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IL-33 Promotes Growth and Liver Metastasis of Colorectal Cancer in Mice by Remodeling the Tumor Microenvironment and Inducing Angiogenesis

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Abstract

Liver metastasis is the major cause of death from colorectal cancer (CRC). Understanding its mechanisms is necessary for timely diagnosis and development of effective therapies. Interleukin-33 (IL-33) is an IL-1 cytokine family member that uniquely functions as a cytokine and nuclear factor. It is released by necrotic epithelial cells and activated innate immune cells, functioning as an alarmin or an early danger signal. Its role in invoking type 2 immune response has been established; however, it has contrasting roles in tumor development and metastasis. We identified IL-33 as a potentially upregulated cytokine in a highly metastatic murine CRC cell line and examined its role in tumor growth and metastasis to the liver. IL-33 was transgenically expressed in murine and human adenocarcinoma and carcinoma cell lines and their growth and spontaneous metastasis to the liver were assessed in orthotopic models of CRC in wild-type C57Bl/6 and *Il33* knockout mice. The results showed that increased expression of IL-33 in CRC cells enhanced their tumor take, growth, and liver metastasis. Tumor- rather than host-derived IL-33 induced the enhanced recruitment of CD11b⁺ GR1⁺ and CD11b⁺F4/80⁺ myeloid cells to remodel the tumor microenvironment by increased expression of mobilizing cytokines, and tumor angiogenesis by activating endothelial cells. IL-33 expression was elevated in patient tumor tissues, induced early in adenoma development, and activated by pro-inflammatory cytokines derived from the tumor microenvironment. The data suggest that tumor-derived IL-33 modulates the tumor microenvironment to potentially promote colon carcinogenesis and liver metastasis, underscoring its potential as a therapeutic target.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

colon cancer; alarmin; myeloid cells; inflammation; cytokines

INTRODUCTION

Colorectal cancer (CRC) is a major cause of cancer-related death [1,2]. The main cause of death is liver metastasis. Following resection of localized tumor, the 5-year survival rate is 95%, but is less than 12% after metastasis has occurred [2]. Metastasis is the end result of multiple-complicated steps that are often asymptomatic [3]. At the time of diagnosis, approximately 25% of CRC patients present with metastases, while autopsies reveal that up to 70% of patients have liver metastasis [2]. Development of new treatments and methods for early detection are necessary for effective disease management and improved patient survival. These require understanding of the biological mechanisms that drive metastatic development.

IL-33 is an IL-1 cytokine [4] expressed in endothelial and epithelial cells [5,6], that uniquely functions as a cytokine and a nuclear factor. It is a pro-inflammatory alarmin released upon necrosis and in response to viruses [7,8], potently invoking Th2 immune response in inflammatory diseases [9]. IL-33 binds to a membrane-bound receptor complex consisting of suppression of tumorigenicity 2 (ST2L) and IL-1R accessory protein (IL1RAP) to activate NF- κ B and MAPK signaling [4], and to a soluble receptor, sST2 [10].

Accumulating but conflicting evidence has implicated IL-33 in carcinogenesis. It is elevated in sera of patients with hepatocellular carcinoma, gastric cancer, and non-small cell lung cancer, correlating with poor prognosis [11–13], upon UV irradiation of squamous cell carcinomas [14], and in metastatic pancreatic carcinoma [15]. IL-33/ST2 facilitated the accumulation of immunosuppressive cells to promote breast cancer progression and metastasis [16], development of oncogene-induced cholangiocarcinoma [17], and invasiveness of head and neck squamous cell carcinoma [18], while loss of ST2 suppressed metastatic breast cancer [16]. In contrast, IL-33 inhibited the growth of B16 melanoma, Lewis lung carcinoma, and 4T1 cells by activating CD8⁺T and NK cells [19]. Blockade of IL-33 attenuated mucositis induced by CPT-11 chemotherapy [20]. Importantly, increased IL-33 expression correlated with colorectal cancer malignancy and lung metastasis [21,22]. More recently, Maywald et al. [23] demonstrated that IL-33 was important for intestinal polyposis in *Apc*^{Min/+} mice, a model of early CRC adenomatous polyposis, by activating subepithelial myofibroblasts and mast cells in the tumor stroma. These cells express extracellular matrix components, growth factors, proteases, and cytokines that were shown to activate the tumor stroma and promote polyp development upon loss of the tumor suppressor adenomatous polyposis coli (*APC*) gene in intestinal epithelial cells [23]. The role of IL-33 in liver metastasis of CRC has not been fully elucidated.

In this study, we used an orthotopic model of invasive CRC and spontaneous liver metastasis to show that IL-33 was over-expressed in CRC cells that were highly metastatic to the liver. Increased IL-33 expression enhanced tumor growth and angiogenesis, and potently increased liver metastasis. IL-33 was elevated in adenomas of *Apc*^{Min/+} mice during early

CRC development, as also shown by Maywald et al., and our data suggest that it is induced in tumor epithelium by pro-inflammatory molecules that may be secreted by activated stromal cells. Tumor-, rather than host-derived IL-33 increased the recruitment of myeloid-derived suppressor cells and macrophages by activating the expression of cytokines that mobilize these cells to create a pro-tumorigenic stromal environment. In all, these observations underscore its role in remodeling the tumor microenvironment and its potential as a novel and viable therapeutic target to treat CRC and reduce liver metastasis.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Transfection

The Balb/c-derived CT26 and the C57BL/6-derived MC38 mouse colon adenocarcinoma cell lines and their derivatives were cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Pen/Strep, Mediatech, Manassas, VA) at 37°C and 5% CO₂ in a humidified atmosphere. The human colon cancer cell lines HCT116, HT29, Caco2, RKO, COLO205, HCT115, LOVO, MOSER, and SW480 were purchased from American Type Culture Collection (ATCC), and maintained in RPMI-1640 medium supplemented with 10% FBS.

Mouse and human *Il33* cDNAs were from Origene (Rockville, MD) and cloned into pcDNA 3.1 vector to generate pcDNA3.1-mIl33 and pcDNA3.1-hIl33 plasmids, respectively. MC-38, CT-26, and HCT-116 cells were stably transfected with pcDNA3.1 (Vector) or pcDNA3.1-mouse or human IL-33 using Lipofectamine 2000 (Invitrogen, Grand Island, NY) following manufacturer's instructions. Stable transfectants were selected in media containing 100 µg/mL Zeocin (Invitrogen, Grand Island, NY).

Mice

C57BL/6J and Balb/c(ByJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and *Il33^{tm1(KOMP)Vlcg} (Il33^{-/-})* mice from the University of California Davis Knock Out Mouse Project Repository (Davis, CA) but were bred and maintained at the Mouse Experimentation Core Facility of the Center for Colon Cancer Research (CCCR) at the University of South Carolina (USC). Animal procedures were performed according to the guidelines and approval of the University of South Carolina Institutional Animal Care and Use Committee.

Mouse Models of CRC Growth and Liver Metastasis

In the orthotopic model, spontaneous liver metastasis was assessed after cecal implantation of CRC cells as previously described [24]. Briefly, CRC cells were harvested and washed with PBS just prior to implantation. Eight-week-old syngeneic male mice were anesthetized with 2% isoflurane in oxygen. A midline incision was made to expose the cecum. A total of 2×10^6 cells in 10–15 µL PBS were injected into the cecum subserosa using a 33-gauge micro-injector (Hamilton Company, Reno, NV). The injection site was sealed with tissue adhesive (3M, St. Paul, MN) and washed with 70% alcohol and PBS to prevent leakage. The cecum was replaced in the peritoneal cavity, and the abdominal wall and skin closed with

6-0 polyglycolic acid sutures (CP Medical, Portland, OR). In vivo tumor growth was assessed by subcutaneous injection of 2×10^6 CRC cells suspended in 100 μ L of PBS into the flank of syngeneic male mice. Mice were sacrificed after 6 weeks in both procedures. In the splenic liver metastasis model, 8-week-old male C57BL/6, IL-33 knockout, or Balb/c(ByJ) mice were anesthetized with isoflurane. A lateral incision was made to expose the spleen. A total of 2×10^5 cells in 10 μ L PBS were injected into the spleen with a 33-gauge micro-injector. The injection site was sealed with tissue adhesive, and washed with PBS to prevent leakage. The spleen was replaced and the abdominal wall and skin closed with 6-0 polyglycolic acid sutures. Three weeks after implantation, mice were sacrificed. Tumors, livers, and spleens were collected, then weighed, and processed for further analysis.

Patient Tissues

Frozen patient tissues representing normal adjacent non-cancer sections and tumors classified as Stages I, II, III, and IV were obtained from the USC Center for Colon Cancer Research Tissue Bank. Tissues were thawed and fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.2 (PFA), and processed for histology.

Histology

Tumor-bearing mice were humanely sacrificed and the entire intestine, primary cecal tumors, and livers were excised and fixed in 4% PFA. Tissues were embedded in paraffin, 5 μ m sections obtained, and stained with hematoxylin and eosin (H&E) (VWR, West Chester, PA) for visual examination. The stained slides were reviewed and screened for representative tumor regions by a pathologist.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded (PPFE) tissue sections were deparaffinized, rehydrated, and then incubated in a microwave oven with 0.01 M citrate buffer, pH 6.0 for 10 min for antigen retrieval. Endogenous peroxidases were blocked with 3% H_2O_2 for 15 min. Nonspecific epitopes were blocked with normal horse serum (Jackson ImmunoResearch, West Grove, PA) for 1 h. The sections were incubated overnight at 4°C with antibodies against one of the following proteins: LOX, MMP9, MMP2, LYVE1, VEGF, and S100A8 (all from Abcam, Cambridge, MA), S100A9, IL-33 (R&D Systems, Minneapolis, MN), cleaved Caspase-3 (Cell Signaling Technology, Danvers, MA), Ki67 (OriGene Technologies, Rockville, MD). This was followed by incubation with secondary antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) for 1 h at room temperature. Antigen signals were detected using the 2-Solution Diaminobenzidine (DAB) Kit (Invitrogen, Frederick, MD), counterstained with hematoxylin, mounted in Acrymount (StatLab, McKinney, TX), and visualized under a light microscope.

Immunofluorescence

PPFE sections were subjected to antigen retrieval as described above, then incubated with antibodies against CD31, α -SMA, S100A8/9, GR1, CD45, or F4/80 (all from Abcam, Cambridge, MA). Samples were washed three times with PBS-Tween, followed by incubation with a fluorescence-conjugated secondary antibody (1:1000, Invitrogen). Nuclei

were stained with 1:20 000 dilution of 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen) and then mounted. Slides were viewed using an Olympus fluorescence microscope.

Cell Proliferation Assay

Ten thousand cells in 2 mL of DMEM with 10% FBS were plated per well in 6-well plates. The numbers of cells were counted after incubation for 2–6 days at 37°C. Alternatively, cell proliferation was measured using the WST-1 Cell Proliferation reagent (Clontech, Mountain View, CA) following manufacturer's instructions. Assays were performed in triplicate and repeated three times.

Western Blotting

Sera from tumor bearing mice and total protein extracts were analyzed by immunoblotting as described previously [24]. Primary antibodies against the following proteins were used as probes: MMP9, MMP2, VEGF, IL-1 β , β -actin, PCNA (all from Abcam), IL-6, TNF α , β -catenin, Vimentin, cleaved Caspase-3 (all from Cell Signaling Technology), Albumin (Santa Cruz Biotechnology, Santa Cruz, CA), and IL-33 (R&D Systems).

RNA Isolation and Gene Expression Analysis

Total RNA was isolated from cells and tissues using RNeasy RNA Isolation kit (Qiagen) and complementary cDNA was synthesized using iSCRIPT cDNA Synthesis Kit (Bio-Rad). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on an iCycler iQ5 PCR Thermal Cycler using SYBR green supermix (Bio-Rad). Validated gene specific primers were obtained from RealTimePrimers (Elkins Park, PA). Mouse β -actin and human *GAPDH* were used for normalization. Assays were run in five replicates.

Microarray analyses were performed using the Agilent platform. Single cell suspensions from freshly isolated CT26 or CT26-FL3 tumors obtained from four mice each were briefly grown in cell culture for 24 h to remove stromal cells. Total RNA was extracted from harvested cells and the quality assessed in an Agilent 2100 Bioanalyzer. Samples with RNA Integrity Numbers (RIN) of 8–10.0 were used. Total RNA was amplified and labeled with Cy3 fluorescent dye using Agilent's Low Input Quick Amp Labeling Kit (Agilent, Wilmington, DE). Labeled RNA was purified using RNeasy Mini Kit from Qiagen (Qiagen, Valencia, CA) and dye incorporation and cDNA yield were assessed. Labeled cDNA samples were hybridized to Agilent Whole Mouse Genome Microarrays 4 $\times$ 44 K (Agilent, Wilmington, DE) using Agilent's Gene Expression Hybridization Kit (Agilent, Wilmington, DE). After washes and drying, arrays were scanned in an Agilent B Scanner. All procedures were performed according to manufacturer's instructions. Microarray data were extracted with Agilent Feature Extraction software version A8.5.3, and normalized intensities were analyzed using GeneSpringGX software (Agilent). KEGG Pathway analysis was performed using the GeneSifter software (Geospiza).

Matrigel Invasion and Wound Healing Assays

Invasion and migration properties were measured using the Matrigel transwell invasion and wound healing assays as previously described [24]. Experiments were performed in triplicate and repeated three times.

Isolation of Single Cells From Tumors

Tumors were dissected free of necrotic areas, connective tissue, and blood clots then rinsed three times with cold (4°C) DMEM containing 1% FBS and 2% Pen/Strep. Tumors were sliced into 1–3 mm³ fragments then incubated in DMEM containing collagenase type I (200 U/mL), DNase (270 U/mL), and hyaluronidase type IV (35 NF U/mL) (Sigma, St. Louis, MO) for 30 min at 37°C. The resulting cell suspension was kept on wet ice, filtered through a 70 µm nylon cell strainer (BD Biosciences, Bedford, MA), and washed twice with PBS. Single cells were resuspended at 10⁷ cells/mL and analyzed by fluorescence-activated cell sorting (FACS).

FACS Analysis

Single-cell suspensions from MC38-Vector or MC38-IL33 tumors were incubated with APC-conjugated CD11b, FITC-conjugated anti-Gr1, PE-conjugated anti-F4/80, or eFluor450-conjugated anti-CD45 (all from eBioscience, San Diego, CA). One million cells were incubated with antibodies in 100 µL wash buffer (PBS with 0.1% BSA) at 4°C for 1 h. Cells were washed twice with ice-cold wash buffer and fixed with 1% paraformaldehyde. FACS analyses were performed on a Beckton Dickinson LSRII flow cytometer and analyzed with FACSDiva 8.0 software (BD Biosciences).

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test when two value sets were compared, and one-way ANOVA followed by Dunnett's test when the data involved three or more groups. *P* < 0.05, *P* < 0.01, or *P* < 0.001 were considered statistically significant and indicated in the figures by *, **, or ***, respectively.

RESULTS

IL-33 Is Strongly Upregulated in Highly Metastatic CT26-FL3 Cells

The CT26-FL3 CRC cell line was isolated from CT26 parental cells by in vivo selection in an immunocompetent orthotopic model of CRC liver metastasis (Figure 1A) [24]. CT26-FL3 is 10-fold more metastatic to the liver as compared to CT26 cells [24]. To identify genes that may promote liver metastasis, gene expression in the poorly versus highly metastatic cells were compared by microarray analysis. The most altered pathways expressed genes involved in inflammation, immune responses, and cell signaling (Table 1). Genes in the cytokine–cytokine receptor interaction pathway were most highly upregulated, and were of interest because they encode molecules that mediate immune responses or cell–cell communication that may facilitate liver metastasis. Unsupervised hierarchical cluster analysis of altered genes in this pathway (fold change ≥ 2.0 between CT26 and CT26-FL3 cells) showed that *Il33* was the most up-regulated (37.3-fold higher, Figure 1B and Supplemental Figure S1A) and among the top 10 most differentially expressed genes (Supplemental Table S1) in CT26-FL3 as compared to CT26 cells (*P* < 0.0001). Intracellular IL-33 levels were higher in CT26-FL3 (Supplemental Figure S1B), and serum levels were higher in mice bearing CT26-FL3 tumors as compared to mice with CT26 or no tumors

(Figure 1C). Sections from CT26-FL3-derived cecal tumors showed darker staining for IL-33 (Figure 1C). Thus, IL-33 expression at the mRNA and protein levels was significantly elevated in the highly metastatic CT26-FL3 cells and in tumors that they generated in vivo.

IL-33 Expression Is Elevated in Human CRC

We analyzed patient tissues from the USC CCCR Tissue Bank that were taken from tumors classified as Stages I, II, III, or IV and adjacent normal colon tissue, and found increased IL-33 staining in tumor epithelial cells in all stages (Figure 1F). *IL33* mRNA levels were significantly higher in Stage I–III CRC tumor tissues as compared to adjacent normal colonic tissue ($n > 10$) (Figure 1D). However, while its expression in Stage IV tumors was lower compared to Stages I–III, IL-33 expression was still significantly higher than that in non-tumor cells. Of the human patient samples analyzed, tissues classified as well, moderately, and poorly differentiated adenocarcinomas were distributed across Stages I–IV. Thus, on the basis of tumor grade, IL-33 mRNA levels, as measured by RT-PCR, were highly elevated in tumor tissues as compared to levels in adjacent non-tumor tissues. Furthermore, expression levels of full length (~30 kDa) and processed IL-33 (~19 kDa, enclosed in rectangle) were elevated, although at different levels, in human CRC cell lines (Figure 1E). The lower molecular weight bands observed in CaCo2 and Colo205 might represent breakdown products or reduced or oxidized forms of processed IL-33 similar to those described by Cohen et al. [25]. Taken together, increased expression of *IL-33* found in highly metastatic CT26-FL3 cells and tumors was reflected in CRC patient tissues and in human CRC cell lines, suggesting a role in malignancy and potentially, metastasis of CRC. These data are consistent with recent findings by Cui et al. [21] and Maywald et al. [23] indicating that IL-33 expression was induced in human CRC patient tissues.

IL-33 Enhanced Tumor Growth in an Immunocompetent Orthotopic Model of CRC

To assess the role of IL-33 in tumor growth and metastasis, we used the MC-38 murine colon adenocarcinoma cell line that exhibited low tumor take and rare liver metastasis when implanted into the cecum of immunocompetent C57Bl/6 mice. In previous studies, expression of IL-33 in human CRC cells enhanced their growth in subcutaneous xenografts in nude mice [22] and IL-33 was necessary for normal polyp development in *Apc^{Min/+}* mice [23]. However, subcutaneous CRC xenografts do not metastasize to the liver and adenomas in *Apc^{Min/+}* mice rarely become invasive and do not metastasize to the liver or other organs. In this study, we used a reliable orthotopic model of CRC liver metastasis [24] in an immunocompetent host to examine the impact of IL-33 on tumor growth and spontaneous metastasis to the liver. MC38-IL33 cells expressing *IL-33* and MC38-Vector control cells were generated (Supplemental Figure S2A). Two independent clones, MC38-IL33-1 and MC38-IL33-2, expressed ~60-fold higher *IL33* mRNA levels as compared to control and untransfected cells (Supplemental Figure S2B); however, intracellular IL-33 protein levels were only twofold higher (Supplemental Figure S2C). Analysis of cell culture media showed significantly higher levels of IL-33 in media from MC38-IL33 as compared to MC38-Vector cells, indicating that IL-33 was secreted (Supplemental Figure S2D). Expression of the ST2 receptor was approximately twofold higher in MC38-IL33 than in MC38-Vector cells (Supplemental Figure S2E) which was similarly observed in CT26-FL3 as compared to CT26 cells (Supplemental Figure S1A). MC38-IL33 proliferated faster than MC38-Vector

cells in culture (Figure 2A). After cecal implantation, 80% (16/20) of mice bearing MC38-IL33 developed primary tumors compared to 25% (5/20) of mice bearing MC38-Vector cells (Figure 2D). More importantly, MC38-IL33 tumors weighed approximately five times heavier than MC38-Vector tumors (Figure 2B). Similar growth rates were observed when cells were injected into the flank of C57Bl/6 mice (Figure 2C). Serum levels of IL-33 were higher in mice bearing MC38-IL33 tumors compared to mice bearing MC38-Vector tumors (Figure 2G). Furthermore, enforced expression of *IL33* caused faster proliferation of human HCT-116 CRC cells compared to cells transfected with empty vector (Supplemental Figure S5A).

Sections from MC38-IL33 tumors exhibited higher expression of the proliferative marker Ki-67, compared to MC38-Vector tumors; however, there was no difference in levels of the apoptotic marker cleaved caspase-3 (Figure 2F). Western blot analyses of total protein extracts of cells grown in culture showed higher levels of the proliferative marker PCNA in MC38-IL33, but no difference in cleaved caspase 3 between these cell lines (Figure 2E). Thus, IL-33 increased tumor proliferation but did not affect cell apoptosis. Collectively, these data show that IL-33 enhanced the growth of CRC cells in vitro and tumors in vivo.

IL-33 Promotes Liver Metastasis

We assessed the effect of IL-33 on the ability of tumors cells to spontaneously metastasize to the liver. Six weeks after cecal implantation, 25% (5/20) of mice with MC38-IL33 tumors developed liver metastasis, 2.5-fold higher than mice with MC38-Vector tumors (2/20) (Figure 3A). Using a splenic model of liver metastasis, 70% (14/20) of mice receiving MC38-IL33 compared to 30% (6/20) of mice receiving MC38-Vector developed liver metastasis 3 weeks after injection. More importantly, livers from mice bearing MC38-IL33 tumors were significantly heavier and exhibited multiple large cauliflower-like nodular metastatic lesions compared to mice bearing MC38-Vector tumors (Figure 3B and C). Therefore, IL-33 potentially promotes liver metastasis of CRC.

To begin to elucidate the mechanisms by which IL-33 enhanced liver metastasis, sections from cecal tumors were analyzed for markers typically associated with metastasis. There were no differences between tumors from MC38-Vector or MC38-IL33 cells with respect to expression of MMP9 and MMP2, biomarkers associated with invasion, or lysyl oxidase (LOX) that is essential in hypoxia-induced metastasis [26] (Figure 3D). Similarly, there were no significant differences in the invasive and migratory properties of MC38-Vector and MC38-IL33 cells based on matrigel transwell invasion and wound healing assays, respectively (Supplemental Figure S3A and B). Moreover, there were no morphological differences between these cells (data not shown), that might indicate alterations in epithelial to mesenchymal transition (EMT), an essential process in metastasis [27]. These findings were confirmed by Western blot analysis of total protein extracts from MC38-IL33 and MC38-Vector cells for biomarkers associated with invasion (MMP9, MMP2) and EMT (Beta-Catenin, Vimentin) (Supplemental Figure S3C). Thus, the ability of IL-33 to promote liver metastasis did not result from enhanced properties of the cancer cells to invade, migrate, undergo EMT, or induce hypoxia.

Interestingly, microvessel density in MC38-IL33 cecal tumors was approximately threefold higher than in MC38-Vector tumors (Figure 3E), indicating significant generation of microvessels. Immunofluorescence staining of tumor sections for the vascular markers CD31, LYVE1, and α -SMA, indicated that the neomicrovessels included blood and lymphatic vessels, suggesting very active angiogenesis and lymphangiogenesis occurring in MC38-IL33 tumors (Figure 3F). α -SMA is also expressed by tumor associated fibroblasts or myofibroblasts, indicating that IL-33 may promote their recruitment into the tumor microenvironment, where they can contribute to angiogenesis [28] and enhanced metastatic potential of colon cancer cells. Since VEGF is a critical signal for stimulating angiogenesis [29], we measured its mRNA expression in MC38-IL33 and MC38-Vector cells. Surprisingly, *Vegf* was significantly lower in MC38-IL33 cells (Supplemental Figure S3D) suggesting that IL-33 might also enhance angiogenesis in MC38-IL33 tumors possibly by an alternative mechanism. Recent studies showed that IL-33 can induce pro-inflammatory activation of endothelial cells to promote angiogenesis in vascular diseases [30,31]. IL-33 might enhance angiogenesis in MC38-IL33 tumors by similar mechanisms.

In summary, IL-33 increased liver metastasis of CRC and induced neomicrovessel formation in nascent tumors. Increased metastasis was not a consequence of acquiring enhanced metastatic capabilities by the tumor cells. Since most of the IL-33 was secreted (Figure 2G and Supplemental Figure S2D), its impact might be in mobilizing factors to the tumor microenvironment where they can interact with tumor cells to increase their proficiency for liver metastasis

IL-33 Remodels the Tumor Microenvironment

To examine the influence of IL-33 on the tumor microenvironment, sera from mice bearing MC38-IL33 or MC38-Vector cecal tumors were analyzed by Western blotting. Serum levels of IL-6, IL1 β , and TNF- α were higher in mice with MC38-IL33 compared to mice with MC38-Vector tumors or underwent sham surgery (Figure 4A). These cytokines are known to stimulate CRC proliferation, angiogenesis, and tumor dissemination [32]. However, there were no differences in mRNA levels of *Il6* and *Il1 β* , while *Tnf- α* only moderately increased in MC38-IL33 compared to MC38-Vector cells (Figure 4B). White blood cell counts (WBCs) were similar in peripheral blood of mice bearing MC38-IL33 or MC38-Vector tumors or sham control mice (Supplemental Figure S4A), indicating that tumor-derived IL-33 did not induce a systemic immune response that might result in elevated serum cytokine levels in mice with MC38-IL33 tumors. These results suggest that the tumor-promoting cytokines were derived from external sources and were not induced by IL-33 in the tumor cells and then secreted into circulation.

To determine how IL-33 might modulate the tumor microenvironment, stromal cells in cecal tumors were analyzed by FACS. We found significantly higher infiltration by CD45⁺ leukocytes into MC38-IL33 as compared to MC38-Vector tumors (30% vs. 15%, respectively) (Figure 4C). The majority of these (~80%) also expressed the CD11b myeloid cell marker (Supplemental Figure S4B). There was no difference in the number of cells expressing lymphoid markers (mainly T cells and B cells) (data not shown). Further analysis of the CD11b⁺ subsets revealed a significant twofold higher infiltration by CD11b⁺F4/80⁺

macrophages and CD11b⁺Gr1⁺ myeloid-derived suppressor cells (MDSCs) in MC38-IL33 as compared to MC38-Vector tumors (Figure 4C). This was confirmed by immunofluorescence staining of cecal tumor sections with antibodies against CD45, Gr1, and F4/80 (Supplemental Figure S4C). Thus, IL-33 enhanced the recruitment of CD11b⁺F4/80⁺ macrophages and CD11b⁺Gr1⁺ MDSCs into primary tumors.

We measured the expression of chemokines that promote recruitment of CD11b⁺F4/80⁺ and CD11b⁺Gr1⁺ MDSCs into the tumor microenvironment where they can contribute to myeloid cell-mediated angiogenesis or metastasis [33–37]. The results showed that MC38-IL33 tumors expressed significantly higher levels of *Csf1*, *Csf2*, *Ccl2*, *Ccl5*, and *Cxcl1* mRNA compared to MC38-Vector tumors by 2-, 7-, 4-, 6-, and 11-fold, respectively (Figure 4D). Similar results were found in HCT-116 cells expressing IL-33 (Supplemental Figure S5B). Therefore, induction of these chemokines by IL-33 might provide the underlying basis for enhanced recruitment of CD11b⁺F4/80⁺ and CD11b⁺Gr1⁺ myeloid cells.

Serum levels of S100A8, S100A9, and VEGF were also elevated in mice with MC38-IL33 tumors (Figure 4A). S100A8/9 are pro-inflammatory cytokines released by myeloid cells that are recruited by tumor-derived CXCL1 to enhance cancer cell survival and promote tumor progression and metastasis [37–40]. We found that MC38-IL33 tumors were infiltrated by a greater number of cells expressing S100A8/9 and VEGF (Figure 4E), likely accounting for their elevated serum levels. Data obtained from co-staining tumor sections with antibodies against Gr1 and F4/80 showed that S100A8/9 co-localized with CD11b⁺Gr1⁺MDSCs (Figure 4F, top panel), and VEGF with F4/80⁺ macrophage cells (Figure 4F, bottom panel). Therefore, tumor-derived IL-33 enhanced the recruitment of myeloid cells that secrete S100A8/9 and VEGF to promote tumor angiogenesis and metastasis.

Host-Derived IL-33 Does not Contribute to CRC Malignancy

To determine the role of host-derived IL-33 in tumor growth or metastasis, MC38-IL33 or MC38-Vector cells were implanted into the cecum or spleen of *Il33*^{-/-} knockout or wild-type C67BL/6 mice. There were no significant differences in primary cecal tumor growth after 6 weeks or liver metastases at 3 weeks after splenic injection of CRC cells in *Il33*^{-/-} or wild-type mice (Figure 5A and B). FACS analysis of MC38-Vector cecal tumors showed similar infiltration by CD45⁺, CD11b⁺F4/80⁺, or CD11b⁺Gr1⁺ MDSCs in the presence or absence of host *Il33* (Figure 5C). While these numbers were significantly higher in MC38-IL33 tumors, as seen in Figure 4C, they were not altered by the loss of *Il33* in host mice (data not shown). Furthermore, WBCs in peripheral blood from tumor-bearing *Il33*^{-/-} or C67BL/6 mice were similar, indicating that host *Il33* did not affect systemic immune responses (Figure 5D and E). Taken together, these data showed that tumor- rather than host-derived IL-33 dictated the malignancy of tumor phenotype, mobilization of cells into the microenvironment, and metastasis into the liver.

Role of IL-33 in Early Colorectal Tumorigenesis

Inflammation plays an important role in CRC development and patients with long-term chronic gut inflammation can develop CRC [41]. To assess the involvement of IL-33 in early

stage CRC, MC38 cells were treated with IL-1 β , TNF α , and IL-6, molecules that have been shown to promote CRC development [42,43]. They induced *Il33* mRNA levels by six, two, and fivefold, respectively (Figure 6A). Similar results were found in HCT-116 (Supplemental Figure S5C) and in CT26 cells for *Il1 β* and *Il6* (Figure 6B), suggesting that inflammatory mediators in the colon can induce *Il33* expression in tumors. We measured *Il33* mRNA in intestinal adenomas of *Apc^{Min/+}* mice, a model of early stage CRC [44]. Previous studies showed that IL-1 β , TNF α , and IL-6 were elevated in the intestinal mucosa during polyp development in these mice [45]. mRNA levels of these cytokines and *Il33* were measured in adenomatous polyps and tumor-free intestinal regions in *Apc^{Min/+}* mice and in intestinal tissues of wild-type mice. *Il1 β* , *Tnfa*, and *Il6* mRNA expression were significantly higher in intestinal tissues of *Apc^{Min/+}* compared to wild-type mice (Figure 6C). Importantly and consistent with previous findings [23], *Il33* mRNA and protein levels were dramatically higher in polyps compared to non-tumor or normal intestinal tissues of *Apc^{Min/+}* or wild-type mice (Figure 6D and E). Since overexpression of *Il33* in cancer cells did not induce expression of *Il1 β* , *Tnfa*, or *Il6* (Figure 4B), these data suggest that elevated levels of at least these cytokines in the tumor microenvironment may account in part for increased *Il33* expression in intestinal tumor cells. These in vivo results are consistent with those from in vitro treatment of MC38, CT26, and HCT-116 cells with these pro-inflammatory cytokines. Therefore *Il33* is induced very early during polyp development, presumably in response to increased inflammation during colorectal tumorigenesis.

DISCUSSION

Liver metastasis is the final and most devastating stage of CRC malignancy and our goal was to identify potential diagnostic markers or therapeutic targets to alleviate the impact of this disease in patients. In this study, we provide functional evidence that IL-33 potentially promoted primary tumor growth and liver metastasis in a mouse model of advanced carcinoma and spontaneous liver metastasis. We compared global gene expression of two isogenic murine CRC cell lines and identified *Il33* as a highly expressed gene in CT26-FL3 cells, that were 10-fold more metastatic to the liver than parental CT26 cells, suggesting a role in liver metastasis. Protein and mRNA levels of IL-33 were elevated in patient CRC tissues, as also reported by others [21–23], where in some cases, it correlated with advancing stage and poor prognosis. Consistent with being a “danger” signal, *Il33* was upregulated early in pre-malignant CRC as observed in adenomas of *Apc^{Min/+}* mice (this study and Maywald et al.). Enforced expression in mouse MC38 and CT26 or human HCT116 CRC cell lines increased their proliferation in vitro and in vivo. IL-33 increased primary tumor take of MC38 cells in the cecum and their spontaneous metastasis to the liver in immunocompetent hosts, producing multiple metastatic nodules. When injected into spleen, MC38-IL33 produced more and larger lesions in the liver compared to MC38-Vector tumors.

The mechanisms by which IL-33 is induced in adenomas early in CRC development are not fully understood. Our data showed that pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α induced its mRNA levels in CRC cells. In contrast, over-expression of IL-33 had little to no effect on the mRNA levels of these cytokines in CRC carcinoma cells. However, abrogation of IL33 in *Apc^{Min/+}* adenomas reduced expression of IL-6 but not TNF α or IL1 β [23]; this might indicate that regulatory loops in intestinal polyps may be altered as tumors

progress into carcinomas, as also suggested by Chen et al. [18]. Previous studies showed that cytokines and factors that have been implicated in polyposis can activate IL-33 expression, although at different levels [23]. Furthermore, in *Apc^{Min/+}* polyps, IL-33 was localized to epithelial cells with elevated nuclear levels of β -catenin, suggesting that active Wnt signaling in these cells might contribute to its induction in intestinal epithelial cells [23,46].

Because IL-33 is an alarmin that induces activation and recruitment of host immune cells [47,48], our findings support the notion that tumor-derived IL-33 promotes tumor growth and metastasis by remodeling the tumor microenvironment rather than by altering the intrinsic properties of tumor cells to invade, migrate, or undergo EMT. It was speculated, based on the intensity of immunohistochemical staining of IL-33 in patient tissues, that IL-33 may increase the invasive properties of CRC cells during progression from adenoma to carcinoma [21]. However, using invasion and migration assays, we showed that over-expression of IL-33 in CRC cell lines did not increase their invasive or migratory properties. It possible, however, that in vivo, increased invasiveness of cancer cells may be mediated by IL-33 through their interaction with cells recruited into the tumor microenvironment. Chen et al. [18] showed that IL-33 from cancer-associated fibroblasts (CAFs) regulated the invasiveness of head and neck squamous cell carcinomas and their expression of IL-33 through paracrine mechanisms. Here, we showed that elevated IL-33 in CRC cells increased the proficiency of tumor cells in recruiting CD11b⁺ F4/80⁺ macrophages and CD11b⁺ GR1⁺ MDSCs by inducing the expression of soluble factors that mobilize these cells into the tumor stroma. Interactions between tumor and these cells through secreted soluble factors, including IL-33, might enhance the invasiveness of CRC cells in vivo. Once recruited into the tumor stroma, macrophages and MDSCs secrete pro-inflammatory cytokines that may further stimulate tumor expression of *Il33*. Induction of *Il33* by pro-inflammatory cytokines has been reported in metastatic pancreatic cancer cells to modulate expression of its ST2 receptor [15], acting through both paracrine and autocrine mechanisms to increase growth and metastasis. We further show that recruited MDSCs and macrophages express S100A8/9 and VEGF molecules that are known to establish the pre-metastatic niche [38,39] and promote tumor angiogenesis [33], respectively. In a breast cancer model, the IL33/ST2 axis was also shown to promote growth and metastases by recruitment of MDSCs and activated macrophages [16]. We propose a model wherein IL-33 can serve as a potent amplifier of signaling pathways that link the inflammatory microenvironment of tumorigenesis to a sustained tumor-promoting amplification loop that results in increased tumor growth and proliferation, angiogenesis, and liver metastasis (Figure 6F). Consistent with this model, Maywald et al. [23] showed that IL-33 promoted polyp development in *Apc^{Min/+}* mice by activating the tumor stroma through recruitment of myofibroblasts and mast cells. Furthermore, Lim et al. [49] showed that recruitment of myeloid cells was critical for the establishment of liver metastasis. Our data suggest that tumor-derived IL-33 might be a pivotal instigator and promoter of this process.

Once induced, endogenous IL-33 can potentially promote tumor growth by acting as a nuclear factor to activate NF- κ B and MAPK signaling pathways that enhance proliferation [50]. In xenografts, IL-33 dramatically increased nascent microvessel formation and endothelial cell accumulation. IL-33 has been implicated as a potent stimulator of angiogenesis and VEGF expression in vascular disease [31]. Here, we show that IL-33 may

promote cancer angiogenesis and vasopermeability independent of VEGF, possibly by increasing endothelial cell proliferation, migration, or tubular network formation [30,31]. Although ST2 levels were rarely detected in endothelial cells in *Apc*^{Min/+} adenomas [23], it is possible that elevated IL-33 from the engrafted carcinomas might induce ST2 expression in endothelial cells, enhancing their recruitment to the tumor stroma where they can contribute to enhanced angiogenesis. Further studies are needed to verify this notion. Our data also showed that CD11b⁺F4/80⁺ macrophages recruited into the microenvironment expressed VEGF, while Maywald et al. [23] showed that IL-33 induced VEGF in myofibroblasts in *Apc*^{Min/+} polyps. These observations suggest that stromal-derived VEGF produced by direct or indirect induction of stromal cells by IL-33 can boost tumor vasculogenesis to increase the development of thin-walled blood vessels during early angiogenesis, α -SMA⁺ blood vessels, and lymphatic vessels in the tumor. Thus, multiple mechanisms may act collaboratively to enhance tumor angiogenesis at various stages in tumor development. It is also interesting to note that blood vessels in MC38-IL33 tumors are larger and appear to resemble the appearance of vasculature in colorectal liver metastases following antiangiogenic therapies [51,52] where vasculature remodeling was shown to enhance tumor growth by providing escape from anti-VEGF therapy. Collectively, these observations suggest that metastasizing cells with upregulated IL-33 possess intrinsic capabilities that allow them to better survive, proliferate, and colonize the liver environment upon extravasation, that may also aid in their escape from the primary tumor site or anti-angiogenic therapies.

Previous studies have reported conflicting roles of IL-33 in tumor growth and metastasis [16,18,53]. IL-33 was shown to promote tumor growth and/or metastasis of colon [22,23], pancreatic [15], breast [16] cancers, and chronic myeloid leukemia [54] by recruiting immunosuppressive cells into the microenvironment or by autocrine regulation of its ST2 receptor in tumor cells. In contrast, enforced expression of secreted IL-33 in melanoma and breast cancer cells lacking ST2 receptors inhibited tumor growth and metastases by recruiting CD8⁺T and NK cells [19,53]. The former suggests that blockade of IL-33 or its ST2 receptor could inhibit tumor growth or metastasis. The latter suggests that IL-33 might enhance the efficacy of anti-cancer immunotherapy by promoting type 1 cancer-eradicating immune responses [19]. IL-33 might exert different effects based on its concentration, primary target cells in the models used, expression of ST2 in tumor or target cells, the composition of the local tumor microenvironment, or the source of IL-33. Our studies showed that tumor-derived IL-33 was pivotal for its effect on CRC tumorigenesis and liver metastasis. Loss of host *Il33* had no impact on tumor growth, metastasis, or mobilization of myeloid cells. In contrast, studies have shown that host ST2 receptor was critical for IL-33-mediated recruitment of immune cells and its effects on tumor growth and metastasis [16,19,20,22,23]. Regardless of its pro- or anti-tumorigenic effects, IL-33 mainly functions by altering the tumor immune microenvironment.

In summary, we showed that IL-33 is induced early in CRC development and accelerated tumor growth and liver metastasis by mobilizing CD11b⁺ F4/80⁺ macrophages and CD11b⁺ GR1⁺ MDSC cells to remodel the tumor microenvironment and by potentially activating microvessel development and angiogenesis independent of VEGF. In turn, myeloid-derived factors sustain elevated *IL-33* expression in tumors and secrete factors that prime the pre-

metastatic niche and promote angiogenesis to facilitate liver metastasis. Because IL-33 is activated early, it has potential as a diagnostic marker, and pharmacologic inhibition of IL-33 or its ST2 receptor could be a viable strategy to prevent or treat CRC, liver metastasis, or cancer angiogenesis. The soluble sST2 may be used as a decoy receptor to deplete serum IL-33 concentration and block tumor progression. In other cancers, IL-33 may be used to manipulate the inflammatory tumor microenvironment to enhance the effectiveness of cancer immunotherapy. Understanding the breadth of its role in tumor growth and metastasis will help realize its full potential in anti-cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CRC	colorectal cancer
IL	interleukin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	mitogen-activated protein kinase
qRT-PCR	quantitative real-time PCR
FACS	fluorescence activated cell sorting
KEGG	Kyoto encyclopedia of genes and genomes
MMP	matrix metalloproteinases
LOX	lysyl oxidase
EMT	epithelial to mesenchymal transition
VEGF	vascular endothelial growth factor
LYVE	lymphatic vessel endothelial hyaluronan receptor
SMA	smooth muscle actin
TNF	tumor necrosis factor

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Figure 2.

IL-33 enhanced tumor growth. (A) Proliferation of MC38-Veector and MC38-IL33 cells in tissue culture. (B and C) Weights (grams) of primary tumors derived from MC38-Veector or MC38-IL33 cells 6 weeks after cecal implantation (B) or subcutaneous injection (C). *** $P < 0.001$. (D) Incidence of primary tumor growth in cecum of C57Bl/6 mice implanted with MC38-Veector or MC38-IL33 cells ($n = 20$). (E) H&E images (400 $\times$) and primary cecal tumor sections stained with anti Ki67 and cleaved caspase-3 antibodies. (F) Immunoblot analysis of total protein extracts from MC38-Veector and MC38-IL33 clones with antibodies against PCNA and cleaved caspase-3. (G) Serum levels of IL-33 in mice bearing MC38-Veector or MC38-IL33 tumors analyzed by ELISA.

Figure 3.

IL-33 promotes liver metastasis of CRC. (A) Incidence of liver metastasis 6 weeks after cecal implantation and (B) macroscopic liver metastases 3 weeks after splenic injection of MC38-Vector or MC38-IL33 cells. (C) Weights of livers from mice in (B) $^{**}P < 0.01$. (D) Primary cecal tumor sections stained with antibodies against IL-33, LOX, MMP9, and MMP2 (400 $\times$). (E) Microvessel density in cecal tumors from MC38-Vector and MC38-IL33 cells, (top panel 40 $\times$, bottom panel 100 $\times$), $^{***}P < 0.001$. (F) cecal tumor sections stained with H&E (400 $\times$), or antibodies against LYVE1 (200 $\times$), CD31 or α -SMA (100 $\times$) to detect microvessel formation.

Figure 4.

IL-33 mobilizes immune cells into the tumor microenvironment. (A) Serum levels of IL-6, IL-1β, TNFα, S100A8, S100A9, VEGF, and IL-33 in mice 6 weeks after cecal injection of MC38-Vector, MC38-IL33 cells or sham injection. See Supplemental Figure S6 for quantitation. (B) Expression of *Il6*, *Il1β*, and *Tnfα* in MC38-Vector and MC38-IL33 cells. **P*<0.05; ns, not significant (C) Representative flow cytometry (FC) plots of CD45⁺, CD11b⁺F4/80⁺, and CD11b⁺GR1⁺ cells in tumor stroma of mice bearing MC38-Vector or MC38-IL33 tumors. ****P*<0.001, ***P*<0.01. (D) Fold changes in *Csf1*, *Csf2*, *Csf3*, *Ccl2*, *Ccl5*, and *Cxcl1* mRNA levels in MC38-IL33 over MC38-Vector cells. (E) Primary cecal tumor sections stained with antibodies against S100A8, S100A9, and VEGF. (F) Co-localization of S100A8/9 with Gr1⁺ cells and VEGF with F4/80⁺ cells in primary cecal tumors.

Figure 5.

Host-derived IL-33 is not necessary for CRC malignancy. (A) Weights of MC38-Vector or MC38-IL33 tumors cecal tumors in wild-type (WT) or *Il33*^{-/-} mice. (B) Liver weights from WT or *Il33*^{-/-} mice 3 weeks after splenic injection of MC38-Vector or MC38-IL33 cells. (C) Representative FC plots of CD45⁺, CD11b⁺F4/80⁺, and CD11b⁺GR1⁺ cells in tumor stroma of WT or *Il33*^{-/-} mice 6 weeks after cecal implantation of MC38 cells. (D) Peripheral white blood cell (PWBC) count (gray area indicates normal physiologic range) and (E) Percentages of lymphocytes, monocytes, and neutrophils in PWBCs (pink, blue, and green areas indicate normal physiologic ranges, respectively) from tumor bearing WT and *Il33*^{-/-} mice.

Figure 6.

IL-33 is induced by inflammation early in colorectal tumorigenesis. *Il33* mRNA expression in (A) MC38 cells and (B) CT26 cells treated with IL-1 β (10 ng/mL), TNF α (5 ng/mL), and IL-6 (10 ng/mL) for 24 h. *** P <0.001. (C) Fold increase in *Il-1 β* , *Tnf α* , and *Il-6* mRNA expression in intestine of *Apc*^{Min/+} mice over normal intestine of WT mice. ** P <0.001. (D) Relative *Il33* mRNA expression in adenomatous polyps compared to non-tumor intestine in *Apc*^{Min/+} and WT mice. (E) Immunohistochemical analysis of polyps in *Apc*^{Min/+} and normal intestine in C57BL/6 mice with anti-IL-33 antibodies (200 $\times$). (F) Model depicting the role of IL-33 in tumor growth and metastasis of CRC (see text for discussion).

Table 1
Top 10 affected pathways between CT26 and CT26-FL3 cells based on the Z-score by KEGG pathway analyses.

KEGG Pathway	List	Up	Down	Gene Set	z-score (Up)	z-score (Down)
Cytokine-cytokine receptor interaction	60	37	23	247	8.22	0.72
Focal adhesion	54	12	42	197	1.15	6.93
Amoebiasis	38	13	25	114	3.65	5.47
Jak-STAT signaling pathway	31	22	9	147	6.28	-0.88
Toll-like receptor signaling pathway	27	20	7	97	7.8	-0.32
ECM-receptor interaction	25	2	23	84	-0.92	6.53
Cytosolic DNA-sensing pathway	14	13	1	51	7.33	-1.61
Steroid biosynthesis	13	1	12	18	0.23	9.13
African trypanosomiasis	10	9	1	30	6.82	-0.96
Terpenoid backbone biosynthesis	8	0	8	14	-0.81	6.74