Deficiency of the immunostimulatory cytokine IL-21 promotes intestinal neoplasia via dysregulation of the Th1/Th17 axis

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\textbf{ABSTRACT}

IL-21 has reported activity in promoting both Th1 and Th17 immune responses. Its role in sporadic human colorectal cancer is unknown. We aimed to delineate the role of IL-21 in a model of sporadic intestinal tumorigenesis. We found that in APC\textsuperscript{MIN–/–} mice, ablation of IL-21 increased intestinal tumorigenesis. Expression of pro-inflammatory Th17-associated genes, including RORC and IL-17A, was increased in the intestine in the absence of IL-21, while expression of antitumor Th1-associated genes Tbet, IFN\textgamma, granzyme B, and perforin was decreased. Similarly, the IL-21-deficient APC\textsuperscript{MIN–/–} mouse intestines had fewer infiltrating T cells as well as decreased effector memory T cells, NK cells, and granzyme B-expressing cells. Finally, our data suggest that IL-21 impairs Th17 immune responses as mesenteric lymph nodes from IL-21-deficient mice had increased IL-17A expression, and naive helper T cells from IL-21-deficient mice were more prone to differentiate into IL-17A-secreting cells.

\textbf{Introduction}

Human cancers consist not only of neoplastic cells but also a microenvironment of immune cells and mediators. In colorectal cancer, the concentration of tumor-infiltrating T cells and genes associated with T cells is associated with metastasis and survival. While high expression of molecules associated with the Th1 cytotoxic pathway is associated with improved outcomes, expression of molecules associated with the Th17 pathway is associated with aggressive tumor biology.\textsuperscript{1}

IL-21 is involved in the proliferation of T and B cells as well as the cytotoxic activity of T and NK cells.\textsuperscript{2,3} IL-21 was initially viewed as a potential anticancer treatment for its enhancement of the Th1 pathway\textsuperscript{4,5} without a compensatory increase in regulatory T cells (Tregs).\textsuperscript{6,7} More recently, however, IL-21 has been reported to be associated with the promotion of Th17 pathway inflammation,\textsuperscript{8,9} and its inhibition has been associated with improvement in colitis-associated colorectal cancer.\textsuperscript{10,11} The immune mechanisms in the development of colitis-associated colorectal cancer and sporadic colorectal cancer likely differ. In sporadic colorectal cancer, there is a balance between tumor-promoting inflammation and antitumor immunity, whereas in colitis-associated colorectal cancer, the immune system appears to have a mostly inflammatory and pro-tumorigenic role.\textsuperscript{12} The goal of this study was to delineate the role of IL-21 in sporadic intestinal carcinogenesis.

\textbf{Materials and methods}

\textbf{Mice}

C57BL/6J (WT), C57BL/6J-Apc\textsuperscript{MIN/J} (APCMIN–/–) mice (Jackson Laboratories), and B6.129S-I21\textsuperscript{tm1Lex/Mmucd} (IL-21–/–, IL-21KO) mice (Mutant Mouse Resource and Research Center) were used. IL-21–/–, APC\textsuperscript{MIN–/–} (IL-21KO-APCMIN–/–) mice were generated through breeding. Protocols were approved by the VA Boston Healthcare System Institutional Animal Care and Use Committee.

\textbf{Quantification of polyps}

Murine small intestine was flushed and divided into three segments. Polyps were counted and measured at their greatest diameter. Tumor load was calculated by summing the maximum diameter of all polyps.\textsuperscript{13,14}

\textbf{Preparation of intestinal leukocytes and flow cytometry}

Ileum was processed per instructions in the mouse Lamina Propria Dissociation Kit (Miltenyi). Cells were then separated with a 40/80 Percoll gradient (GE Healthcare), blocked with anti-mouse CD16/32 (BD Biosciences), and stained with anti-mouse CD45 (clone 30-F11), CD8\textsuperscript{+} (53–67), CD44 (IM7), CD3 (145–2C11), and CD4\textsuperscript{+} (GK1.5) (all BD Biosciences) antibodies as well as anti-mouse NK1.1 (PK136; eBioscience) for 20 min. Cells were washed...
and run on a BD LSRFortessa (BD Biosciences). Data were analyzed with FlowJo v10.0.7.

### Immunohistochemistry

Ileum was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Tissues were deparaffinized in Histo-Clear (National Diagnostics) and rehydrated in ethanol. Antigen retrieval was performed in pH 6.0 sodium citrate for FoxP3, B220, and granzyme B staining or in pH 8.0 EDTA for CD3 staining using a pressure cooker. Staining with anti-mouse FoxP3 (clone FJK-16, eBioscience), CD3 (CD3–12, Abcam), CD8α (53–6.7, R&D Systems), NKp46 (29A1.4, BioLegend), B220 (RA3–6B2, BD Biosciences), and granzyme B (R&D Systems) was performed as previously described.15

### Immunofluorescence

Mouse ileum was fixed in 10% formalin, washed and transferred to 15% sucrose for 1 h and then to 30% sucrose. Tissue was frozen in Optimal Cutting Temperature compound (Sakura Finetek) and cut into 10-μm sections. Slides were incubated at 37°C for 20 min followed by a bath in cold acetone and PBS with Triton-X for 30 min. Slides were blocked with 10% donkey serum (S30, EMD Millipore) for 30 min and briefly placed into cold sodium glycine. Tissue sections were stained with primary antibodies diluted in 10% donkey serum and incubated overnight. Slides were then rinsed with PBS with 0.1% Tween-20. Donkey anti-goat IgG conjugated with Alexa Fluor 488 or Alexa Fluor 647 (Abcam) was applied for 2 h at room temperature. Slides were then rinsed with PBS with 0.1% Tween-20. DAPI (Invitrogen) was applied for 5 min.

### RNA extraction and qRT-PCR

Tissue was frozen at −80°C. Cell lysates were prepared with Qiazol Lysis Reagent (Qiagen) followed by homogenization (Tissue Lyser II, Qiagen). The RNeasy Plus Universal Kit (Qiagen) was used for RNA extraction. cDNA was synthesized using the SuperScript III First Strand Synthesis System (Life Technologies) with oligo-dT as the first strand primer. Reactions were run on the ABI PRISM 7900HT Sequence Detection System (Life Technologies). Relative expression was normalized to GAPDH using the \(2^{-\Delta\Delta Ct}\) method. See Supplementary Table for primers.

### Mouse Th17 polarization

Th17 polarization was performed as previously described.16 In short, naïve splenic CD4+ cells extracted from 6–8-week-old mice with the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi) were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies along with soluble anti-IL-4 and anti-IFNγ antibodies as well as IL-6, TGF-β, IL-23, and IL-1β for 6 d. Cells were then re-stimulated with PMA/ionomycin for 5 h. Brefeldin A was added after 1 h. Fc receptors were blocked and cells labeled with antibodies to surface markers. Cells were then fixed and permeabilized and stained with anti-mouse IL-17A and IFNγ. Flow cytometry was performed as above.

### Statistical methods

All comparisons were made using the Student’s t-test with \(p < 0.05\) considered significant.

### Results and discussion

#### Intestinal polyposis is increased in IL-21KO-APCMIN/+ mice

The APCMIN/+ mouse model is commonly employed to study intestinal carcinogenesis. APCMIN/+ mice develop small intestine tumors due to a germline mutation in Apc. Mutated APC is an important driver of human sporadic colon cancer. While previous studies have examined the role of IL-21 in inflammatory models of intestinal carcinogenesis, to our knowledge, the relevance of IL-21 in spontaneous intestinal tumorigenesis is not known.

We began by comparing ileal IL-21 levels between APCMIN/+ mice and wild-type (WT) mice. We found that IL-21 expression was increased over 4-fold in polyp-bearing APCMIN/+ ileum of 15-week-old mice (Fig. 1A). We also noted an approximately 16-fold increase in IL-21 protein in the polyp-bearing jejunum of APCMIN/+ mice at 15 weeks (Fig. S1A). As IL-21 has been implicated in both tumor-promoting and antitumor responses, we crossed APCMIN/+ mice with IL-21KO mice to further...
delineate the role of IL-21 in intestinal tumorigenesis. IL-21KO-APC\(^{MIN/+}\) mice had an increased number of polyps as well as an increased tumor load at 15 weeks (Figs. 1B and C), indicating that IL-21 may function in suppressing tumor growth. An increase in adenoma burden could also be seen in IL-21KO-APC\(^{MIN/+}\) mice at 8 weeks of age, although the overall polyp burden was much lower (Fig. S2). Similarly, 8-week old APC\(^{MIN/+}\) mice appeared to have increased expression of ileal IL-21 compared with WT animals, although the magnitude of the difference was much lower than that seen for the 15-week-old animals and was not statistically significant, possibly due to the lower adenoma load (Fig. S1B). The decreased adenoma burden seen in IL-21-deficient animals is in line with previous work showing that colon cancers engineered to overexpress IL-21 exhibit decreased growth and were even found to regress in subcutaneous tumor challenge models.\(^4\),\(^17\) Our results, however, contradict previous studies examining the relationship between IL-21 and intestinal tumorigenesis induced by dextran sulfate sodium (DSS) and azoxymethane (AOM).\(^10\),\(^11\),\(^18\) DSS and AOM are commonly used to mimic colitis-associated colon cancer. Notably, one study used AOM alone in APC\(^{MIN/+}\) mice.\(^18\) The differences between the APC\(^{MIN/+}\) and the AOM-APC\(^{MIN/+}\) model could conceivably be related to the fact that in the AOM-APC\(^{MIN/+}\) model, colon tumors were examined, whereas in the APC\(^{MIN/+}\) model, the preponderance of the adenomas develop in the small intestine. More likely, however, the differences are related to administration of AOM, which has been shown to induce colitis-associated crypt changes in the colon.\(^19\) It should also be noted that our experiments as well as the AOM-APC\(^{MIN/+}\) and AOM-DSS studies primarily relied on mice with a germ-line IL-21 deficiency. Given the well-characterized influences of IL-21 on several immune cell populations, the effect of the absence of IL-21 during development of the immune system cannot be assessed despite the fact that IL-21KO mice do not have an overt immunologic phenotype under homeostatic conditions. One study utilizing the AOM-DSS model obtained similar results regardless of whether the authors used IL-21KO mice or WT mice treated with an IL-21-blocking antibody.\(^11\)

**Th17-associated genes are upregulated and Th1-associated genes downregulated in IL-21KO-APC\(^{MIN/+}\) ileum**

As IL-21 has been implicated in multiple T helper pathways, most importantly the Th1 and Th17 pathways, we next sought to assess differences in T helper differentiation associated with IL-21 deficiency as a possible cause of the increased polyposis. We examined T helper cell gene expression profiles in the intestine of IL-21KO-APC\(^{MIN/+}\) and APC\(^{MIN/+}\) mice using qRT-PCR (Fig. 2 and Fig. S3). IL-21KO-APC\(^{MIN/+}\) ileum was found to have decreased expression of Th1-related genes such as the transcription factor Tbet as well as the cytokine IFNg and the effector cytotoxic molecules granzyme B and perforin. Previous studies have demonstrated a role for IL-21 in the release of granzyme B and perforin by T and NK cells.\(^20\) Similarly, our data are in line with another study where in the AOM-APC\(^{MIN/+}\) model of intestinal polyposis, mice lacking IL-21 were found to have a reduction in the fraction of both perforin and granzyme B producing NK and CD8\(^+\) cells among tumor-infiltrating lymphocytes.\(^18\)

In contrast to the downregulation of the Th1-associated gene profile in IL-21 deficient mice, we found increased expression of pro-inflammatory Th17 genes with a greater than 2-fold increase in expression of the prototypical Th17 transcription factor ROR\(\gamma\) and a greater than 5-fold increase in expression of the eponymous cytokine IL-17A at 15 weeks. The role of IL-17A in promoting carcinogenesis in the APC\(^{MIN/+}\) model has previously been demonstrated,\(^16\),\(^21\) and genes associated with Th17 polarization have been correlated with decreased survival in human colorectal cancer.\(^1\)

![Figure 2](image-url) **Figure 2.** Deficiency of IL-21 in APC\(^{MIN/+}\) mice intestine favors increased expression of Th17-associated transcripts and a decrease in Th1-associated transcripts. Gene expression was determined by qRT-PCR on 15-week-old mouse ileum (n = 4 per group).
Nevertheless, IL-21 has been shown to enhance Th17 polarization \textit{in vitro} when combined with other cytokines. Thus, it was surprising that deficiency of IL-21 was associated with decreased Th17 polarization in this model. Our results are also surprising given that reduced expression of IL-17A was seen in tumors from IL-21-deficient AOM-APC\textsuperscript{MIN/+} mice.\textsuperscript{18} As discussed further above, we believe the differences in the APC\textsuperscript{MIN/+} and the AOM-APC\textsuperscript{MIN/+} models are likely related to the use of AOM, which has been shown to induce inflammatory changes.

Expression of genes associated with the Th2 and Treg pathways was essentially unchanged between the groups.

**IL-21KO-APC\textsuperscript{MIN/+} mouse intestine has fewer infiltrating T cells, effector memory T cells, B cells, NK cells, and granzyme B-expressing cells with an increased CD4\textsuperscript{+}/CD8\textsuperscript{+} ratio**

Given the decreased expression of Th1-associated genes in the poly-polarizing intestine of IL-21-deficient APC\textsuperscript{MIN/+} mice, we next explored for alterations in lymphocyte number and phenotype relevant to a Th1 bias. We first evaluated the number of T cells and B cells in poly-polarizing ileum using immunohistochemistry. T cells were decreased over a third in IL-21KO-APC\textsuperscript{MIN/+} compared with APC\textsuperscript{MIN/+} mice. The number of B cells was also decreased in IL-21KO-APC\textsuperscript{MIN/+} mice, although the total number of infiltrating B cells was low in both cases. There was no alteration in Treg number (Fig. 3A).

We next analyzed single-cell preparations of poly-polarizing ileum by flow cytometry to further characterize T cell populations in relation to IL-21 deficiency. We first explored the CD4\textsuperscript{+}/CD8\textsuperscript{+} T cell ratio. A lower ratio represents a higher number of cytotoxic CD8\textsuperscript{+} T cells in relation to helper CD4\textsuperscript{+} T cells and has been correlated with a good prognosis in colorectal cancer.\textsuperscript{22} Consistent with the hypothesis that IL-21 promotes a Th1 bias and favors antitumor immunity, in this model, the CD4\textsuperscript{+}/CD8\textsuperscript{+} T cell ratio was more than two times higher in the IL-21KO-APC\textsuperscript{MIN/+} mice intestines as compared with that of APC\textsuperscript{MIN/+} mice (Figs. 3B and C).

We also looked at the effector memory CD8\textsuperscript{+} T cell population in the adenoma-bearing ileum as a marker of a robust antitumor immune response. The percentage of effector memory (i.e., CD44\textsuperscript{+}) CD8\textsuperscript{+} T cells was almost twice as high in APC\textsuperscript{MIN/+} as compared with IL-21KO-APC\textsuperscript{MIN/+} intestine (Fig. 3D). As NK cells, like cytotoxic CD8\textsuperscript{+} T cells, can function as an important effector of Th1 responses and antitumor immunity, we similarly examined the effect of IL-21 deficiency on the number of infiltrating NK cells. NK cells were decreased in the poly-polarizing ileum of
IL-21KO-APCMIN/+ compared with APCMIN/+ mice in a similar proportion to total T cells (Fig. 3E).

The cytotoxic function of both CD8+ T cells and NK cells is mediated by the enzyme granzyme B. IL-21 has been shown to upregulate granzyme B in these cells. Thus, we investigated whether the number of granzyme B-expressing cells in the polyp-bearing ileum of APCMIN/+ mice was altered by IL-21 deficiency. Compared to the APCMIN/+ mouse intestine, that of the IL-21KO-APCMIN/+ mouse had approximately half as many cells staining for granzyme B on immunohistochemistry (Figs. 3F and G). To investigate which cells are responsible for any granzyme B-mediated cytotoxicity in APCMIN/+ mice, we doubly stained tissue sections for granzyme B and cell type markers by immunofluorescence. We found that over half of the granzyme B-staining cells also co-stained for CD3, indicating that T cells were the major producers of granzyme B with a smaller contribution from B cells and NK cells (Fig. 3H).

Our finding of alterations in the profile of infiltrating leukocytes indicating that IL-21 promotes a cytotoxic antitumor response in colorectal cancer is in line with prior studies and further explains our gene expression results. The role of IL-21 in the expansion of T cells and NK cells has been previously demonstrated in vitro.2,6 Mice overexpressing IL-21 have increased populations of effector memory T cells,5,6,20 and IL-21 can enhance the cytotoxic activity of T and NK cells via release of granzyme B and perforin.20 In experiments where IL-21 was overexpressed in subcutaneous tumor challenge models, IL-21 was found to increase infiltration of T and NK cells4 and to enhance lysis of tumor cells by splenocytes.17 Furthermore, increased expression of IL-21 has been correlated with dramatically improved disease-free survival in human colorectal cancer.23

**Deficiency of IL-21 increases the tendency to polarize to the Th17 phenotype**

While IL-21 is well known to enhance cytotoxic lymphocyte responses and antitumor immunity, multiple studies, using predominantly in vitro experiments, have indicated that IL-21 not only promotes, but also sustains the Th17 pathway.24-26 As our data appeared to conflict with these earlier reports, we went on to further investigate the potential relationship between loss of IL-21 and Th17 differentiation in vivo. We first aimed to assess for differences in baseline RORγt and IL-17A expression related to IL-21 deficiency in mesenteric lymph nodes draining the intestine using qRT-PCR. We found that IL-17A expression was increased by 30-fold in IL-21KO mice. RORγt expression also appeared to be increased 2-fold, although the difference was not statistically significant (Fig. 4A). We next sought to determine whether the absence of IL-21 influences the ability of naive CD4+ T cells to polarize to the Th17 phenotype. Naïve T cells from spleens of WT and

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**Figure 4.** Deficiency of IL-21 results in an increase in the Th17 phenotype. (A) Comparative expression of RORγt and IL-17A in mesenteric lymph nodes from IL-21KO as compared with that from wild-type (WT) mice as determined by qRT-PCR (n = 5 per group) *p < 0.05. (B) Percent of naive splenic CD4+ cells expressing IL-17A after polarization toward the Th17 phenotype as determined by flow cytometry with intracellular staining. (C) Representative density plots of IL-17A- and IFN-γ-expressing cells after polarization are shown. Gating was performed on live, single cells.
IL-21KO mice were subjected to Th17 polarizing conditions and expression of IL-17A was measured. Naïve CD4+ T cells from IL-21KO mice proved to be more easily polarized to the Th17 pathway with approximately 1.5 times the number of IL-17A-producing cells after polarization compared with those from WT mice (Figs. 4B and C).

These results may help to better understand the role of IL-21 in helper T cell differentiation, particularly with regard to Th17 responses. The exact role of IL-21 in the Th17 pathway has been controversial. Several in vitro studies using human and mouse cells have proposed that IL-21 is involved in amplification of the Th17 differentiation loop.24,27,28 It has even been argued that IL-21 is necessary for the development of Th17 cells.29,30 Subsequent in vitro studies, however, demonstrated that IL-6 with or without TGF-β is sufficient to drive Th17 polarization of naïve CD4+ mouse T cells deficient in IL-21 or its receptor IL-21R. These studies also showed that Th17-mediated mouse experimental autoimmune encephalomyelitis (EAE), is preserved in the absence of IL-21 and IL-21R deficiency of IL-17A, but not the pro-

In summary, we are the first to demonstrate that in a mouse model of spontaneous intestinal tumorigenesis, IL-21 impairs tumor growth and is associated with enhancement of T and NK cell proliferation and an infiltrating leukocyte profile suggesting cytolytic activity. In addition, we discovered that deficiency of IL-21 is associated with enhancement of the Th17 axis both in sporadic intestinal tumorigenesis and in healthy mice. Together, these results offer new information on the role of IL-21 in sporadic intestinal carcinogenesis as well as on the role of IL-21 in Th1 and Th17 responses.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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