Interleukin 33 Signaling Restrains Sporadic Colon Cancer in an Interferon-γ–Dependent Manner

Moritz F. Eissmann1, Christine Dijkstra1, Merridee A. Wouters1, David Baloyan1, Dmitri Mouradov2,3, Paul M. Nguyen3,4, Mercedes Davalos-Salas5, Tracy L. Putoczki3,4, Oliver M. Sieber2,3,6,7, John M. Mariadason5, Matthias Ernst1, and Frederick Masson1

Abstract

Interleukin 33 (IL33) is an inflammatory cytokine released during necrotic cell death. The epithelium and stroma of the intestine express large amounts of IL33 and its receptor St2. IL33 is therefore continuously released during homeostatic turnover of the intestinal mucosa. Although IL33 can prevent colon cancer associated with inflammatory colitis, the contribution of IL33 signaling to sporadic colon cancer remains unknown. Here, we utilized a mouse model of sporadic colon cancer to investigate the contribution of IL33 signaling to tumorigenesis in the absence of preexisting inflammation. We demonstrated that genetic ablation of St2 enhanced colon tumor development. Conversely, administration of recombinant IL33 reduced growth of colon cancer cell allografts. In reciprocal bone marrow chimeras, the concurrent loss of IL33 signaling within radioresistant nonhematopoietic, and the radiosensitive hematopoietic, compartments was associated with increased tumor burden. We detected St2 expression within the radioresistant mesenchymal cell compartment of the colon whose stimulation with IL33 induced expression of bona fide NF-κB target genes. Mechanistically, we discovered that St2 deficiency within the nonhematopoietic compartment coincided with increased abundance of regulatory T cells and suppression of an IFNγ gene expression signature, whereas IL33 administration triggered IFNγ expression by tumor allograft-infiltrating T cells. The decrease of this IFNγ gene expression signature was associated with more aggressive disease in human colon cancer patients, suggesting that lack of IL33 signaling impaired the generation of a potent IFNγ-mediated antitumor immune response. Collectively, our data reveal that IL33 functions as a tumor suppressor in sporadic colon cancer.

Introduction

Accumulating evidence links gastrointestinal malignancies to inflammation, a correlation highlighted by an increased incidence of colon cancer in patients with inflammatory bowel disease (1, 2). We and others have shown that excessive activation of the STAT3 signaling pathway through the inflammatory cytokines interleukin (IL)-6 and IL11 licenses the development and progression of colon cancers that harbor bona fide driver mutations (3, 4). However, when and how inflammation prevents tumorigenesis remains unclear. The inflammatory cytokines that elicit antitumor immune responses and prevent colon tumor development remain obscure.

IL33, an unconventional cytokine, functions as a danger-associated molecular signal that is released upon necrotic cell death (5, 6). IL33 supports Th2 immune polarization to mediate allergic reactions and parasite immunity. However, IL33 also has potential to promote Th1 immune responses and CD8+ T cell and NK cell cytotoxicity during viral infection (7–11). IL33 signals through a receptor heterodimer comprising an IL33-specific St2 subunit that is encoded by the Il1rl1 gene and the IL-1RAP subunit (12–14). St2 is expressed in many immune cell types, including type II innate lymphocytes (ILC2; ref. 15), regulatory T cells (Treg; refs. 16–19), Th2 CD4+ T cells (20), CD8+ T cells (21), mast cells (22), as well as nonhematopoietic cells including fibroblasts, endothelial, and epithelial cells (23–26).
Increased IL33 expression has been associated with poor prognosis in gliomas (27), ovarian (28), head and neck (29), gastric cancers (30), and other malignancies. Likewise, IL33 expression is increased in tumors of colon cancer patients (23, 31, 32) and IL33 signaling affected development of colitis-associated colon cancer in mice (31, 33). Although colitis is underpinned by extensive mucosal cell death and associated with prominent IL33 release, IL33 is also detected during the continuous homeostatic renewal of the intestinal lining that enhances antitumor activities. However, it remains unknown whether IL33 release during tissue homeostasis, and thus during initiation of sporadic colorectal cancer (CRC), exacerbates tumorigenesis or enhances antitumor activities.

Here, we have analyzed the role of IL33 signaling in a mouse model of sporadic CRC in the absence of overt inflammation or colitis. Using adoptive bone marrow (BM) transfers, we found that the lack of IL33 signaling resulting from the concurrent deficiency of the IL33 receptor St2 within the nonhematopoietic and hematopoietic cell compartments enhanced colon tumorigenesis. We showed that St2 deficiency in mesenchymal cells of the colon led to a decrease in a protective IFNγ gene expression signature and that attenuated expression of this gene signature correlated with disease progression in colon cancer patients. Finally, IL33 treatment reduced tumor growth in a transplantable colon cancer model, a phenomenon associated with increased frequency of tumor-infiltrating IFNγ-positive CD4+ and CD8+ T cells. Collectively, our data argue that IL33 promotes an immune protective mechanism that helps guard against the development of sporadic colon cancer.

Materials and Methods

Mice
St2−/− mice have been previously described (13) and were maintained on a BALB/c background, and age- and sex-matched wild-type (WT) BALB/c mice were used as controls. For MC38 allograft experiments age- and sex-matched littermates from the C57BL/6 background were used (14). All mice were used at 6 to 8 weeks of age and were maintained under specific pathogen-free conditions in accordance with the guidelines of the Walter and Eliza Hall Institute of Medical Research and the Austin Health Animal Ethics Committees.

Colon cancer model
Sporadic colon cancer was induced by intraperitoneal injections of azoxymethane (AOM; Sigma, 10 mg/kg) once weekly for 6 consecutive weeks as described (3). Mice were monitored for tumor development for up to 20 weeks after the last AOM injection.

Transplantable colon cancer model
MC38 colon cancer cells (1 × 10⁶) were injected subcutaneously into the hind flank of either WT or St2−/− mice. Tumor volume was determined via caliper measurement and calculated with the formula volume = (length × width²)/2. At the experimental endpoint, MC38 allograft tumors were dissected and tumor mass determined.

For the IL33 treatment experiment, the cohort of MC38 tumor-bearing WT mice was randomly divided into two treatment groups when the average tumor volume reached 50 mm³. Group 1 received 50 μg/kg recombinant murine IL33 (BioLegend) and group 2 PBS daily via intraperitoneal injections.

MC38 cells were not authenticated within the past year. Cells were freshly frozen from liquid nitrogen and used for transplantation within 2 to 3 weeks for all experiments performed.

BM chimeras
BM chimeras were established by reconstituting lethally irradiated (2 × 5 Gy) WT or St2−/− recipient mice with 5 × 10⁶ to 10 × 10⁶ BM cells from either WT or St2−/− mice and allowed to reconstitute for 8 weeks (3).

Flow cytometry
Fluorochrome-conjugated antibodies directed against the following antigens were used for analysis by flow cytometry: CD16/ CD32 (clone 93), CD4 (clone GK1.5), EpCAM (clone G8.8), Foxp3 (clone FJK-16s), and Gata3 (clone TWAJ), all from eBioscience RORγt (clone Q31-378), CD62L (clone MEL-14), and IFNγ (clone XMG1.2), all from BD Biosciences; CD3 (clone REA641), CD90.2 (clone S450-REA641), CD45.2 (clone S450-REA641), CD32 (clone 93), CD4 (clone GK1.5), EpCAM (clone G8.8), Foxp3 (clone FJK-16s), and Gata3 (clone TWAJ), all from eBioscience RORγt (clone Q31-378), CD62L (clone MEL-14), and IFNγ (clone XMG1.2), all from BD Biosciences; CD3 (clone REA641), CD90.2 (clone S450-REA641), CD45.2 (clone S450-15-2) from the WEHI monoclonal antibody laboratory. Propidium iodide, SytoxBlue (Invitrogen) or Fixable viable dye eFluor560 (eBioscience) stains were used to exclude dead cells. For intracellular staining of transcription factors the eBioscience Fixation/Permeabilization kit and for intracellular IFNγ detection the BD Cytotox/Cytoperm Plus kit including a Golgi-Stop incubation step were used according to the manufacturer’s instructions.

Quantitative RT-PCR
Total RNA from frozen tissues was extracted using TRIzol Reagent (Life Technologies). RNA from cells was purified using RNaseasy kits Mini Plus or Micro Plus (both from Qiagen) according to the manufacturer’s instructions. Reverse transcription of RNA isolated from frozen tissues was performed from 2 μg RNA using the High Fidelity cDNA synthesis kit (Applied Biosystems/ Roche). Reverse transcriptase of RNA isolated from cells was performed using Superscript III first-strand synthesis system (Invitrogen). Real-time PCR was performed using the SensiMix SYBR no-Rox kit (Bioneer). Analyses were done in triplicate and mean normalized expression was calculated with Hprt or Gadd45 as the reference gene. Refer to Supplementary Table S1 for oligonucleotide sequences used.

Mesenchymal cell isolation and cytokine stimulation
Tumor-free colons and tumors were excised and digested enzymatically as previously described (34). CD45+/EpCAM+/CD90fluorescent mesenchymal cells were then FACS-sorted using FACS ARIA III (BD Biosciences). Sorted cells (2 × 10⁵) were cultured in 96-well, flat-bottom plates in RPMI medium containing 10% heat-inactivated fetal calf serum and stimulated for 16 hours with murine IL33 (20 ng/mL; R&D Systems).

Bioplex cytokine assay
Cytokines levels from cell culture supernatants were quantified using the Bio-Plex Pro Mouse Cytokine Assay (Bio-Rad).

Tissue preparation and processing
Tumors and adjacent colon tissues were resected, weighed and then snap frozen for subsequent RNA or protein isolation.
Alternatively, entire colons were cleaned, rolled, and fixed in 10% neutral buffer formalin for histological analysis as described (3).

Immunohistochemistry

Hematoxylin/eosin and Alcian blue stainings of formalin-fixed paraffin-embedded tissue slides were performed as previously described (3). Staining for apoptosis (Cell Death Detection Kit, Roche) were performed according to the manufacturer's instructions. For all other immunohistochemical stains, the blocking of endogenous peroxidases was performed by incubation in 3% hydrogen peroxide (v/v; LabServ). For Ki67 and CD3 staining, heat-induced antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6, Thermo Scientific) and sections were stained with anti-Ki67 (Thermo Fisher, clone SP6) or polyclonal anti-CD3 (Abcam), respectively. For CD8 staining, heat-induced antigen retrieval was performed in buffer containing 12 mmol/L EDTA, 0.05% Tween, pH 9, and stained with anti-CD8 (eBioscience, clone 4SM15). For F4/80 staining, antigen retrieval was performed in 0.1% trypsin, 3% acetic acid buffer, followed by staining with anti-F4/80. Staining was detected using HRP-conjugated secondary antibodies (Dako) and the liquid diaminobenzidine (DAB) substrate chromogen system (Dako), before counterstaining with hematoxylin. Slides were scanned using an Aperio Digital Pathology Slide Scanner (Leica) and image analysis was performed using the Fiji image analysis software.

Immunofluorescence staining

Immunofluorescence staining for St2 and IL33 on 6xAOM-treated formalin-fixed paraffin-embedded tissue slides was performed using the Opal 5-Colour IHC kit (PerkinElmer, Inc. NEL795001KT). Heat-induced antigen retrieval was performed in buffer containing 12 mmol/L EDTA, 100 mmol/L Tris, 0.05% Tween, pH 9, and endogenous peroxidases were blocked in 3% hydrogen peroxide (v/v; LabServ). The sections were blocked with TNB Buffer (0.1 mol/L Tris–HCl, 0.15 mol/L NaCl, and 0.5% Blocking Reagent; PerkinElmer, Inc.) prior to staining with polyclonal goat anti-mouse St2 (R&D Systems, catalog # AF1004) and polyclonal goat anti-mouse IL33 (R&D Systems, catalog # AF3626). A polyclonal HRP-conjugated secondary antibody (Sigma) in combination with Opal Fluorophore 520 (PerkinElmer, Inc.) was used for detection of staining. Antibody stripping was performed as described in aforementioned heat-induced antigen-retrieval method before counterstaining with Spectral DAPI (PerkinElmer, Inc.) and mounting with HardSet Antifade Medium (Vector Laboratories). Sections were scanned with the Vectra 3.0 Imaging System (PerkinElmer, Inc.) and imaged using the PhenoChart v1.0.4 software (PerkinElmer, Inc.).

Western blotting

For analysis of NF-κB activation, NIH-3T3 murine embryonic fibroblasts were stimulated with murine IL33 (20 ng/mL, R&D Systems). Whole cell protein lysates were prepared in RIPA lysis buffer supplemented with protease and phosphatase inhibitor tablets (Roche) with a TissueLyser II (Qiagen). Protein lysates (30 μg) were loaded on 4% to 12% Bis-Tris polyacrylamide gels and transferred to nitrocellulose membranes using an iBlot apparatus (Invitrogen). Proteins were visualized using an enhanced chemiluminescence detection system (Chemidoc XRS+, Bio-Rad). Antibodies were used to detect α-tubulin (Abcam, clone DM1A), total p65 (Cell Signaling Technology, clone D14E12), or phospho-p65 (Cell Signaling Technology, clone 93H1).

RNA sequencing

Total RNA was extracted from snap-frozen tumors collected from BM chimeras using TRIzol Reagent (Life Technologies). Extracted RNA was then further purified using RNasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Libraries were generated using the Illumina TrueSeq RNA sample preparation kit according to the manufacturer's instructions. Three biological replicates were generated and sequenced for each group of BM chimeras by subjecting 1 μg of RNA to single-end sequencing on an Illumina HiSeq 2500 instrument at the Australian Genome Research Facility. RNA-Seq raw and alignment data are publicly available at NCBI gene expression omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) using the accession number GSE109694.

Bioinformatic analysis

The quality of RNA-Seq reads was checked with FastQC. Untrimmed reads were aligned to the GRCh38/mm10 build of the Mus musculus genome using the TopHat aligner with Bowtie2. Gene wise counts were obtained using HTSeqCount. Genes were discarded for further downstream analysis if they failed to achieve at least 0.5 counts per million in at least two samples. Differential expression analysis was performed using the Bioconductor R package edgeR on the remaining set of 14,389 genes. Normalization was performed using the TMM method. Dispersion was estimated using a generalized linear model with a common component as well as a trended component using a bin-spline method. Averages of the three biological replicates of each group were taken and z scores calculated for each gene across the four groups. These were plotted with the R package heatmap.2. Biological processes analysis was performed using Metacore (Thomson Reuters) bioinformatics analysis tool.

Analysis of the TCGA datasets was performed using the R statistical suite on 363 CRC samples. Raw RNASeq counts and tumor stage information was retrieved via the GDC Data Portal (https://portal.gdc.cancer.gov/). Only transcripts where the provided Ensembl IDs mapped to HGNC-approved gene nomenclature were included. Genes were further filtered from downstream analysis if their expression failed to reach at least 1 count per million in at least 5% of samples. Differential expression analysis was performed using the voom/limma R package on the remaining set of 17,021 genes. We used TMM to normalize RNA-Seq data. Human homologs of the mouse genes were retrieved from The Jackson Laboratory. Gene set enrichment analysis (GSEA) was performed using limma.

Statistical analysis

Statistical analyses were performed using the Prism 6 software, Student t test for comparison of groups of samples of equal variance, and t test with Welch correction to compare groups of samples with significantly different variance.

Results

IL33 signaling suppresses the development of sporadic colon cancer

The majority of sporadic CRC in humans arises as a consequence of mutations in the canonical Wnt/β-catenin signaling

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pathway and occurs in the absence of pathologies characterized by overt inflammation. To examine the contribution of IL33 signaling to sporadic colon tumorigenesis, we challenged WT (BALB/c) and Il1rl1−/− mice (referred to as St2−/− mice thereafter) once per week with the alkylating mutagen AOM for 6 consecutive weeks (refs. 3, 4; Fig. 1A). St2−/− mice harbored a larger number of colonic tubular adenomas than their WT counterparts (Fig. 1B). Tumors from St2−/− mice remained noninvasive and were histologically indistinguishable from WT mice (Fig. 1C).

To understand the mechanism(s) accounting for the difference in tumor number, we assessed tumor cell proliferation (Ki67+ cells) and apoptosis (Apoptag). We detected similar numbers of Ki67+ cells (Fig. 1D) and Apoptag+ cells (Fig. 1E) in tumors from WT and St2−/− mice. We then performed quantitative RT-PCR analysis for Il33 (F) and Il1rl1 (exons 10–11, specific for membrane-bound isoform) expression (G) from tumors or adjacent normal colon tissues from 6×AOM-treated WT and St2−/− mice. Data were normalized against expression of Gapdh.

Figure 1.

St2 deficiency promotes the development of sporadic colon cancer. Sporadic colon cancer was induced by weekly administration of AOM over 6 consecutive weeks (6×AOM) to WT and St2−/− mice and analysis occurred 9–15 weeks after the last AOM injection depending on the onset of tumor development. A, Schematic of the experimental procedures. B, Representative whole mounts of the colons of WT and St2−/− mice following 6×AOM treatment 15 weeks after last AOM injection (left), and enumeration of the total number of resulting tumors per mouse (right). C and D, Representative sections from formalin-fixed and paraffin-embedded colons stained for hematoxilin–eosin (C, scale bar, 100 μm) and Ki67 or Apoptag (D; scale bar, 25 μm). E, Enumeration of positive staining cells from D expressed as the total number of Ki67+ cells (left) or Apoptag+ cells (right) per mm² of tumor area. F and G, Quantitative RT-PCR analysis for Il33 (F) and Il1rl1 (exons 10–11, specific for membrane-bound isoform) expression (G) from tumors or adjacent normal colon tissues from 6×AOM-treated WT and St2−/− mice. Data were normalized against expression of Gapdh. B–G, Data were pooled from three independent experiments and shown as mean ± SEM, with each symbol representing an individual mouse. Data were analyzed using unpaired Student t test (E) or unpaired t test with Welsh correction (B, F). *, P < 0.01; **, P < 0.001; n.s., not significant; Student's t test with Welsh correction (B, F).
Tumor burden was analyzed when disease reached ethical endpoints, with each symbol representing an individual mouse. Data were analyzed using 6\(\times\)AOM for 6 consecutive weeks. Independent of the genotype of the reciprocal, adoptive BM transfer, challenged thereafter with 6 lethally irradiated WT or St2-responsive compartment that antagonizes tumor development.

Because St2 is expressed in cells of both hematopoietic and nonhematopoietic origins, we next aimed to identify the IL33 signaling in hematopoietic and nonhematopoietic cells. To explore this discrepancy further, we used colons of Lgr5\textsuperscript{CreERT2} mice (35) to FACS-sort Lgr5\textsuperscript{+} colonic epithelial cells and quantify St2 expression by qRT-PCR and flow cytometry. St2 was not detected in epithelial Lgr5\textsuperscript{+} stem cells of the small intestine express St2, and IL33 signaling within this cell population is associated with increased capacity to differentiate into Paneth and goblet cells (18). To explore this discrepancy further, we used colons of Lgr5\textsuperscript{CreERT2} mice (35) to FACS-sort Lgr5\textsuperscript{+} (eGFP\textsuperscript{+}) and Lgr5\textsuperscript{−} (eGFP\textsuperscript{−}) EpCAM\textsuperscript{−}/CD45\textsuperscript{−} crypt epithelial cells and quantify St2 expression by qRT-PCR and flow cytometry. St2 was not detected in epithelial Lgr5\textsuperscript{+} stem cells from the colon (Supplementary Fig. S3A). Likewise, colons from St2-deficient mice had neither decreased expression of the goblet cell marker Muc2 nor reduced mucus production when compared with WT counterparts (Supplementary Fig. S3B and S3C). Although Il1rl1 transcripts could be detected in EpCAM\textsuperscript{−}/CD45\textsuperscript{−} stromal cells, we failed to detect St2 protein by flow cytometry immediately following enzymatic digestion of colonic tissues (Supplementary Fig. S4A). To ensure that enzymatic digestion would not reduce a weak membrane-associated St2 signal.

IL33 triggers NF-κB signaling in colon mesenchymal cells

Because our results suggest that the lack of IL33 signaling in the nonhematopoietic compartment contributes to the increased tumor burden, we next aimed to identify the cell type(s) within this compartment that could respond to IL33 signals. Epithelial cells (CD45\textsuperscript{−}/EpCAM\textsuperscript{−}) and stromal cells (CD45\textsuperscript{+}/EpCAM\textsuperscript{−}) were FACS-sorted and the expression of Il1rl1 was analyzed by quantitative RT-PCR. We detected Il1rl1 transcripts encoding all St2-encoded isoforms as well as those encoding the membrane-bound St2 isoform (19) in stromal cells but not in epithelial cells (Fig. 3A). Others have shown that Lgr5\textsuperscript{+} epithelial stem cells of the small intestine express St2, and IL33 signaling within this cell population is associated with increased capacity to differentiate into Paneth and goblet cells (18). To explore this discrepancy further, we used colons of Lgr5\textsuperscript{CreERT2} mice (35) to FACS-sort Lgr5\textsuperscript{+} (eGFP\textsuperscript{+}) and Lgr5\textsuperscript{−} (eGFP\textsuperscript{−}) EpCAM\textsuperscript{−}/CD45\textsuperscript{−} crypt epithelial cells and quantify St2 expression by qRT-PCR and flow cytometry. St2 was not detected in epithelial Lgr5\textsuperscript{+} stem cells from the colon (Supplementary Fig. S3A). Likewise, colons from St2-deficient mice had neither decreased expression of the goblet cell marker Muc2 nor reduced mucus production when compared with WT counterparts (Supplementary Fig. S3B and S3C). Although Il1rl1 transcripts could be detected in EpCAM\textsuperscript{−}/CD45\textsuperscript{−} stromal cells, we failed to detect St2 protein by flow cytometry immediately following enzymatic digestion of colonic tissues (Supplementary Fig. S4A). To ensure that enzymatic digestion would not reduce a weak membrane-associated St2 signal.

IL33 Promotes a Protective IFNγ Response in Colon Cancer

**Figure 2.**

St2 deficiency in the radiosensitive cell compartment promotes colon tumor development. Tumor burden of number of WT and St2\textsuperscript{−/−} irradiated hosts following reciprocal, adoptive BM transfer, challenged thereafter with 6\(\times\)AOM and assessed 20–22 weeks after the last AOM administration depending on the onset of the disease in each of the independent experiments. A and B, Number of tumors per mouse (A) and the total tumor area per mouse (B). C, Size distribution of 6\(\times\)AOM-induced colonic tumors of mice analyzed above. Data were pooled from two (B, C) or four (A) independent experiments and shown as mean ± SEM. Tumor burden was analyzed when disease reached ethical endpoints, with each symbol representing an individual mouse. Data were analyzed using unpaired Student t test. ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, not significant.
IL33 stimulation induces expression of NF-κB target genes in colon-derived mesenchymal cells. A, Quantitative RT-PCR analysis of Il1rl1 mRNA expression encoding all isoforms (exons 7–8) or membrane-bound isoform (exons 10–11) in EpCAM<sup>+</sup>/CD45<sup>−</sup> epithelial cells and EpCAM<sup>+</sup>/CD45<sup>−</sup> mesenchymal cells isolated from uninfected colon (“colon”) and tumors (“tumor”) of WT mice undergoing the 6x AOM protocol or left untreated (“UT”). Data were derived from 2 to 4 individual mice per cohort and were normalized against expression of Hprt. Data are representative of two (untreated colon) or one (colon and 6x AOM colon and tumor samples) independent experiments. Mean ± SEM of the technical replicates are shown. B, Mesenchymal cells (CD45<sup>−</sup>/EpCAM<sup>+</sup>/CD90<sup>−</sup>FSC<sup>−</sup>) were isolated from the colon of WT mice and cultured in vitro for 16 hours. FACS plots show St2 and CD90 expression after 16 hours in vitro culture and is representative of three independent experiments. Mean ± SEM with symbols representing independent experiments. Data were analyzed using paired Student t test. **, P < 0.01; *, P < 0.05. C, Protein quantification following multiplex cytokine assay in the supernatant of cultured mesenchymal cells in D. Mean ± SEM with symbols representing independent experiments. Data were analyzed using unpaired Student t test. ***, P < 0.001; **, P < 0.01; *, P < 0.05. E, Immunoblotting analysis of NIH-3T3 murine embryonic fibroblasts stimulated with murine IL33 (20 ng/mL).

below detection, we analyzed St2 expression on FACS-sorted EpCAM<sup>−</sup>/CD45<sup>−</sup> stromal cells following an overnight in vitro culture. Membrane-bound St2 was expressed in about 10% of Sca1<sup>−</sup>/CD90<sup>low</sup>/FSC<sup>−</sup>/EpCAM<sup>−</sup>/CD45<sup>−</sup> cells consistent with their mesenchymal origin (Fig. 3B; Supplementary Fig. S4B). However, these cells were not derived from endothelial cell lineage, because CD31 expression remained restricted to Sca1<sup>+</sup>/CD90<sup>low</sup> cells (Supplementary Fig. S4C).

Because IL33 activates NF-κB signaling in other cell types such as mast cells (36), we then asked whether IL33 also induced NF-κB target genes in colon mesenchymal cells. We found that IL33 stimulation induced expression of Ccl2 (encoding Mcp-1), Ccl5 (encoding Rantes), and Csf2 (encoding GM-CSF) both at the mRNA and protein level (Fig. 3C and D). Consistent with this, IL33 could drive the activation of the canonical NF-κB signaling pathway in NIH-3T3 fibroblasts, as indicated by the rapid phosphorylation of the p65 subunit and concurrent degradation of IκBα (Fig. 3E). Collectively, these data indicated that mesenchymal cells in the colon respond to IL33 by activating NF-κB signaling and leading to the production of inflammatory cytokines that could confer an antitumor immune response.

St2 deficiency leads to increased Treg infiltration within colon tumors

Our data suggested a link between an increased tumor burden and a decreased immune activation and antitumor immune response possibly arising from the absence of IL33-mediated NF-κB activation in mesenchymal cells. Because IL33 stimulation...
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Figure 4.
Loss of IL33 signaling in both the radiosensitive and the radioresistant cell compartments promotes Treg infiltration within colon tumors. A–C, Flow cytometric analysis of enzymatically digested tumors collected from 6xAOM-treated BM chimeric mice generated as in Fig. 2. Representative FACS plots show St2 and Foxp3 expression on gated CD4+/CD3+/CD90+/CD45-/EpCAM+ T cells (A). Data show Foxp3+/CD4+ Treg among CD45+/EpCAM- hematopoietic cells (B, left), and ratio of Treg to EpCAM+/CD45+ tumor cells (B, right). Frequency of St2- cells among Foxp3+/CD4+ Tregs within the spleen or tumors from the indicated BM chimeras (C). A–C, Bars show mean ± SEM; symbols represent individual mice. D–G, Flow cytometric analysis of enzymatically digested colon tumors from 6xAOM-treated WT or St2-/- mice. Representative FACS plots showing intracellular expression of Gata3 and Rorγ in Foxp3+/CD4+ Tregs (D). Frequency of Gata3+ cells within Foxp3+/CD4+ Tregs (E). Quantification of Foxp3 expression as geometric mean fluorescence (GMFI) in Foxp3+/CD4+ Tregs (F). Frequency of Rorγ+ (Gata3+) within Foxp3+/CD4+ Tregs (G). Data are representative of three independent experiments and shown as mean ± SEM. Data were analyzed using unpaired Student t-test. *, P < 0.05; n.s., not significant.

of colonic mesenchymal cells resulted in the production of chemokines supporting myeloid cell migration (i.e., Ccl2 and Ccl5), we analyzed the abundance of myeloid cells within colon tumors. We detected similar numbers of infiltrating F4/80+ macrophages and mast cells between WT and St2-/- mice, suggesting that migration of myeloid cells may not be affected by St2 deficiency (Supplementary Fig. S5A–S5D). Similarly, the numbers of colon tumor-infiltrating CD8+ T cells remained comparable between 6xAOM-challenged WT and St2-/- mice (Supplementary Fig. S5E and S5F).

Because we observed a trend of increased abundance of total CD3+ T cells in St2-deficient tumors (Supplementary Fig. S5G and S5H), we postulated that this could arise from an increase in CD4+ T cells, of which 30% to 70% account for Foxp3+ regulatory T (Treg) cells (Fig. 4A). Indeed, we observed an increased frequency of tumor-infiltrating Foxp3+ Tregs in
tumors of chimeric mice, where neither the hematopoietic nor the nonhematopoietic compartments could respond to IL33 signals (Fig. 4B and C). In contrast, the frequency and total number of Tregs in colon of mice that had not undergone the 6xAOM challenge remained unaffected by St2 deficiency (Supplementary Fig. S6A and S6B).

Consistent with previous reports (16, 37), we found a high proportion (28.5%) of St2-expressing Tregs within the colon of naïve mice (Supplementary Fig. S6C), and this was further increased in the colon tumors (Fig. 4C). Although St2 deficiency increased the accumulation of tumor-infiltrating Tregs, it did not affect the differentiation of these cells as the expression of the transcription factors Foxp3, Gata3 or Rorγt remained unchanged (Fig. 4D–G). From these data, we concluded that St2 deficiency increased the frequency of tumor-infiltrating Tregs, but affected neither their differentiation status nor the recruitment of CD8+ T cells and myeloid cells into the tumors.

Nonhematopoietic cell responsiveness to IL33 mounts IFNγ gene expression signature

To elucidate the molecular mechanisms underpinning the tumor-suppressing effect of IL33 signaling in sporadic colon cancer, we performed whole transcriptome analysis of tumors collected from each of the four cohorts of the 6xAOM-treated BM chimeras. We compared the genes differentially expressed (DE) between chimeras with an identical hematopoietic cell compartment grafted in either WT or St2-deficient hosts (WT→WT vs. WT→St2+/−, or St2+/−→WT vs. St2−/−→St2−/−). We conducted the analyses and comparison of DE genes between chimeric mice with the same host genotype but reconstituted with either a WT or a St2-deficient hematopoietic system. Although comparison among the chimeras with St2-deficient hematopoietic systems only revealed 13 DE genes, the corresponding comparison between chimeras with St2-deficient nonhematopoietic compartments identified 195 DE genes. From these genes, we derived a core signature comprising 63 downregulated genes and 2 upregulated genes that remained DE regardless of whether or not the hematopoietic system could respond to IL33 (Fig. 5A and B). This latter signature was enriched in genes known to regulate immune activation, in particular type I/II IFN signaling (Ifng, Ifr1, Ifr7, Stat1, Stat2, and Socs1), IFN-regulated genes (Cxcl9, Cxcl10, Cxcl11, Ifng1, Ido1, and Isg32), and antigen presentation (B2m, Tap1, Tap2, Pomp8, and Pomp9; Fig. 5C and D). We found that Ifng expression was reduced in St2-deficient hosts (Fig. 5D); we could not detect expression of type I IFNs (Ifna and Ifnb) in any of the four cohorts of chimeric mice. This suggested that the attenuated interferon gene expression signature observed in St2-deficient hosts most likely arose from a reduction in Ifng gene expression.

Decreased IL33-dependent gene signature correlates with colon cancer progression

Our results argued that IL33 signaling was required to promote an IFN-dependent antitumor immune response in mice. We therefore wanted to validate whether this IL33-dependent IFN-γ gene signature correlated with attenuated disease progression in CRC patients. To this end, we made use of The Cancer Genome Atlas (TCGA) datasets in order to analyze the expression of the 40 human orthologs corresponding to the 65 mouse genes that make up the St2-dependent core gene signature in mice. We then correlated these data with the four stages of CRC progression in humans (i.e., stage I, confined to the bowel wall; stage II, penetrating the bowel wall; stage III, involvement of lymph nodes; stage IV, tumor has spread to distant organs). Our analysis revealed a global downregulation of the St2-core gene signature in late stages of CRC (stage III/IV) compared with early stages (stage I/II; Fig. 6A), including IFNγ target genes such as the transcription factor IRF1; IFNγ-induced chemokines CXCL10 and CXCL11; or genes involved in antigen presentation, such as TAP1, CD274, PSMB9, and PSMB10 (Fig. 6B). GSEA confirmed a significant enrichment (P < 0.0005, as determined by ROAST) of those St2-dependent genes that were downregulated in late stages compared with early stages (Fig. 6C).

Collectively, our data indicate a tumor-suppressing role for IL33-mediated NF-κB signaling in nonhematopoietic cells that can promote immune activation through an IFNγ-dependent gene expression signature, which is predictive of colon cancer aggressiveness in patients.

IL33 administration reduces colon cancer development

Thus far, our data suggested that IL33 signaling conferred resistance to colon cancer development by promoting an antitumor immune response. We therefore asked whether IL33 could provide a therapeutic benefit against the development of colon tumor. For this we established subcutaneous MC38 colon cancer cell allografts in WT mice before commencing systemic treatment with recombinant IL33. Consistent with our observations on sporadic development of endogenous colon tumors in St2-deficient mice, we observed reduced tumor burden, associated with an increased proportion of tumor-infiltrating T cells in the IL33-treated cohort when compared with the vehicle-treated cohort (Fig. 7A–C). IL33 treatment increased the proportion of IFNγ+ cells among tumor-infiltrating CD4+ and CD8+ T cells (Fig. 7D and E). Collectively, our results suggest that IL33 treatment boosted an antitumor immune response against colon cancer.

Discussion

We used reciprocal BM chimeras and whole transcriptome analysis to uncover a tumor suppressor role for IL33 signaling in mesenchymal cells of the colon that coincided with a decrease in Ifng expression and the associated signature of IFNγ-target genes in the absence of St2. Conversely, IL33 administration increased the expression of IFNγ by T cells in MC38 implanted mice and led to reduced tumor burden, in line with reports showing that IL33 could promote IFNγ expression by CTLs in vitro and in a transplantable tumor model (38, 39). Because IFNγ-receptor 1 has been reported to be expressed by colon tumor cells and IFNγ protected against intestinal tumor development (40, 41), our findings suggest that the increased tumor burden in St2−/− mice is likely to result from a decrease in IFNγ signaling in colon tumors. Our observation that BM chimeras in which St2 expression was abrogated in both the radiosensitive and radioresistant cell compartments showed the highest tumor burden suggests a collaborative effect between IL33 signaling between these two compartments. In line with this, Treg frequency was increased in tumors from fully St2-deficient BM chimeric mice. In a mouse model of T-cell–induced colitis, St2 deficiency in Treg was associated with reduced Treg survival, a decrease in Foxp3 expression, and a loss of Treg suppressive function (16). By contrast, our results indicated that despite the high abundance of St2-expressing Tregs in colon tumors, the loss of St2 expression in these cells did not diminish Treg frequency within the tumor or...
Figure 5.
Loss of IL33 signaling in the radioresistant cell compartment leads to a decrease in IFN gene signature and immune activation within colon tumors. Whole genome expression analysis of tumors collected from BM chimeras generated as in Fig. 2. A, Distribution of commonly and uniquely differentially expressed genes between tumors collected from the indicated BM chimeras (red—upregulated, blue—downregulated). B, Relative expression level (z score) of the 65 DE genes commonly regulated in BM chimeras in which St2 deficiency was restricted to the radioresistant compartment as indicated in A. C, Metacore analysis of biological processes for the 65 DE genes selected as in B. D, Normalized expression counts of selected genes in tumors from the indicated BM chimeras as determined by RNAseq. Mean ± SEM normalized expression counts with each symbol representing an individual mouse.
the unaffected colon. Likewise, our data did not reveal any alteration in Treg differentiation status as the expression of Foxp3, Gata3 or Rorγt remained unchanged in the absence of St2 expression. Increased Treg frequency could have various causes and reflects either an increased TGFβ-dependent conversion of conventional CD4+ T cells into Treg, increased local Treg proliferation dependent on IL2 or IL15 or an increased migration of Treg cells to the tumor bed.

Figure 6.
Downregulation of IL33-regulated gene signature is associated with disease progression in colon cancer patients. Gene expression analysis of the 363 tumors samples from the CRC TCGA dataset was performed. A, Heat map illustrating relative expression level (z score) of the 40 orthologs from the St2-dependent gene signature (as defined in Fig. 5B) across the four stages of disease progression in CRC patients (stage I, n = 59; stage II, n = 139; stage III, n = 114; and stage IV, n = 51). B, Boxplots of normalized gene expression (log2 scale) by stage of CRC. Data show median ± upper and lower quartiles. Symbols represent outlier patients. C, Enrichment analysis of the 40 ortholog gene set in early stages (stage I/II) vs. late stages of CRC (stage III/IV).
IL33 Promotes a Protective IFNγ Response in Colon Cancer

Because our results suggested a dominant effect of St2 deficiency in the radioresistant cell compartments, we analyzed the pattern of St2 expression within the nonhematopoietic compartments. Consistent with previous reports, St2 transcripts were detected in CD45−/EpCAM− mesenchymal cells but not in epithelial cells (23, 42). IL33 stimulation of mesenchymal cells isolated from the colon induced NF-κB signaling and expression of associated target genes including the chemokines Ccl2, Ccl5, and the cytokine Csf2. Indeed, activation of NF-κB signaling in colon-derived fibroblasts suppressed the development of both sporadic and colitis-associated colon cancer (43). In addition, fibroblast-specific ablation of the NF-κB activator IκKB led to an increase in Treg numbers within colon adenomas akin to our observations in St2-deficient mice (43). We speculate that the decrease in Ifn gene signature observed upon the deficiency of IL33 signaling in mesenchymal cells occurs via a decrease in NF-κB signaling, which would enable Treg infiltration and attenuate immune activation. Thus, our data imply that IL33/St2-dependent engagement of NF-κB signaling in colon mesenchymal cells restrains the development of tumors in the colon.

Our results complement those by Malik and colleagues, which also showed increased tumor burden in a colitis-associated colon cancer model in IL33-deficient mice (33). However, these authors attributed their findings to a reduction of IgA-secreting plasma cell frequency in the absence of IL33, leading to communicable dysbiosis and increased severity of experimentally induced colitis. Indeed, these authors identified the release of IL10 as the mechanism that promoted tumor development (33). We surmise from our data that the IL33/St2 signaling axis may restrain colon tumorigenesis by two different mechanisms. As described here, during early stage of mutagen-induced, sporadic tumorigenesis, the most prevalent setting in humans, the continuous release of...
IL33 that occurs as a consequence of the normal renewal of the colonic mucosa may facilitate an IFN-dependent antitumor immune response during tissue homeostasis. On the other hand, the increased release of IL33 during colitis, and the ensuing inflammatory response, may help to restore mucosal immunity and prevent dysbiosis, thereby reducing the production of IL1α, IL6 and other inflammatory cytokines that promote survival and proliferation of genetically damaged epithelial cells. Our observations can also explain the apparent discrepancy between our findings and the observation of reduced polyposis in IL33-deficient ApcMin mice, in which spontaneous loss-of-heterozygosity of the Apc tumor suppressor gene in Lgr5+ intestinal epithelial stem cells triggers tumor formation in the small intestine (23). Indeed, our data suggested that epithelial cell compartments in the colon and the small intestine display different abilities to respond to IL33: The Lgr5+ stem cells in the colon, but not as shown here those in the colon, express ST2 and are responsive to intestinal overexpression of IL33 (18).

In conclusion, our study demonstrated that the homeostatic release of IL33 by dying colon epithelial cells in the absence of preexisting chronic or acute inflammation protects against the initiation and development of sporadic colon cancer. Whether IL33 plays a pro- or antitumorigenic role during progression from adenoma to carcinoma and ultimately metastatic disease remains unclear. However, we showed here that the downregulation of the expression of the ST2-dependent gene signature identified in our mouse model of colon cancer correlated with disease progression in CRC patients. Indeed, IL33 exhibits a tumor suppressive role in CRC patients as well as in mouse colon carcinoma cell lines (44). Therefore, our results suggest that therapeutic intervention aiming at enhancing IL33 signaling could be of benefit for the treatment of CRC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Dependent Manner
− γ- Interferon-Interleukin 33 Signaling Restrains Sporadic Colon Cancer in an Interferon-γ-Dependent Manner

Moritz F. Eissmann, Christine Dijkstra, Merridee A. Wouters, et al.


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