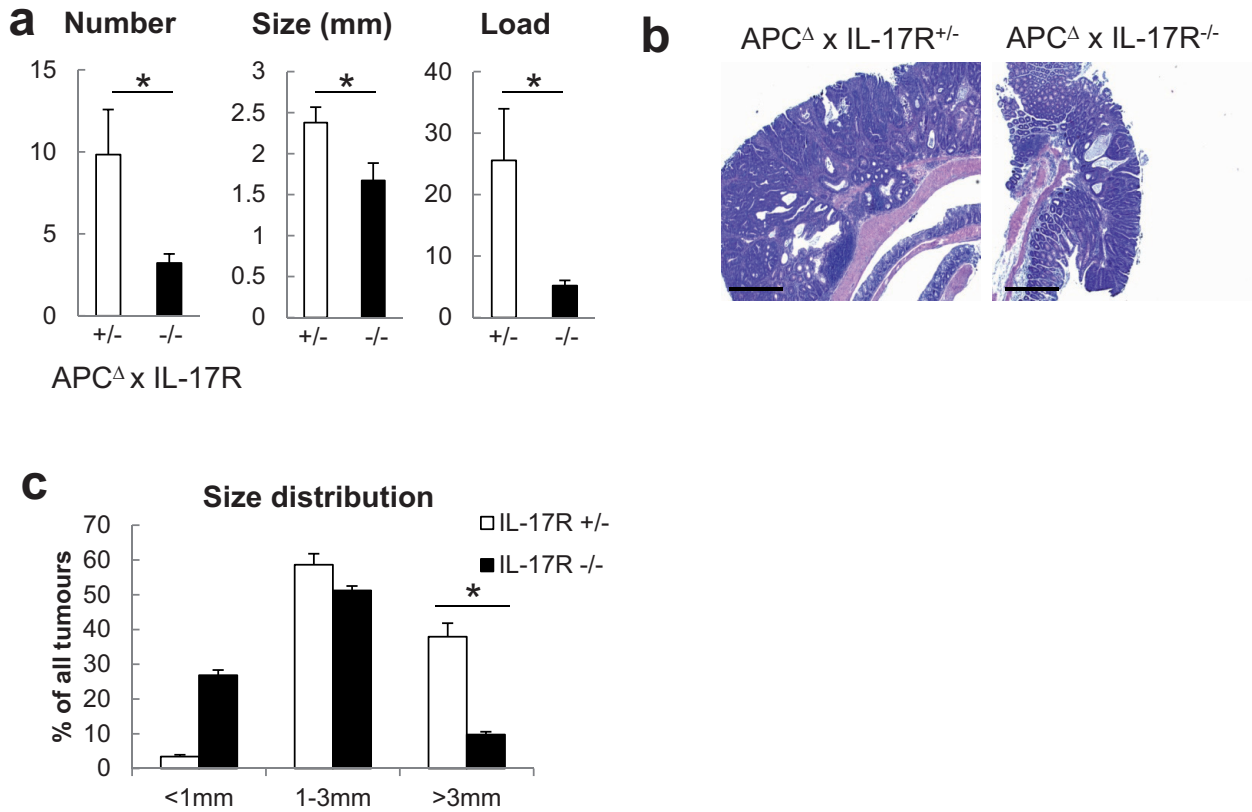
Supplementary Figure 2. IL-23 signaling promotes colorectal tumour development and growth.
a: Colonic tumours from 4.5 months old *Il23r-/-*/CPC-APC and control CPC-APC mice were enumerated and measured. n=8, p=0.081, 0.041, 0.024, respectively. **b,c:** Paraffin sections of colons from *Il23-/-*/CPC-APC or control CPC-APC mice were stained with active caspase 3 and E-cadherin antibodies, TUNEL kit or a Ki67 antibody and TUNEL-positive apoptotic or Ki67 positive proliferating cells were quantified. n=12, p=0.001 for proliferation index **(c)**. **d:** Quantification of phospho-STAT3high cells in colonic tumours of *Il23-/-*/CPC-APC or control CPC-APC mice (see Fig. 1g). 4 fields from different sections of 5 independent tumours were counted. p=0.0045. Data represent averages ± s.e.m. *p<0.05. Scale bars, 50 μm.
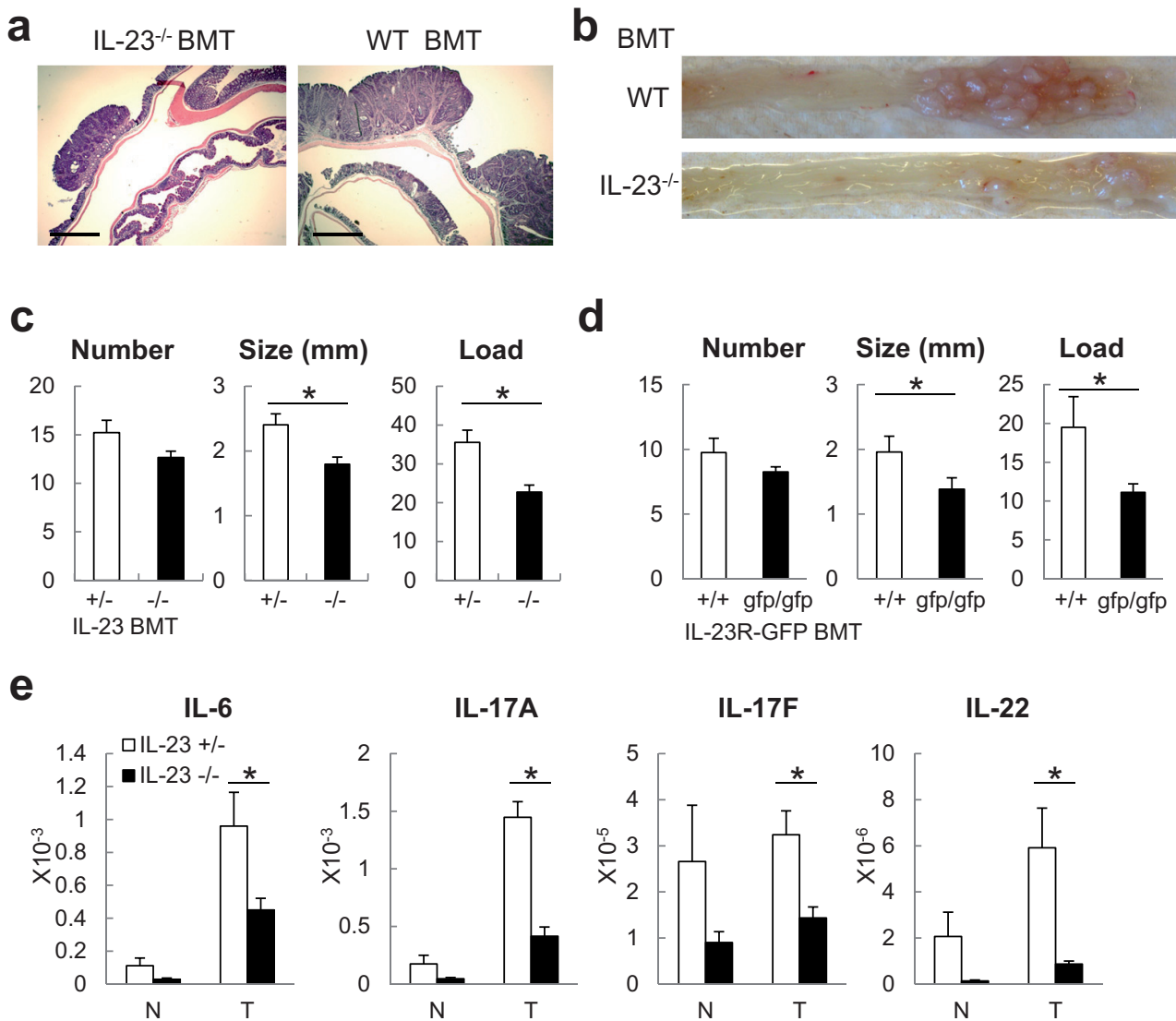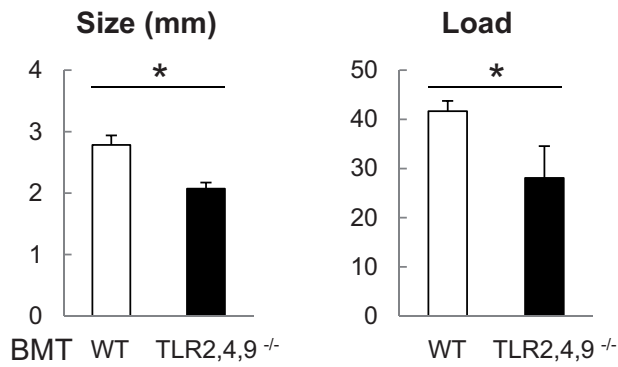
Supplementary Figure 3. The pro-tumourigenic function of IL-23 and IL-23R is exerted within hematopoietic cells.

Six weeks old CPC-APC mice were lethally irradiated (2 x 600 rad) and transplanted with bone marrow from $Il23^{-/-}$, $Il23r^{gfp/gfp}$ or WT donors and analyzed 4-4.5 months later. **a,b:** Representative histology **(a)** and gross appearance of tumour-bearing colons **(b)** from chimeric CPC-APC mice reconstituted with WT or $Il23^{-/-}$ bone marrow. **c,d:** Quantification of tumour development in chimeric CPC-APC mice reconstituted with $Il23^{-/-}$ **(c)** or $Il23r^{gfp/gfp}$ **(d)** bone marrow. n=5, p=0.024, 0.011 for size and load of $Il23^{-/-}$ bone marrow transfer, and 0.023, 0.043 for $Il23r^{GFP}$ bone marrow transfer, respectively. **e:** RT-qPCR analysis of cytokine mRNAs in tumours (T) and adjacent normal colon (N) of chimeric CPC-APC mice reconstituted with control or $Il23^{-/-}$ bone marrow. n=6, p=0.0066, 2.3x10^{-5}, 0.014 and 0.0032, respectively. Data represent averages ± s.e.m. *p<0.05. Scale bars, 500 µm.

a

Number | Size (mm) | Load

APC^{Δ} x IL-17R

b APC^{Δ} x IL-17R$^{+/-}$ APC^{Δ} x IL-17R$^{-/-}$

c Size distribution

□ IL-17R +/-
■ IL-17R -/-

% of all tumours

<1mm   1-3mm   >3mm

Supplementary Figure 4. IL-17R signaling promotes growth and progression of colorectal tumours.
Tumours from 5 months old *Il17ra$^{-/-}$/*CPC-APC and control mice were analyzed. **a:** Tumour number, size and load. n=7, p=0.014, 0.045, 0.011, respectively. **b:** H&E staining of colon sections from CPC-APC mice of the indicated genotypes. **c:** Distribution of tumour sizes among all detectable tumours. n=7, p=0.045 for tumours bigger than 3 mm. Data represent averages ± s.e.m. *p<0.05. Scale bars, 200 µm.

**Supplementary Figure 5. Signaling through TLRs is important for CRC tumour growth.**
Six to seven weeks old CPC-APC mice were lethally irradiated (2 x 600 rad) and transplanted with bone marrow from *Tlr2,4,9-/-* or WT donors and analyzed 4.5 months later. Quantification of tumour size and load in chimeric CPC-APC mice is presented. n=5, p=0.025, 0.049 respectively. Data represent averages ± s.e.m. *p<0.05.
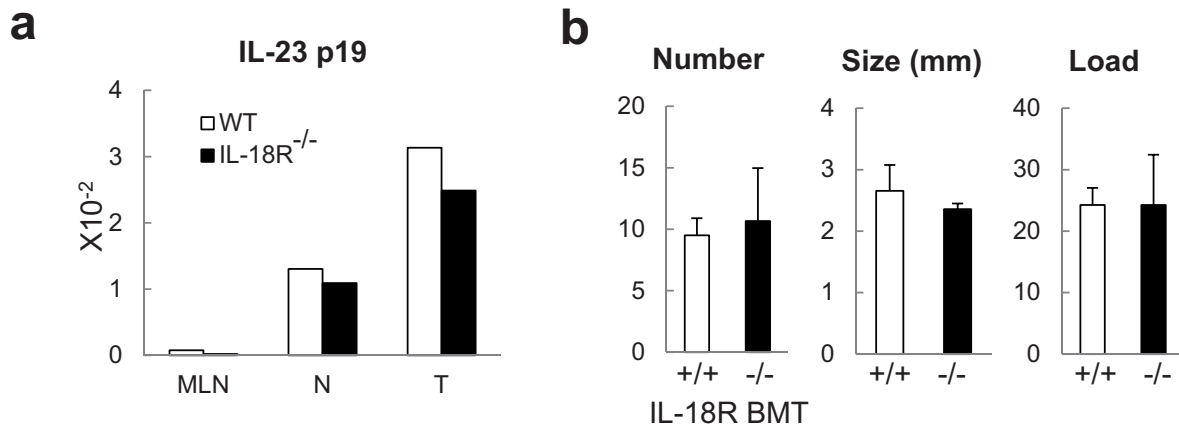
Supplementary Figure 6. IL-18R signaling in hematopoietic cells is not required for IL-23 induction and growth of colorectal tumours.
Six weeks old CPC-APC mice were lethally irradiated and transplanted with *Il18r-/-* or WT bone marrow and sacrificed for analysis 4 months later. **a:** Single cell suspensions from normal colons, tumours and MLN were prepared and CD11b+ cells were sorted by positive selection on magnetic beads. IL-23p19 mRNA expression was measured by RT-qPCR. **b:** Tumour number, size and load were determined. n=5. None of the differences (number, size and load) are significant. Data represent averages ± s.e.m.

a
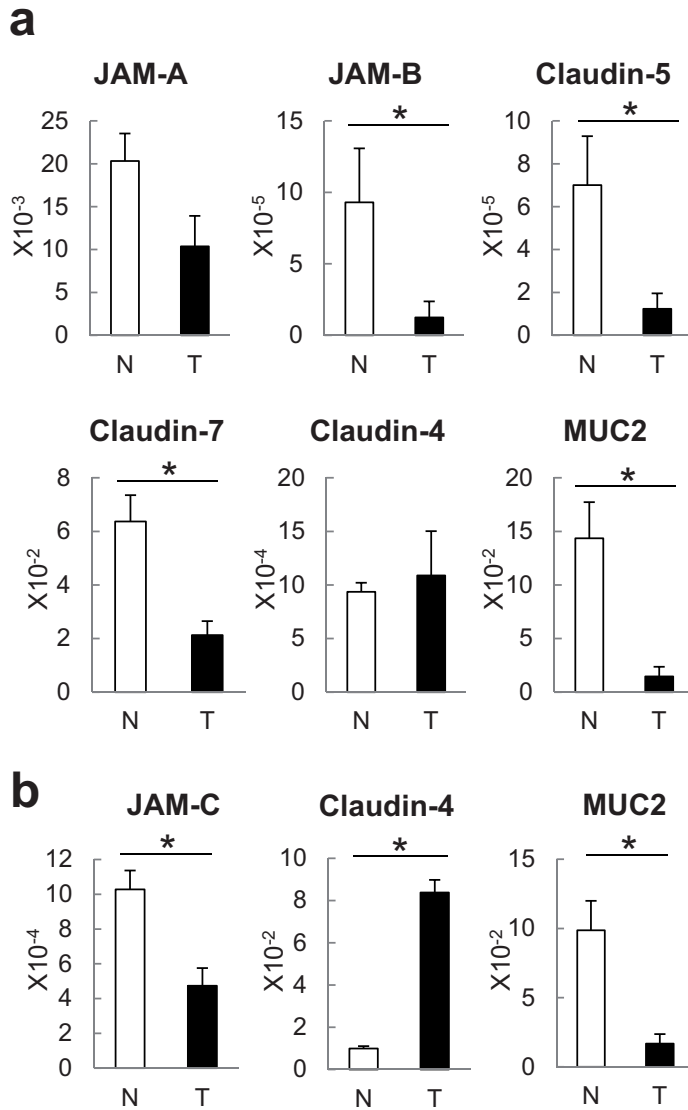
Supplementary Figure 7. Human CRC and mouse colorectal tumours display dysregulation of mRNAs encoding barrier and junctional proteins.

a,b: Total RNA was isolated from frozen tumour (T) and tumour-free (N) colon tissue of CRC patients **(a)** or tumour bearing CPC-APC mice **(b)** and subjected to RT-qPCR analysis of indicated mRNAs. Human: n=7, p=0.078, 0.025, 0.016, 0.025, 0.0065 for JAM-A, JAM-B, claudin-5, claudin-7, and Muc2, respectively; Mouse: n=12, p=0.0012, 0.0035 and 0.0027 for JAM-C, claudin-4 and Muc2, respectively. Data represent averages ± s.e.m. *p<0.05.
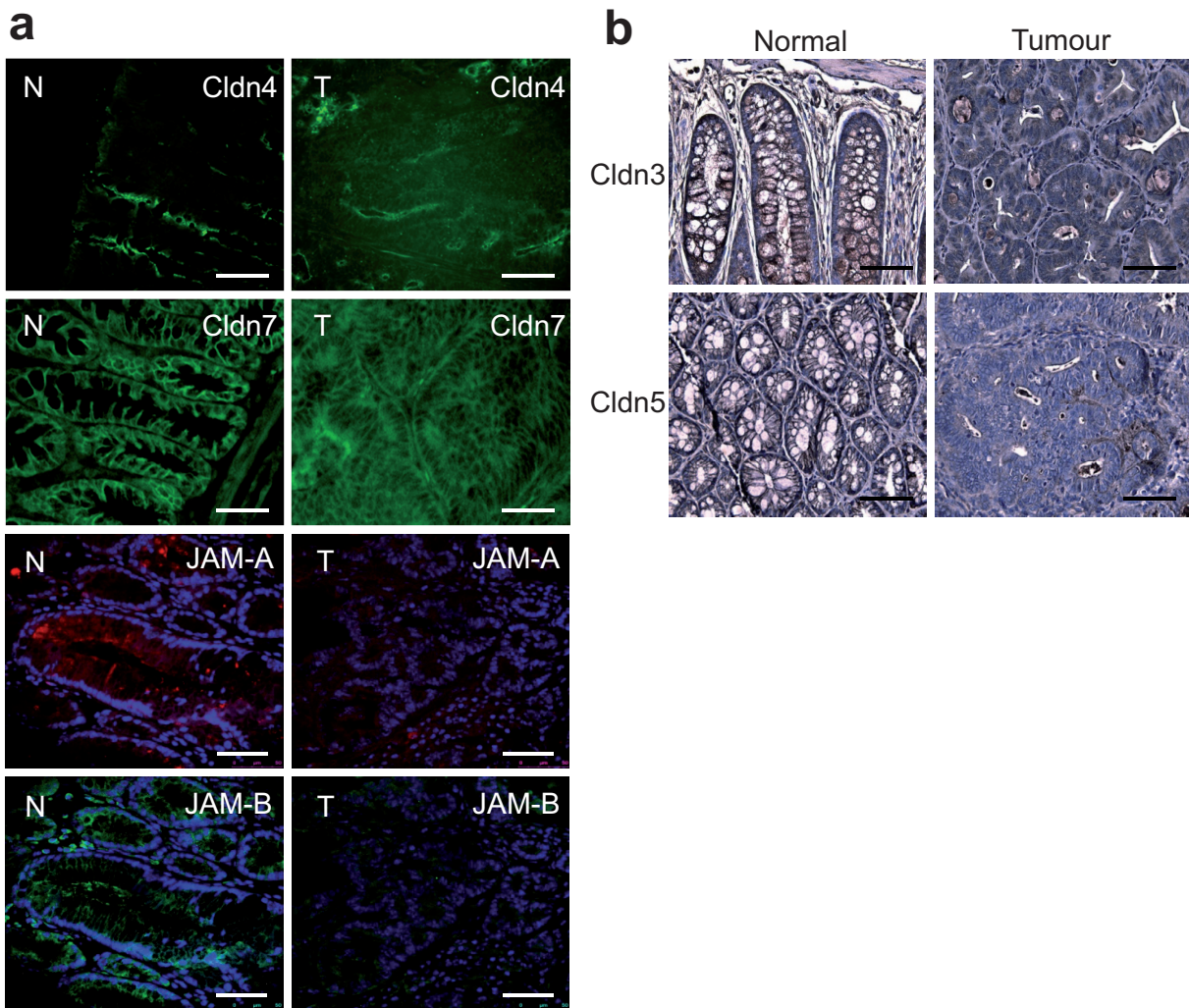
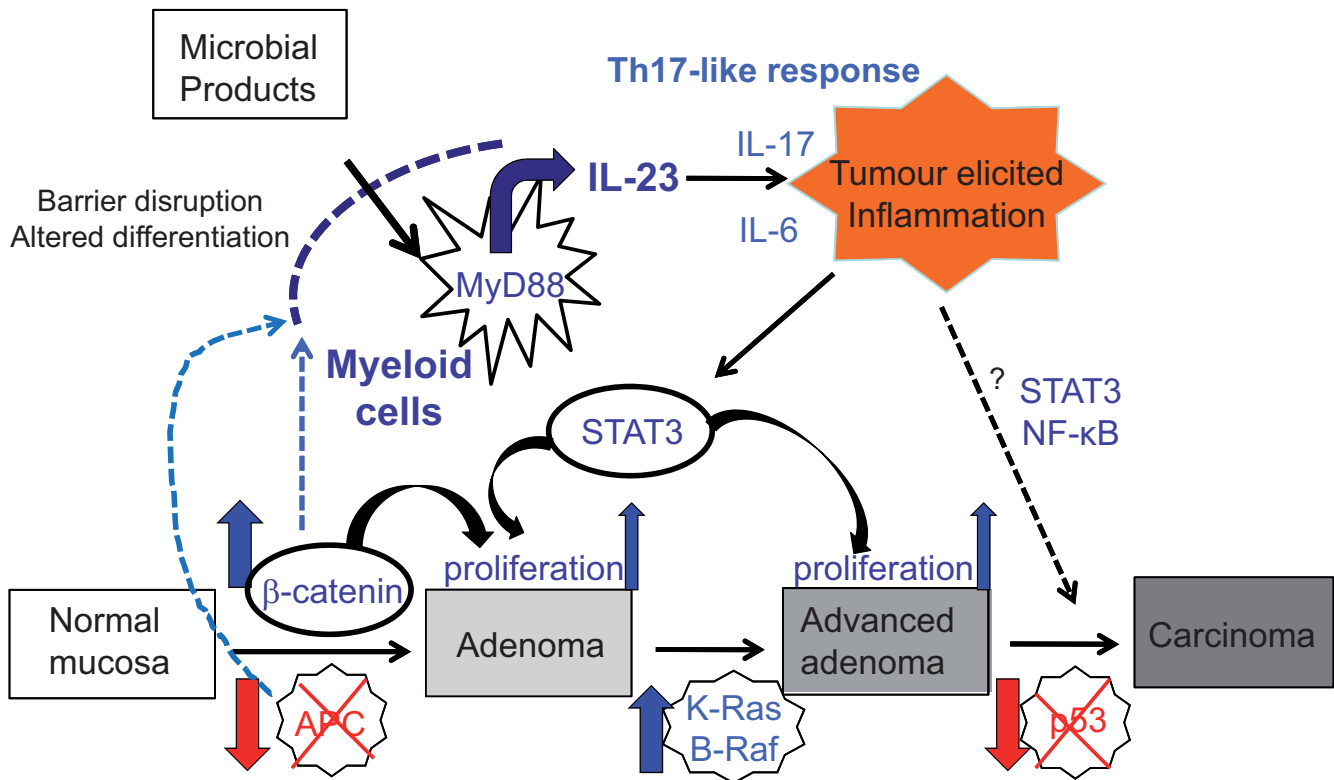**Supplementary Figure 8. Mouse colorectal tumours display dysregulation of barrier and junctional proteins.**
Paraffin-embedded colon sections from CPC-APC mice were stained with antibodies to claudin 4, claudin 7, JAM-A or JAM-B and analyzed by fluorescent microscopy **(a)** or with claudin 3 and claudin 5 specific antibodies **(b)**. Normal (N) and Tumour (T) areas are shown. Scale bars, 50 μm.

a
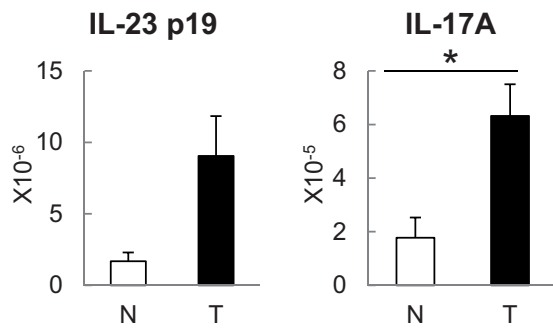
b
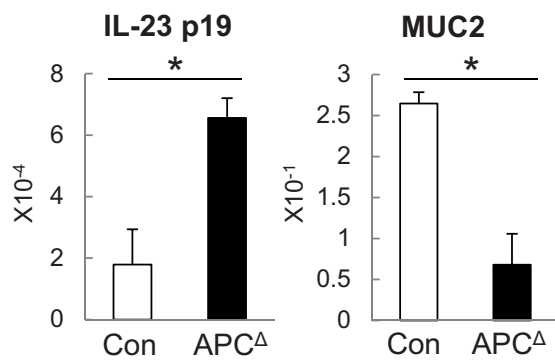
Supplementary Figure 9. Early human adenomas and mouse rapidly transformed colons display upregulation of cytokines and dysregulation of barrier proteins.
Total RNA was isolated from frozen human adenoma tissue removed during colonoscopy (T) and matching tumour-free (N) colon tissue **(a)** or from *Cdx2*[ERT-Cre] x *Apc*[F/F] and control mice injected 3 times with tamoxifen and left for 20 days for rapid colonic transformation **(b)**. RNA was subjected to RT-qPCR analysis of the indicated transcripts. n=5, p=0.056, 0.024 for IL-23p19 and IL-17A in **a**. n=5, p=0.018, 0.014 for IL-23p19 and MUC2 in **b**. Data represent averages ± s.e.m. * p<0.05.

Supplementary Figure 10. Scheme: "Tumour elicited inflammation" is triggered by the classical genetic pathway that drives colorectal tumourigenesis and can further stimulate tumour progression.

Inactivation of tumour suppressor APC is the first step in the classical genetic pathway of colorectal tumourigenesis, leading to persistent activation of β-catenin signaling. We propose that β-catenin signaling in early neoplastic lesions induces altered tissue homeostasis and disruption of the epithelial barrier, including downregulation of mucin 2 expression due to failed goblet cell differentiation and disruption of tight junctions. Tumour-specific deterioration of barrier function allows commensal microflora and microbial products to penetrate the adenomas, activate TLR-MyD88 signaling and induce IL-23 in tumour associated macrophages (TAM). IL-23 regulates tumour elicited inflammation and the Th17-like response, particularly its effector cytokines that include IL-17A and IL-6, to activate transcription factor STAT3, which together with β-catenin enhances the proliferation and growth of adenoma cells. Tumour elicited inflammation further contributes to tumour progression and malignant conversion by multiple mechanisms, some of which many also involve STAT3 activation. Tumour promoting events are in 'blue", tumour suppressors are in 'red'.
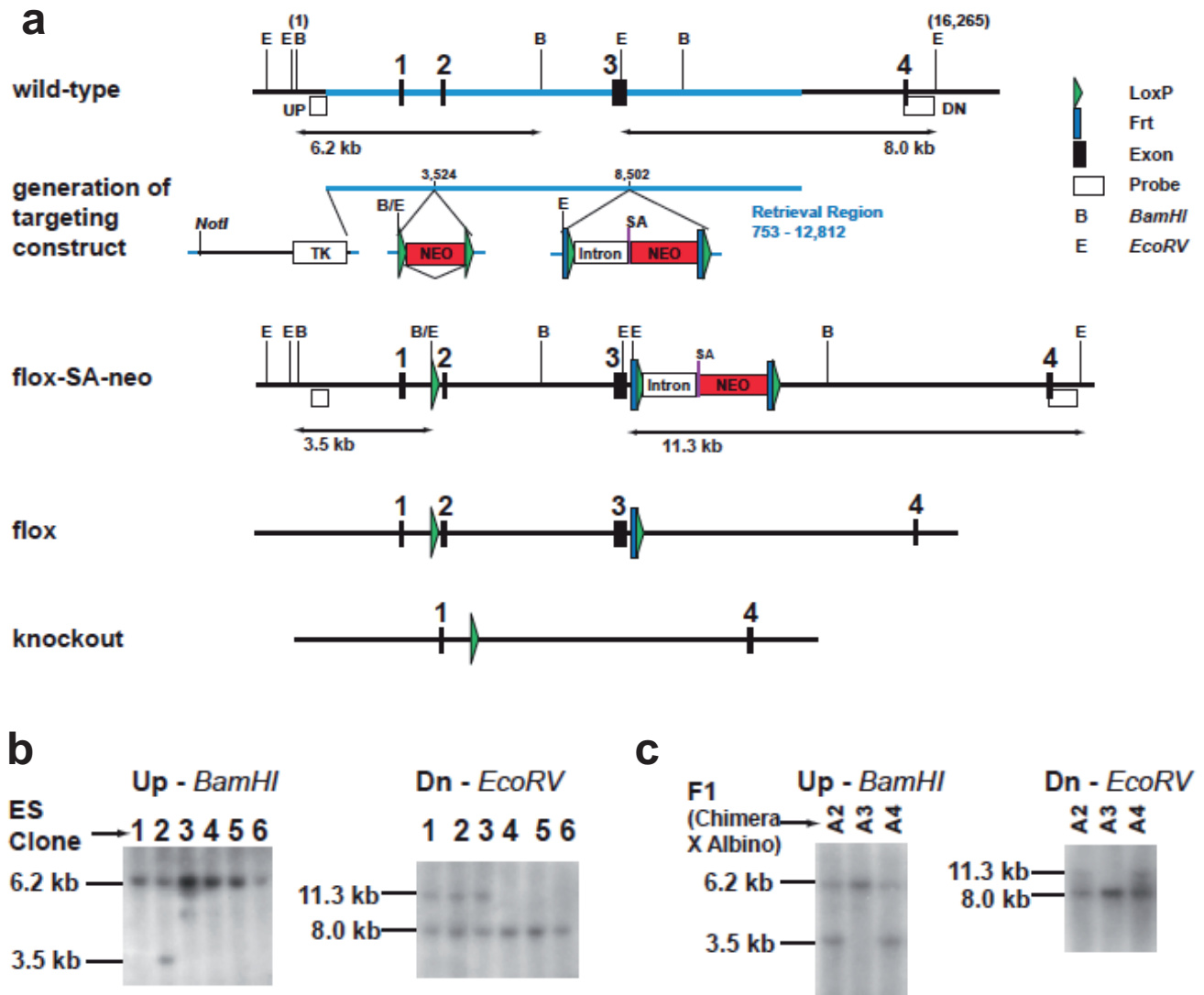
Supplementary Figure 11. Generation of *Il23r*[F/F] and *Il23r*^{-/-} mice.
a: Targeting strategy. Two *LoxP* sites were introduced into introns 1 and 3 of the *Il23r* gene, such that Cre-mediated deletion results in excision of exon2 and exon3, which encode the ligand-binding extracellular portion of the receptor. The targeting construct was introduced into Bruce 4 C57Bl6 ES cells and homologous integrants were used to generate *Il23r*[F/F] mice. **b:** Southern blot screening of Bruce 4 ES clones. Representative clones are shown. **c:** Selected clones were injected into C57BL/6-Albino blastocysts and transferred to pseudopregnant females. Chimeric offspring were bred with C57BL/6-Albino partners to generate F1 mice that were screened for integration by Southern analysis (for more details see Methods). Resulting mice with the *Il23r*[SA-flox-neo] allele were crossed with C57BL/6-FLPe mice to facilitate recombination between *Frt* sequences and generate the *Il23r*[flox] allele. The null allele was generated by crossing *Il23r*[F/F] mice with *CPC-Cre* transgenic mice (for more details see Methods). Complete ablation of IL-23R was confirmed by Q-RT-PCR of spleen and tumour-derived cells.

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