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

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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.

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.

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-

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

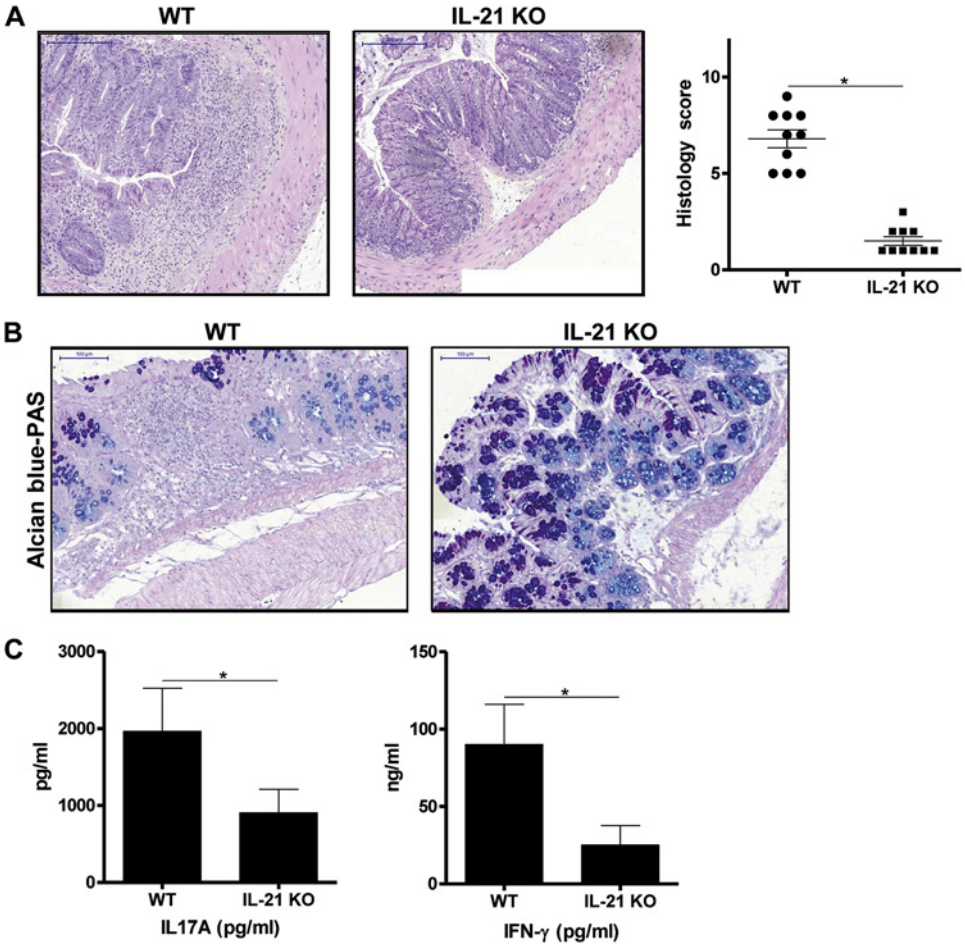

Figure 1 Interleukin (IL)-21-deficient mice are protected from chronic dextran sulphate sodium (DSS)-colitis. (A) H&E staining of representative colon sections and histology score on day 42 of chronic DSS-colitis. Data shown are mean values ± SEM and derived from at least 10 mice per group. Individual 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

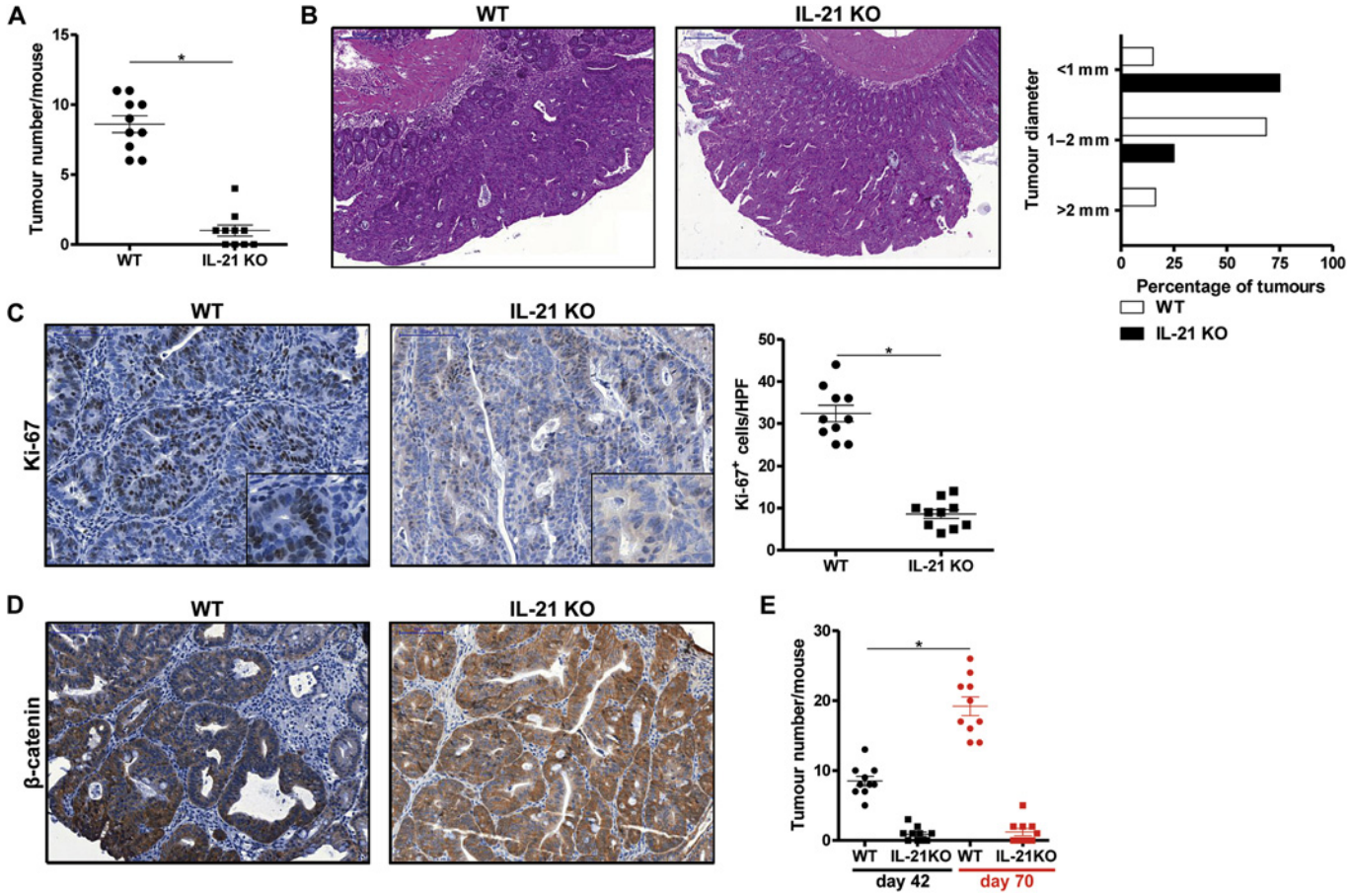

Figure 2 Tumour number is decreased in the absence of interleukin (IL0-21. (A) Number of tumour nodules on day 42 of chronic dextran sulphate sodium (DSS)-colitis with azoxymethane (AOM)-induced tumour growth. Data shown are mean values ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤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 ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤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 ± SEM, derived from at least 10 mice per group. Individual points represent one mouse. *p≤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

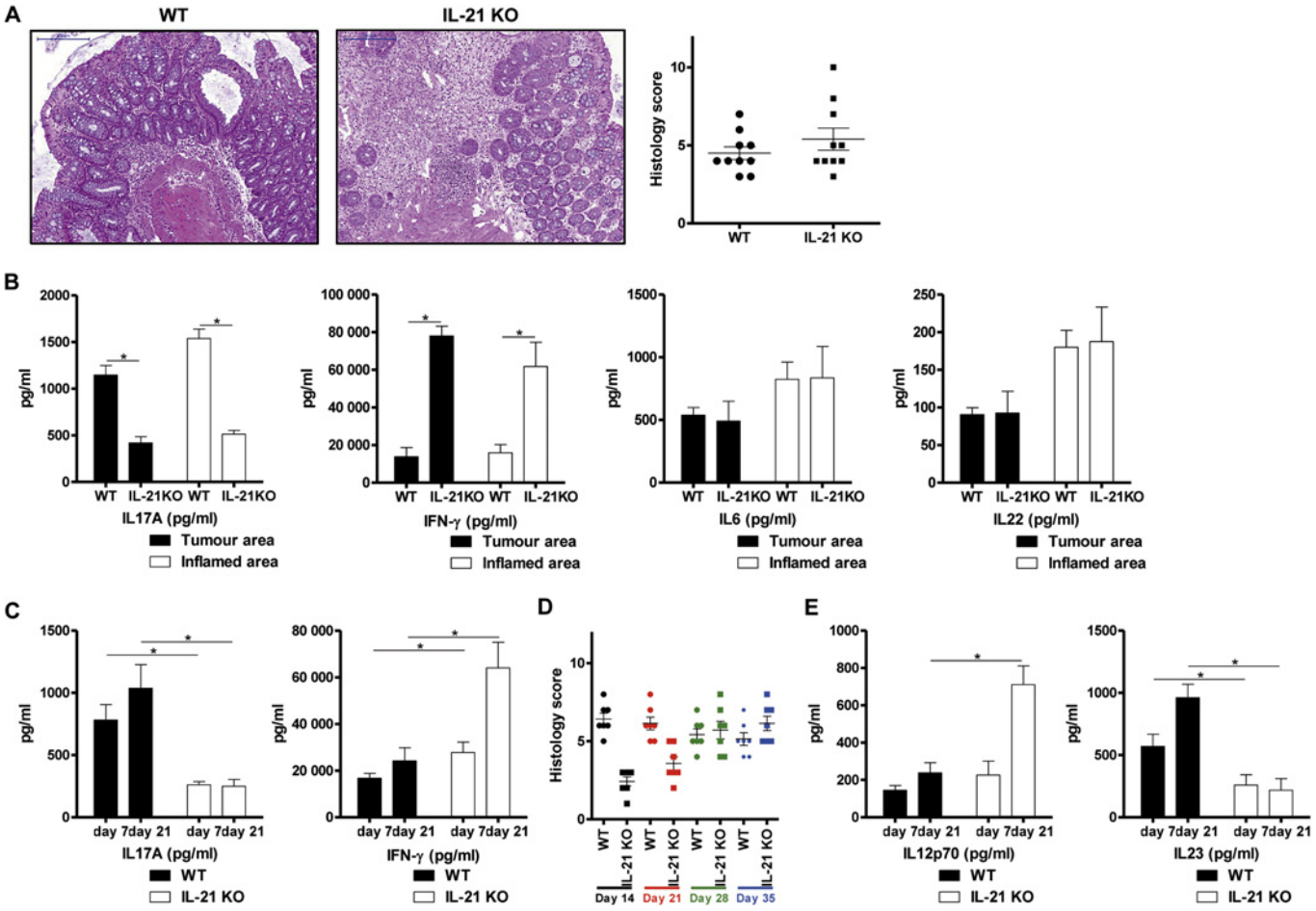

Figure 3 Restored inflammation in interleukin (IL)-21-deficient mice with chronic dextran sulphate sodium (DSS)-colitis and tumorigenesis. (A) H&E staining of representative inflammatory areas of colon sections and histology score on day 42 of chronic DSS-colitis with azoxymethane (AOM)-induced tumour 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.

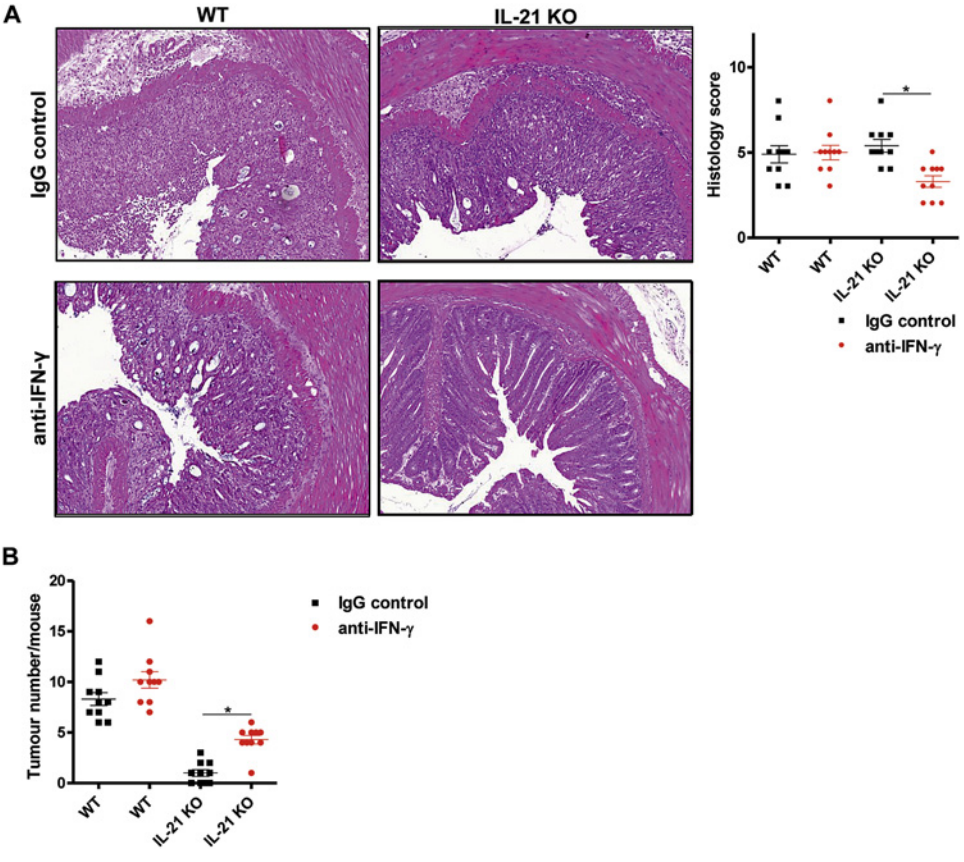

Figure 4 Interferon (INF)γ regulates inflammation and tumour growth in the absence of interleukin (IL)-21. (A) H&E staining of representative colon sections of inflamed areas and histology score on day 42 of chronic dextran sulphate sodium (DSS)-colitis with tumour growth after inhibition of IFNγ. Inhibition 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.

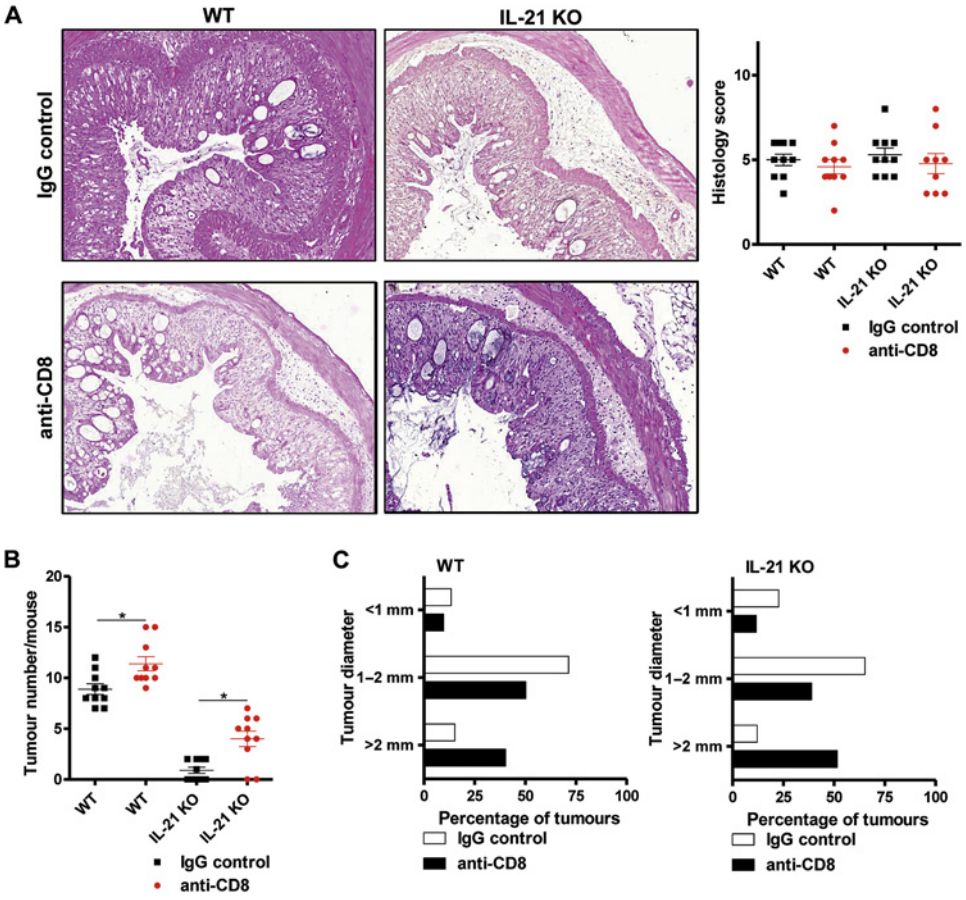

Figure 5 CD8 T cells control tumour growth. (A) H&E staining of representative colon sections of inflamed area and histology score on day 42 of chronic dextran sulphate sodium (DSS)-colitis with tumour growth, after deletion of CD8 T cells. CD8 T cell deletion was started on day 14. Data shown are mean 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

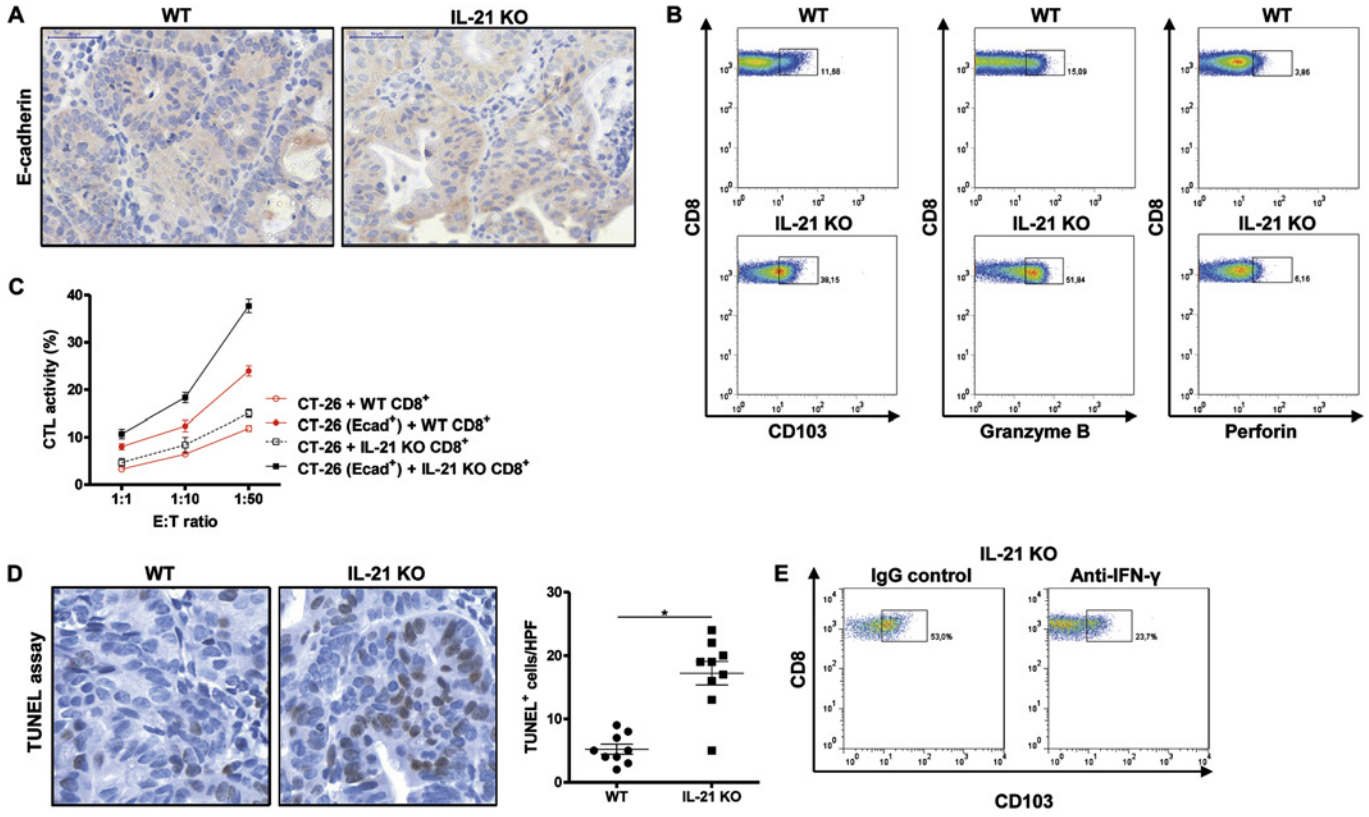

Figure 6 Tumour immunosurveillance increases in the absence of interleukin (IL)-21. (A) E-cadherin staining of representative tumour areas of colon sections on day 42 of chronic dextran sulphate sodium (DSS)-colitis with azoxymethane (AOM)-induced tumour growth. (B) Flow cytometric determination of CD8CD103, 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 cytotoxic 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

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

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