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Mice That Express Human Interleukin-8 Have Increased Mobilization of Immature Myeloid Cells, Which Exacerbates Inflammation and Accelerates Colon Carcinogenesis

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Abstract

BACKGROUND & AIMS—Interleukin (IL)-8 has an important role in initiating inflammation in humans, attracting immune cells such as neutrophils through their receptors CXCR1 and CXCR2. IL-8 has been proposed to contribute to chronic inflammation and cancer. However, mice do not have the IL-8 gene, so human cancer cell lines and xenograft studies have been used to study the role of IL-8 in colon and gastric carcinogenesis. We generated mice that carry a bacterial artificial chromosome that encompasses the entire human IL-8 gene, including its regulatory elements (IL-8Tg mice).

METHODS—We studied the effects of IL-8 expression in APCmin^{+/-} mice and IL-8Tg mice given azoxymethane and dextran sodium sulfate (DSS). We also examined the effects of IL-8 expression in gastric cancer in INS-GAS mice that overexpress gastrin and IL-8Tg mice infected with *Helicobacter felis*.

RESULTS—In IL-8Tg mice, expression of human IL-8 was controlled by its own regulatory elements, with virtually no messenger RNA or protein detectable under basal conditions. IL-8 was strongly up-regulated on systemic or local inflammatory stimulation, increasing mobilization of

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* Authors share co-first authorship.

Conflicts of interest

The authors disclose no conflicts.

Supplementary Material

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immature CD11b⁺Gr-1⁺ myeloid cells (IMCs) with thioglycolate-induced peritonitis, DSS-induced colitis, and *H. felis*-induced gastritis. IL-8 was increased in colo-rectal tumors from patients and IL-8Tg mice compared with nontumor tissues. IL-8Tg mice developed more tumors than wild-type mice following administration of azoxymethane and DSS. Expression of IL-8 increased tumorigenesis in APC^{min}^{+/-} mice compared with APC^{min}^{+/-} mice that lack IL-8; this was associated with increased numbers of IMCs and angiogenesis in the tumors.

CONCLUSIONS—IL-8 contributes to gastrointestinal carcinogenesis by mobilizing IMCs and might be a therapeutic target for gastrointestinal cancers.

Keywords

Colon Cancer; Gastric Cancer; Mouse Model; Immune Regulation

Early-stage colorectal cancer has a 5-year survival rate of ~90%; however, survival is dramatically reduced by local recurrence and metastases.¹ In colon cancer, interleukin (IL)-8 is up-regulated in most tumors and has been associated with poor tumor differentiation, metastasis, and tumor progression.² In vitro, IL-8 is able to stimulate the proliferation and migration of colorectal cancer cell lines,^{2,3} whereas in vivo studies indicate that neutralization of IL-8 can reduce the invasive potential of colonic tumors.⁴ Overexpression of IL-8 has also been observed in gastric cancer, and its expression negatively correlates with prognosis.⁵ Thus, a clearer understanding of the role of IL-8 in early carcinogenesis would help realize the potential of an attractive therapeutic target.

IL-8, also known as CXCL-8, was originally purified from lipopolysaccharide (LPS)-stimulated human monocyte cultures and subsequently shown to induce neutrophil and T-lymphocyte chemotaxis, activate neutrophils, and enhance expression of neutrophil adhesion molecules.⁶ IL-8 can be produced by virtually all nucleated human cells but is primarily expressed in nonepithelial cell types.^{6,7} In inflamed tissues, the primary source of secreted IL-8 appears to be myeloid cells, where the gene is transcriptionally up-regulated by activator protein 1 and nuclear factor κ B.⁸ IL-8 is normally undetectable in healthy tissues, but its expression is strongly up-regulated by proinflammatory cytokines or pathogen-associated factors through the Toll-like receptors in wounded and/or infected tissues.^{8,9}

Previous studies have proposed that secreted IL-8 rapidly recruits and activates neutrophils to inflamed sites by binding with high affinity to two G protein-coupled receptors: CXCR1 and CXCR2.^{10,11} CXCR1 binds IL-8 and the chemokine NAP-2,^{12,13} whereas CXCR2 is more promiscuous and binds to several CXC chemokines, including CXCL1 and CXCL8/IL-8,¹² and is postulated to mediate the angiogenic activity and direct tumor proliferative effects of IL-8.¹³ Moreover, the implication of CD11b⁺Gr-1⁺ immature myeloid cells (IMCs) in tumorigenesis has recently raised the possibility that IL-8 is critical for mobilizing IMCs to the cancer niche. CD11b⁺Gr-1⁺ cells comprise a heterogeneous group that includes myeloid-derived suppressor cells (MDSCs), which have been suggested to contribute to progression of tumorigenesis.^{14,15} However, many cancers express CXCR2, and thus whether IL-8 stimulates tumor growth directly or indirectly through effects on stromal cells has not been clarified.

Despite the primary source of IL-8 is immune cells, studies of IL-8 have primarily been limited to human cancer cell lines because rodents lack the IL-8 gene. Thus, the contribution of stromal or myeloid cell-derived IL-8 in carcinogenesis has not been studied. The homologue of human IL-8 is completely absent from the genome of rodents, and for many years it was assumed that the absence of IL-8 in mice was compensated for by the ligands Cxcl1/KC and Cxcl2, which bind the murine CXCR2 receptor and attract immune cells into inflamed tissues.¹⁶ However, more recently, recombinant IL-8 was shown to play a nonredundant role in proper neutrophil extravasation in the colon of mice with acute *Shigella*-induced colitis.¹⁷ In addition, whereas mice were initially believed to lack the Cxcr1 receptor, the murine Cxcr1 homologue (mCxcr1) has been cloned and shown to be functionally active.^{18,19}

Previously generated IL-8 transgenic (IL-8Tg) mouse models have used constitutive tissue-specific or xenobiotic-inducible promoters that have resulted in high constitutive levels of circulating IL-8 atypical of that normally observed in humans.^{20,21} Thus, the presence of high levels of circulating IL-8 in unchallenged animals, and the absence of physiologic regulation of IL-8 in these earlier models, has limited the conclusions that could be reached regarding IL-8 function. The IL-8 gene is tightly regulated by several distant upstream and downstream promoter elements that are located within at least ~75 kilobases of the surrounding genomic region.²² With this in mind, we developed a physiologically regulated transgene by using a bacterial artificial chromosome (BAC; 166 kilodaltons) encompassing the entire human IL-8 gene. Here, we provide experimental data suggesting that this strategy recapitulates human-like IL-8 expression in appropriate murine tissues in several broadly used models of systemic and local inflammation. Our data also suggest that IL-8 expression exacerbates acute inflammation and promotes acceleration of colonic and gastric carcinogenesis in mice through remodeling of the tumor microenvironment.

Materials and Methods

Isolation and Characterization of a Bacterial Artificial Chromosome

A human bacterial artificial chromosome (hBAC; RPL11-997L11) encompassing the entire *IL-8* gene was purchased through CHORI (Oakland, CA) (Supplementary Figure 2A). To ascertain proper IL-8 gene splicing in mouse cells, the hBAC plasmid (20 μ g) was transfected into mouse dendritic DC2.4 cells that were subsequently treated with 50 ng/mL of mouse IL-1 β . Polymerase chain reaction (PCR) using IL-8 –specific primers in different exons confirmed proper splicing of IL-8 (see Supplementary Materials and Methods for details). Transgenic mice (CBA/C57BL/6) bearing IL-8 hBAC were subsequently generated in the Transgenic Core Facility of Columbia University. hBAC-specific PCR primers were used to genotype hBAC-positive transgenic pups. All animal studies were performed in institutional animal care and use committee-approved facilities at Columbia University.

Analysis of Human Colorectal Cancer

In the present study, colonic tissue biopsy specimens were obtained from patients with colorectal cancer who had undergone surgical resection at Fox Chase Cancer Center (Philadelphia, PA). Identifying information of subjects was removed from biopsy

specimens, and samples were processed for RNA extraction. The experimental protocol was approved by the institutional review board.

See Supplementary Materials and Methods for detailed information on methods.

Results

IL-8 Expression Is Increased in Colorectal Tumors and Contributes to Enhanced Intestinal Carcinogenesis in Humans and IL-8Tg Mice

To confirm that IL-8 plays a role in colon cancer in humans, we examined IL-8 expression in colonic tumors of patients undergoing colorectal cancer resection for stage II or stage III colon cancer. Using real-time quantitative reverse-transcription PCR, we detected significantly increased levels of IL-8 messenger RNA (mRNA) in colonic tumors compared with adjacent normal colonic tissue from the same patients ($n = 10$) (Figure 1A).

Given that mice lack the IL-8 gene, we used a BAC approach to generate transgenic mice expressing human IL-8. The RP11-997L11 BAC encompassing the entire IL-8 gene spanned 166 kilobases of DNA from human chromosome 4 (shown in Supplementary Figure 1A) and was injected into the mouse germline. Germline transmission was achieved in 5 founders, and each line showed a unique autosomal integration site. For one founder line, the unique integration site was directly visualized with fluorescent in situ hybridization (Supplementary Figure 1B). Real-time PCR revealed that 1, 2, and 4 copies of the IL-8 transgene integrated into the chromosome(s) of 3 founder mice, #18, #176, and #180, respectively (data not shown). Progeny of the latter 2 lines, containing 2 and 4 transgene copies, respectively, were used in subsequent experiments.

Unchallenged IL-8Tg mice were indistinguishable in appearance, body weight, behavior, fertility, and life span from their wild-type (WT) littermates. Complete blood cell counts did not reveal any significant difference in hematopoietic parameters at baseline (Supplementary Figure 1C), and histologic examination of the colon and stomach from specific pathogen-free–housed transgenic mice (up to 18 months) showed no inflammatory changes. At baseline, IL-8 mRNA and protein in the gastrointestinal tract (or compensatory increase in MIP-2 or Cxcl1/KC) and circulation were not detectable in transgenic mice. To stimulate IL-8 expression, acute systemic inflammation was induced by LPS and IL-8 measured before and 4 hours after administration of LPS. LPS induced serum IL-8 levels (up to 15 nmol/L) in proportion to the number of transgene copies present (Supplementary Figure 1D), whereas unchallenged transgenic mice had levels below the detectable threshold of the IL-8 enzyme-linked immunosorbent assay (<3 pg/mL).

To examine the contribution of IL-8 to tumorigenesis, colonic tumor number and size were assessed 20 weeks after administration of azoxymethane (AOM) injection and dextran sodium sulfate (DSS) (10 days in drinking water) (Figure 1B). IL-8Tg mice had significantly increased tumors (WT, 1.7 ± 0.2 tumors; IL-8, 5.6 ± 1.8 tumors; $P < .05$) (Figure 1B and C), with most of the increased tumor load attributable to larger tumors (>0.5 cm in diameter). On average, IL-8Tg mice had 3.4 ± 1.5 tumors and WT mice had 0.2 ± 0.2 tumors greater than 0.5 cm in diameter ($P < .01$) (Supplementary Figure 2A). Similarly, *APC^{min}*^{+/-} mice

crossed to IL-8Tg mice developed a greater number of small intestinal tumors than *APC-min*^{+/-} controls (Figure 1F).

Colonic histology from IL-8Tg mice with acute (7 day) colitis revealed increased inflammatory cell infiltrate compared with similarly treated WT controls (Figure 3A and B and Supplementary Figure 2C). A similar trend was observed in IL-8Tg versus WT tumor-bearing mice at later time points (Figure 1E and Supplementary Figure 2C), although the difference in histopathologic scores did not reach statistical significance (Supplementary Figure 2C). Coinciding with enhanced colonic carcinogenesis, human IL-8 (hIL-8) mRNA was significantly increased in the colon (Figure 1D) and circulating IL-8 levels were elevated in tumor-bearing mice (Supplementary Figure 2B). Interestingly, increased carcinogenesis was associated with greater spleen size and increased inflammatory cells in tumor-bearing IL-8Tg versus WT mice (Supplementary Figure 2D and E). Furthermore, we observed increased epithelial proliferation as determined by Ki67⁺ staining in IL-8Tg compared with WT mice (Supplementary Figure 2F).

IL-8 Enhances Helicobacter- and Gastrin-Dependent Gastric Carcinogenesis

Additionally, we performed gastric carcinogenesis studies in IL-8 and WT mice infected with *Helicobacter felis*, a bacterial strain that induces chronic gastritis, dysplasia, and eventually carcinoma.²³ Mice were examined at 6, 12, and 18 months (n = 5 per group) following *H felis* inoculation. At 6 and 12 months postinfection, no differences in histologic scores were observed (data not shown). However, at 12 months, gastric dysplasia was detected only in IL-8Tg mice, and at 18 months postinfection, pseudopyloric metaplasia, foveolar hyperplasia, and dysplasia were significantly increased in the stomach of IL-8Tg mice (Supplementary Figure 3A and B).

Correspondingly, in uninfected WT and IL-8Tg cohorts (controls) and in *H felis*-infected WT mice, serum IL-8 levels remained undetectable during the 18-month period (Supplementary Figure 3C). However, in *H felis*-infected IL-8Tg mice, serum IL-8 levels increased significantly by 6 months postinfection and remained elevated at 12 and 18 months postinfection (Supplementary Figure 3C). Gastric IL-8 mRNA expression was also elevated at baseline in *H felis*-infected IL-8Tg mice versus WT controls and remained elevated over the 6- to 18-month postinfection period.

To investigate the effect of IL-8 in a second model of gastric cancer, we used our hypergastrinemic INS-GAS mice that develop spontaneous corpus gastric tumors.²⁴ Using gastric cancer cell lines, it was previously shown that IL-8 is secreted in direct response to gastrin in vitro.²⁵ Thus, to determine whether IL-8 is a downstream target of gastrin in vivo, INS-GAS mice were crossed with IL-8Tg mice. Consistent with the *H felis* model, double transgenic INS-GAS/IL-8 mice showed accelerated tumor progression and an increased number of invasive tumors (Supplementary Figure 4A and B) compared with INS-GAS mice. At 12 months of age, the serum IL-8 level in INS-GAS/IL-8 mice was significantly increased compared with single IL-8Tg controls (Supplementary Figure 4D). Similarly, gastric IL-8 mRNA expression in INS-GAS/IL-8 mice was significantly increased compared with IL-8Tg controls (Supplementary Figure 4D).

The Predominant Source of IL-8 Is Macrophages and Dendritic Cells

We investigated the cellular source of IL-8 by comparing 3 short-term primary cultures using thioglycolate-mobilized peritoneal macrophages, bone marrow– derived dendritic cells, and colonic epithelial cells. IL-8 secreted into the culture medium following treatment with LPS was then measured by enzyme-linked immunosorbent assay. In the absence of stimulation, low levels of IL-8 (ranging between 50 pg/mL and 5 ng/mL) were detected (Supplementary Figure 5A). However, IL-8 was strongly up-regulated by LPS in a time- and dose-dependent manner (Supplementary Figure 5A), and IL-8 production was much higher (~8-fold greater) in immune cells (ie, macrophages and dendritic cells) than colonic epithelial cells (Supplementary Figure 5A). IL-8 secretion from immune cells peaked at 8 hours (Supplementary Figure 5A), whereas epithelial cells displayed a more gradual response (peak at ~18 to 24 hours) (data not shown). Taken together, these data suggest that myeloid cells are the primary source of IL-8 during the acute inflammatory response in LPS-challenged transgenic mice.

IL-8Tg Mice Show Enhanced Mobilization of IMCs in Acute Inflammation

To characterize the effects of hIL-8 on mobilization of immune cells, we performed fluorescence-activated cell sorting (FACS) analysis of immune cells mobilized upon recombinant hIL-8 injection (100 ng). IL-8 mobilized a significantly higher number of CD11b⁺Gr-1⁺ IMCs compared with saline (16% vs 8%, respectively) (Figure 2A and C). The majority of this myeloid population (~96%) expressed the IMC marker CD31²⁶ (Figure 2A), but these cells were clearly heterogeneous as determined by expression of F4/80⁺, Ly6C, and/or Ly6G (Figure 2A, B, and D). Indeed, detailed FACS analysis of the immune cell subsets revealed a significant increase in both Ly6G⁺Ly6C⁺ IMCs and Ly6G⁻Ly6C⁺ monocytic cells (Figure 2D). As predicted, granulocytic Ly6G⁺Ly6C⁻ immune cells were also mobilized to the peripheral blood to a greater extent than in saline controls, although this increase was not statistically significant.

We further assessed the effects of IL-8 on inflammatory cell mobilization in acute systemic inflammation induced by LPS and acute peritonitis induced by thioglycolate (TTG) broth. LPS was effective in mobilizing a greater number of CD11b⁺Gr-1⁺ IMCs in IL-8Tg versus WT control mice (~70% vs ~30%, respectively) (Figure 2E). Once again, these myeloid cells were predominantly (~90% of IMCs) CD31⁺ (data not shown) and included subsets expressing Ly6C and/or Ly6G (Figure 2F). Interestingly, the significant increase in myeloid subsets in IL-8Tg mice was predominantly due to Ly6G⁺Ly6C⁺ IMCs (Figure 2F). A greater number of Ly6G⁺Ly6C⁻ granulocytic cells were also mobilized in IL-8Tg mice versus WT mice treated with LPS, although this did not reach statistical significance (Figure 2F). Microscopic assessment of the various myeloid cell subsets revealed the characteristic polymorphonuclear morphologic appearance of granulocytic Ly6G⁺Ly6C⁺ immune cells, whereas Ly6G⁺Ly6C⁻ myeloid cells displayed a ring-shaped nuclear structure characteristic of more IMCs (Supplementary Figure 6E) as previously described.²⁶

Similarly, 3 hours after thioglycolate challenge, the number of immune cells was significantly increased in association with elevated IL-8 in the peritoneum of IL-8Tg versus WT mice (Supplementary Figure 5B and C). Accordingly, myeloperoxidase activity

(derived primarily from myeloid cells) was ~1.5-fold higher in the peritoneum of TTG-challenged IL-8Tg versus WT mice (Supplementary Figure 5D). To confirm inflammatory cell mobilization was due to IL-8, we pretreated IL-8Tg mice with an IL-8 neutralizing monoclonal antibody or an unrelated mouse monoclonal immunoglobulin G 2a control antibody before TTG challenge. Pretreatment with IL-8 antibody significantly reduced the number of inflammatory cells in the peritoneum versus isotype-treated IL-8Tg mice (Supplementary Figure 5E). Thus, secreted IL-8 has a nonredundant role in myeloid cell mobilization. Moreover, characterization of these myeloid cells revealed a significantly greater number of CD11b⁺Gr-1⁺ cells in the peritoneum of TTG-treated IL-8Tg versus WT mice, with the majority of this increase due to Ly6G⁺Ly6C⁺ IMCs (Supplementary Figure 5F).

To explore further the mechanism for IL-8 promotion of colonic tumorigenesis, we analyzed immune cells mobilized in acute DSS-induced colitis in IL-8Tg versus WT mice. Not surprisingly, IL-8Tg mice with DSS-induced colitis displayed increased IMCs (characterized by CD11b and Gr-1 cell surface marker expression) within the colon (Figure 3B) and spleen (Figure 3C). A similar trend was observed in the peripheral blood, where we observed increased CD11b⁺Gr-1⁺ cells, although this difference did not reach statistical significance. Similarly, CD11b⁺F4/80⁺ macrophages were significantly increased in the peripheral blood and spleen of DSS-treated IL-8Tg versus WT mice (Figure 3C). The increased IMCs and macrophages in IL-8Tg mice correlated with exacerbation of DSS-induced colitis (ie, increased weight loss) (Figure 3D) and reduced survival (data not shown) compared with WT mice.

To examine further the direct effects of IL-8 on migration of CD11b⁺Gr-1⁺ IMCs, Transwell migration of FACS-sorted CD11b⁺Gr-1⁺ bone marrow– derived IMCs incubated in the presence or absence of IL-8 was assessed (Figure 3E). As an additional control, LPS was used to induce the migration of/activate IMCs. IL-8 significantly increased migration of IMCs when compared with cells incubated in the absence of IL-8 or in the presence of LPS (5 ng/mL) alone (Figure 3E and Supplementary Figure 6C). Taken together, these observations suggest that hIL-8 is effective in mobilizing mouse IMCs both in vivo and in vitro.

IL-8 Mobilizes IMCs and Contributes to Remodeling of the Tumor Microenvironment

IL-8 enhanced mobilization of IMCs and resulted in significantly enlarged spleens in tumor-bearing IL-8Tg compared with WT mice (Supplementary Figure 2D). As a result, we further characterized these cells within the spleen, bone marrow, and colon. Tumor-bearing IL-8Tg mice displayed a significant increase in the number of CD11b⁺Gr-1⁺ IMCs within the spleen and bone marrow (Figure 4A, *left panel*) and an increase in CD11b⁺ cells within the colon in both dysplastic (Figure 4C) and adjacent nondysplastic (Figure 4B) tissues. Further characterization of splenic myeloid cells of tumor-bearing IL-8Tg mice revealed an increase not only in Ly6G⁺ granulocytic cells but in all immature myeloid cell subsets, with the greatest increase attributable to Ly6C⁺Ly6G⁻ and Ly6C⁺Ly6G⁺ cells, although these differences were not statistically different (Figure 4A, *right panel*). Similarly, ~40% of total splenic cells were CD11b⁺Gr-1⁺ in tumor-bearing *APCmin*^{+/-} mice at 16 weeks of age,

whereas IL-8 \times *APCmin*^{+/-} mice displayed an even greater number (~70%) of CD11b⁺Gr-1⁺ cells (Supplementary Figure 6A). The increased IMCs in IL-8 \times *APCmin*^{+/-} mice were predominantly Ly6C⁺ rather than Ly6G⁺ cells (Supplementary Figure 6B). Recruitment of IMCs has also previously been shown to lead to accumulation of cancer-associated fibroblasts and increased tumor angiogenesis.²⁷ In comparing the colonic tumors of our IL-8Tg versus WT mice, we similarly observed increased staining of the cancer-associated fibroblast marker α -smooth muscle actin (SMA), macrophage marker F4⁺/80⁺, and the angiogenesis marker endomucin (Figure 4D–F).

Additionally, we compared immune cell types in colonic tumors of IL-8Tg versus WT mice by microarray analysis of tumors using the gene expression Barcode method²⁸ to determine the presence or absence of genes characteristic of various immune cell types. We identified 3 genes (*Ighm*, *Tsc22d3*, and *Cx3cr*) expressed in immune cells that were expressed in all 3 IL-8Tg mouse colonic tumors but not in any of the WT control tumors examined. Of these, however, only *Ighm* was significantly different in the array experiments ($\log_2FC = 6.13$, $P = 8 \times 10^{-6}$, false discovery rate (fdr) = 0.04) and confirmed by PCR (Table 1). According to the Barcode database, and confirmed by our own reverse-transcription PCR analysis of splenic immune cells, *Ighm* was expressed primarily in B cells (as expected) and less frequently in T cells (Supplementary Figure 7A). Thus, our results suggest that B cells may have a role in the IL-8 –induced immune response and could also contribute to the mechanism of IL-8 –mediated cancer progression, although further work is needed to understand its relevance to tumorigenesis.

IMC-Derived IL-8 Promotes Tumor Growth and Tumor Remodeling

Whereas myeloid cells serve as the primary source of IL-8 (Supplementary Figure 5A), transformed epithelial cells show increased IL-8 expression (Figure 1A and D), and previous studies using human cancer cell lines have implicated only tumor-derived IL-8 in carcinogenesis. To specifically address the contribution of immune cell– derived IL-8 in tumorigenesis, we performed xenograft studies using CT-26 BALB/c mouse colon cancer cells that do not express IL-8.

CT-26 colon cancer cells were coinjected subcutaneously into NOD-SCID mice, with or without bone marrow– derived IMCs (CD11b⁺Gr-1⁺) derived from IL-8Tg mice or WT mice. IMCs from IL-8Tg mice significantly enhanced the growth of xenograft tumors when compared with CT-26 cells implanted alone or in combination with IMCs from WT mice (Figure 5A and B). Indeed, IMCs from IL-8Tg mice expressed IL-8 mRNA on stimulation (ie, with LPS) (Figure 5D and E), and both the CT-26 mouse cancer cells and IMCs expressed the IL-8 receptors *Cxcr1* and *Cxcr2* (Figure 5F). Thus, IL-8 receptors on both IMCs and dysplastic epithelial cells allow for autocrine and paracrine action of IL-8 (Figure 5F and Supplementary Figure 6D).

Interestingly, coimplantation of IL-8 – expressing IMCs led to altered tumor histology, with increased stromal expansion (Figures 5C, 6A, and 6B) and immunohisto-chemical analysis of xenograft tumors confirming the presence of significantly increased cancer-associated α -SMA–positive fibroblasts (Figure 6C), increased epithelial cell (Ki67⁺) proliferation (Figure 6D), and increased endomucin-positive angiogenesis (Figure 6E). These data clearly

implicate IL-8 –producing IMCs in remodeling of the tumor environment and promotion of tumor growth.

Discussion

In this study, we used a BAC encompassing the entire IL-8 gene regulatory elements to generate IL-8 –expressing transgenic mice that recapitulate human physiologic IL-8 expression. Transgenic mouse models expressing constitutively high levels of hIL-8 have previously been reported^{20,21}; however, none have exhibited a physiologic pattern of IL-8 expression with strict inducibility by injury or infection. Here, we show that IL-8 BAC transgenic mice do not exhibit detectable circulating IL-8 at baseline, but following inflammatory injury show significantly increased levels of IL-8 in diseased tissues and the circulation. As in humans, the predominant cellular source of IL-8 in these mice was myeloid, with dramatically lower levels in epithelial cells. Interestingly, our IL-8 BAC transgenic mice displayed heightened inflammatory responses to LPS, TTG-induced peritonitis, DSS-induced colitis, and *Helicobacter* infection. Importantly, IL-8Tg mice displayed significant acceleration of inflammation-associated colonic and gastric carcinogenesis in association with enhanced mobilization of CD11b⁺Gr1⁺ IMCs. Taken together, these data show an important role for stromal-derived IL-8 in the mobilization of IMCs and initiation of gastrointestinal tumors.

We and others have recently shown that CD11b⁺ Gr-1⁺ IMCs play an important role in influencing the tumor microenvironment and promoting tumor progression.^{27,29} In cancer, IMCs have immune suppressive activity that mediates tumor promotion.^{14,15} In this study, we report enhanced mobilization of CD11b⁺Gr-1⁺ IMCs in inflammation and in tumor-bearing IL-8 BAC transgenic mice, supporting a role for these cells in the initiation and progression of gastrointestinal carcinogenesis. Importantly, when implanted with CT-26 colon cancer cells in NOD-SCID mice, IL-8 – expressing IMCs increased tumor growth. These CD11b⁺Gr-1⁺ cells, also labeled MDSCs, negatively regulate immune responses during cancer¹⁵ and are increased 10-fold in the spleen of many mouse tumor models and increased 10-fold in the blood of patients with various cancers.¹⁵ We observed similar increases in splenic MDSC/IMCs in tumor-bearing IL-8Tg mice, pointing to a possible role for IL-8 in expansion of MDSCs. The enhanced immune cell mobilization was almost certainly due to IL-8 because this was observed in 2 independent transgenic lines, and an IL-8 neutralizing antibody partially inhibited this response. Moreover, IL-8 augmented both systemic (ie, LPS-induced) and local (ie, TTG-induced peritonitis, DSS-induced colitis) acute inflammatory responses in rodents to establish a nonredundant role for IL-8 in inflammation and carcinogenesis.

Although previous studies examining the role of IL-8 in inflammation have focused on its effects on mobilization of neutrophils, our data suggest that IL-8 exerts its predominant effect on the expansion and recruitment of CD11b⁺Gr-1⁺ IMCs that coexpress the immature myeloid cell marker CD31 and are morphologically distinct from mature neutrophils. These cells are phenotypically heterogeneous, expressing the granulocytic marker Ly6G, the monocytic marker Ly6C, or a combination of the two. Thus, in the presence of IL-8, IMCs mobilized from the bone marrow early and throughout tumor development influence the

tumor microenvironment to accelerate tumorigenesis. Moreover, the similarity in immune cell profile and gene expression pattern in colonic tumors from IL-8Tg versus WT mice is consistent with enhanced recruitment of IMCs rather than a change in immune cell phenotype, being more important for enhanced tumor initiation and progression. Akin to the recently reported effects of epithelial tumor– derived granulocyte-macrophage colony–stimulating factor in promoting tumor growth via recruitment of IMCs to the tumor, IL-8 similarly promoted tumorigenesis through enhanced mobilization of IMCs.^{15,30–32} Furthermore, on tumor establishment, epithelial-derived IL-8 promotes tumor progression through proangiogenic and pro-proliferative effects. This is consistent with previous in vitro studies using human cancer cells and xenograft tumors showing tumor promotion via IL-8 – induced angiogenesis and epithelial proliferation.¹³

Although we cannot exclude a role for epithelial cell–derived IL-8, our data clearly suggest that inflammatory cell– derived IL-8 plays a key role in the mobilization of CD11b⁺Gr-1⁺ IMCs critical in initiation as well as progression of both colonic and gastric carcinogenesis. Interestingly, the 2 inducers of gastric carcinogenesis (*H felis* infection and gastrin) used also up-regulated IL-8 expression and secretion.³³ In addition, in our xenograft models of cancer, IL-8 – expressing IMCs promoted growth of tumor cells lacking IL-8. We previously defined proinflammatory and tumor-promoting effects of IMCs, independent of their immune suppressive properties.^{27,34} Similarly, here we show that enhanced tumor growth in the presence of IL-8 occurred in association with α -SMA–positive myofibroblast expansion and increased angio-genesis. Thus, increased α -SMA–positive cells in colonic tumors of IL-8Tg mice may be due to enhanced mobilization of CD11b⁺Gr-1⁺ IMCs or a direct effect of IL-8 on myofibroblasts.

In summary, we established a physiologic model of IL-8 expression and for the first time show that endogenous IL-8 enhances acute inflammation by mobilization of predominantly CD11b⁺Gr-1⁺ IMCs and, in turn, accelerates tumorigenesis and remodeling of the tumor microenvironment. Furthermore, myeloid-derived IL-8 contributes to cancer initiation and progression, suggesting that pharmacologic inhibition of IL-8 may be a potential strategy for the prevention and treatment of colon cancer. IL-8 BAC transgenic mice may prove useful for the development of such targeted therapies in inflammatory disease and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Gene microarray accession number: Gene Expression Omnibus number GSE 39273.

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Abbreviations used in this paper

AOM	azoxymethane
BAC	bacterial artificial chromosome
DSS	dextran sodium sulfate
FACS	fluorescence-activated cell sorting
hBAC	human bacterial artificial chromosome
hIL-8	human interleukin-8
IL	interleukin
IL-8Tg	interleukin-8 transgenic
IMC	immature myeloid cell
LPS	lipopolysaccharide
MDSC	myeloid-derived suppressor cell
PCR	polymerase chain reaction
SMA	smooth muscle actin
TTG	thioglycolate
WT	wild-type

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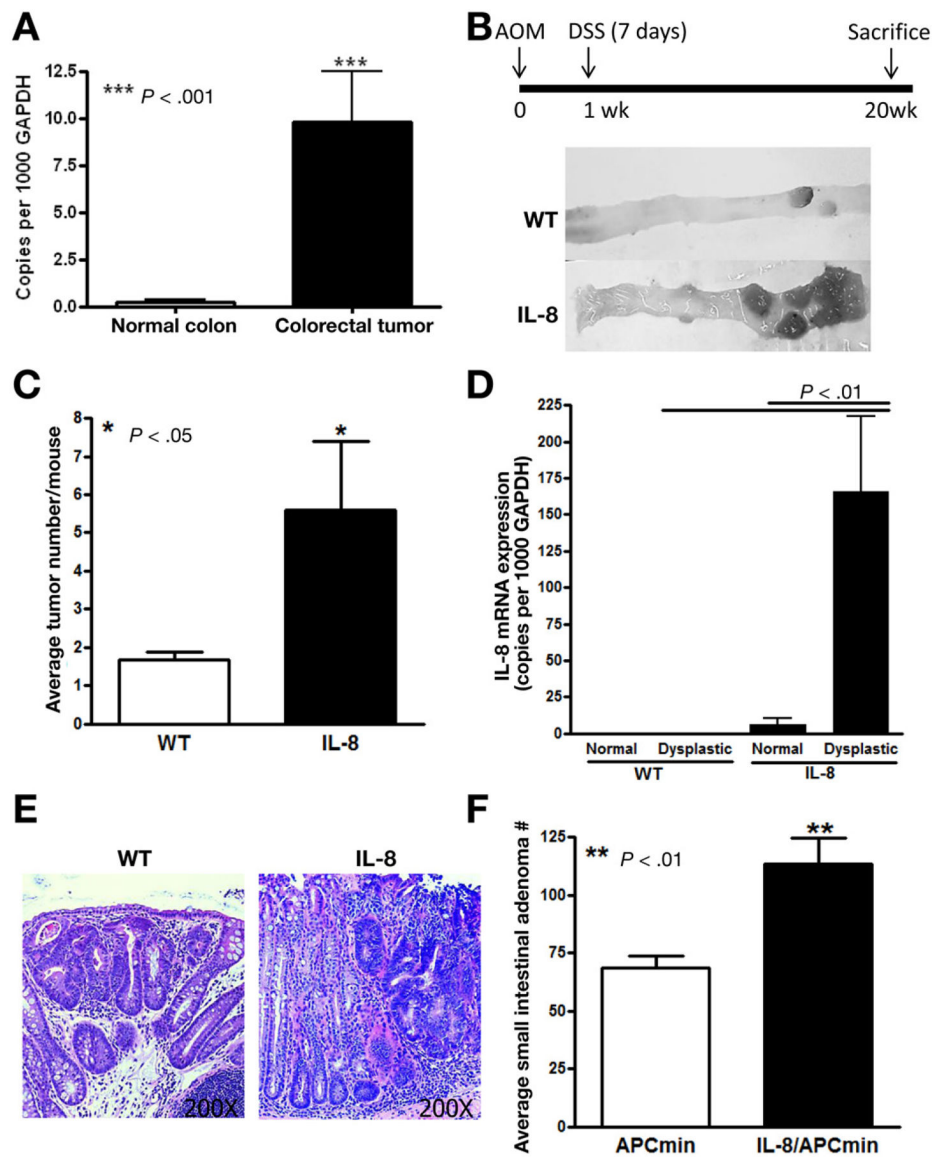


Figure 1.

IL-8 contributes to intestinal carcinogenesis in humans and IL-8Tg mice. (A) IL-8 mRNA expression in colorectal tumors and adjacent histologically normal tissue from patients with colorectal cancer. (B) Representative gross images of colonic tumors from WT versus IL-8Tg mice treated with AOM plus DSS. (C) Colonic tumor number in IL-8Tg versus WT mice. (D) IL-8 mRNA expression in dysplastic versus nondysplastic colonic epithelium from WT and IL-8Tg mice. (E) H&E representative images of colonic tumors from WT and IL-8Tg mice. (F) Small intestinal adenoma number in IL-8 \times *APCmin*^{+/-} transgenic versus *APCmin*^{+/-} mice.

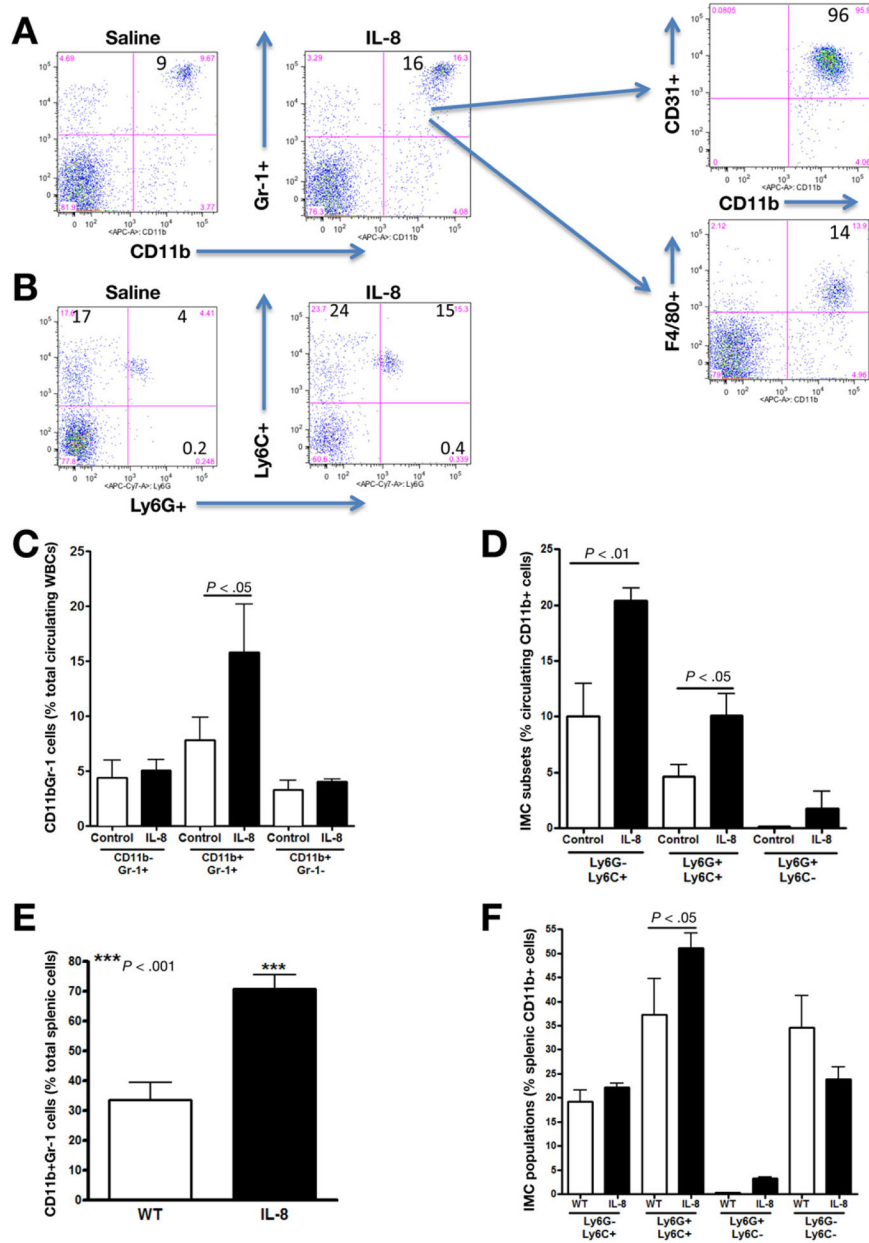


Figure 2. IL-8 mobilizes CD11b⁺Gr-1⁺ IMCs in acute inflammation. (A) Representative FACS plots of circulating CD11b⁺Gr-1⁺ IMCs following recombinant hIL-8 (100 ng) versus saline intravenous injection in WT C57BL/6 mice. FACS analysis of IMCs expressing the immature myeloid cell marker CD31⁺ and macrophage marker F4/80⁺. (B) Representative FACS plot showing Ly6C⁺ and Ly6G⁺ myeloid cell subsets mobilized into the peripheral circulation by hIL-8. (C) Quantitative analysis of CD11b⁺Gr-1⁺ IMCs and (D) Ly6C⁺ and Ly6G⁺ myeloid subsets in IL-8Tg versus WT mice following intravenous injection of hIL-8 (100 μg). (E) Quantitative analysis of CD11b⁺Gr-1⁺ IMCs and (F) Ly6C⁺ and Ly6G⁺

myeloid cell subsets mobilized to the spleen of IL-8Tg versus WT mice following intraperitoneal LPS (5 mg/kg) injection (** $P < .001$ vs WT).

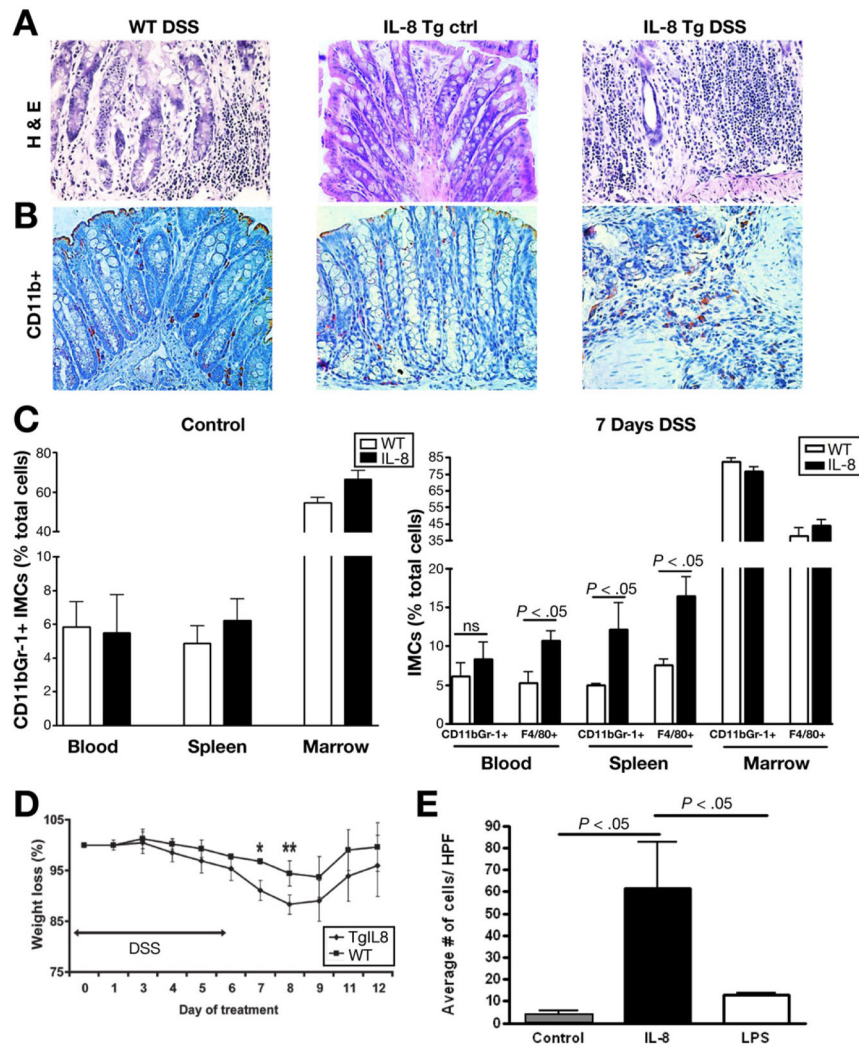


Figure 3. IL-8 mobilizes IMCs in acute colitis and induces migration of IMCs in vitro. (A) H&E staining of colonic tissues from DSS-treated (7 day) WT mice versus untreated or DSS-treated (7 day) IL-8Tg mice. (B) Immuno-staining of CD11b⁺myeloid cells in the colon of DSS-treated (7 day) WT versus untreated or DSS-treated (7 day) IL-8Tg mice. (C) FACS analysis of CD11b⁺Gr-1⁺IMCs and F4/80⁺ macrophages in the peripheral blood, spleen, and bone marrow of IL-8Tg versus WT mice at baseline and following 7 days of treatment with DSS (n = 4 per group). (D) Severity of weight loss in IL-8Tg mice versus WT mice with DSS-induced colitis (*P < .05; **P < .01). (E) Trans-well migration of CD11b⁺ Gr-1⁺ IMCs in the presence or absence of IL-8 (100 ng/mL) or LPS (5 ng/mL); n = 4 per group.

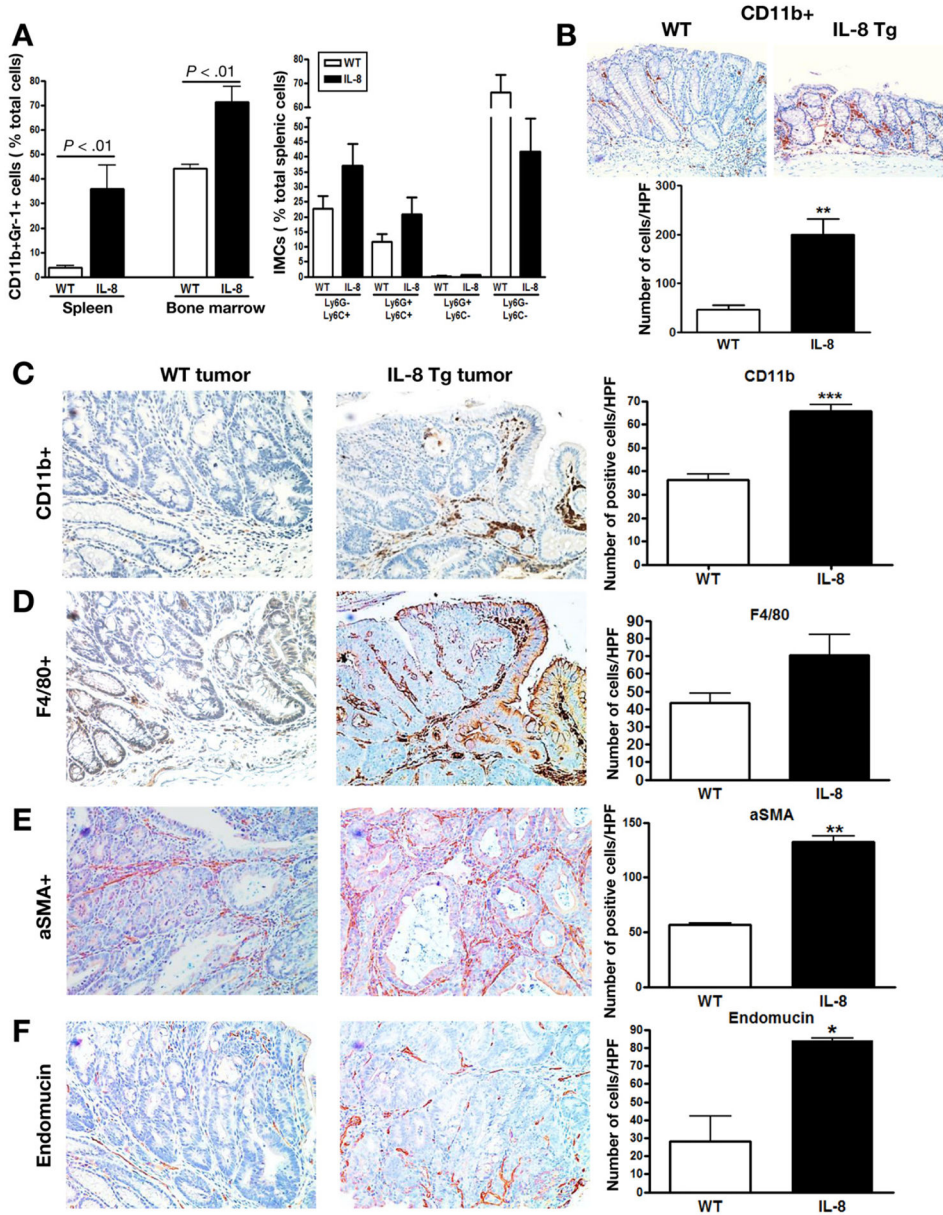


Figure 4. IL-8 mobilizes IMCs in cancer and contributes to remodeling of the tumor microenvironment. (A) Quantitative analysis of splenic CD11b⁺Gr-1⁺ IMCs (*left panel*) and Ly6C⁺ and Ly6G⁺ myeloid cell subsets (*right panel*) in IL-8Tg versus WT tumor-bearing mice 20 weeks after treatment with AOM/DSS. Representative immunostaining of CD11b⁺ myeloid cells in (B) nondysplastic and (C) dysplastic colonic tissues from AOM/DSS-treated WT mice versus IL-8Tg mice. (D) Representative immunostaining of F4/80⁺ tumor-associated macrophages in serial sections of dysplastic colonic tissue from IL-8Tg versus WT mice. (E) Representative immunostaining of α -SMA–positive cancer-associated myofibroblasts in colonic tumors from IL-8Tg and WT mice. (F) Representative immunostaining of endomucin-positive blood vessels in colonic tumors from IL-8Tg and

WT mice. Quantitative analyses of CD11b, F4/80, α -SMA, and endomucin immunostaining in colonic tumors are shown in graphical format next to each representative slide. * $P < .05$, ** $P < .01$, *** $P < .001$ versus respective WT control; n = 5 per group.

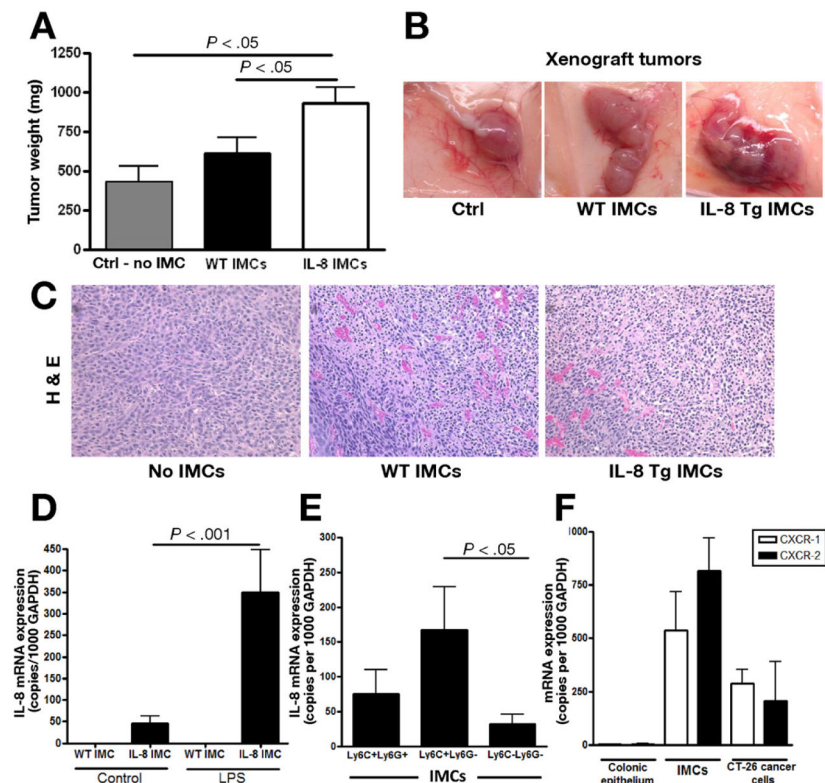


Figure 5. Myeloid cell– derived IL-8 promotes tumor growth. (A) Xenograft tumor weight of CT-26 mouse colonic cancer cells implanted subcutaneously in NOD-SCID mice either alone or combined with IMCs from WT mice or IL-8Tg mice. (B) Representative gross images and (C) H&E microscopic images of the xenograft tumors are shown. (D) IL-8 mRNA expression in IMCs from WT versus IL-8Tg mice cultured in the presence or absence of LPS (5 ng/mL). (E) IL-8 mRNA expression in Ly6C⁺ and/or Ly6C⁻ myeloid subsets isolated by FACS from the spleen of LPS-treated (5 mg/kg intraperitoneally) IL-8Tg mice. (F) CXCR1 and CXCR2 mRNA expression in normal (nondysplastic) colonic epithelial cells or splenic IMCs from WT versus IL-8Tg mice versus CT-26 mouse colonic cancer cells cultured in the presence or absence of LPS (100 ng/mL). n = 5 per group.

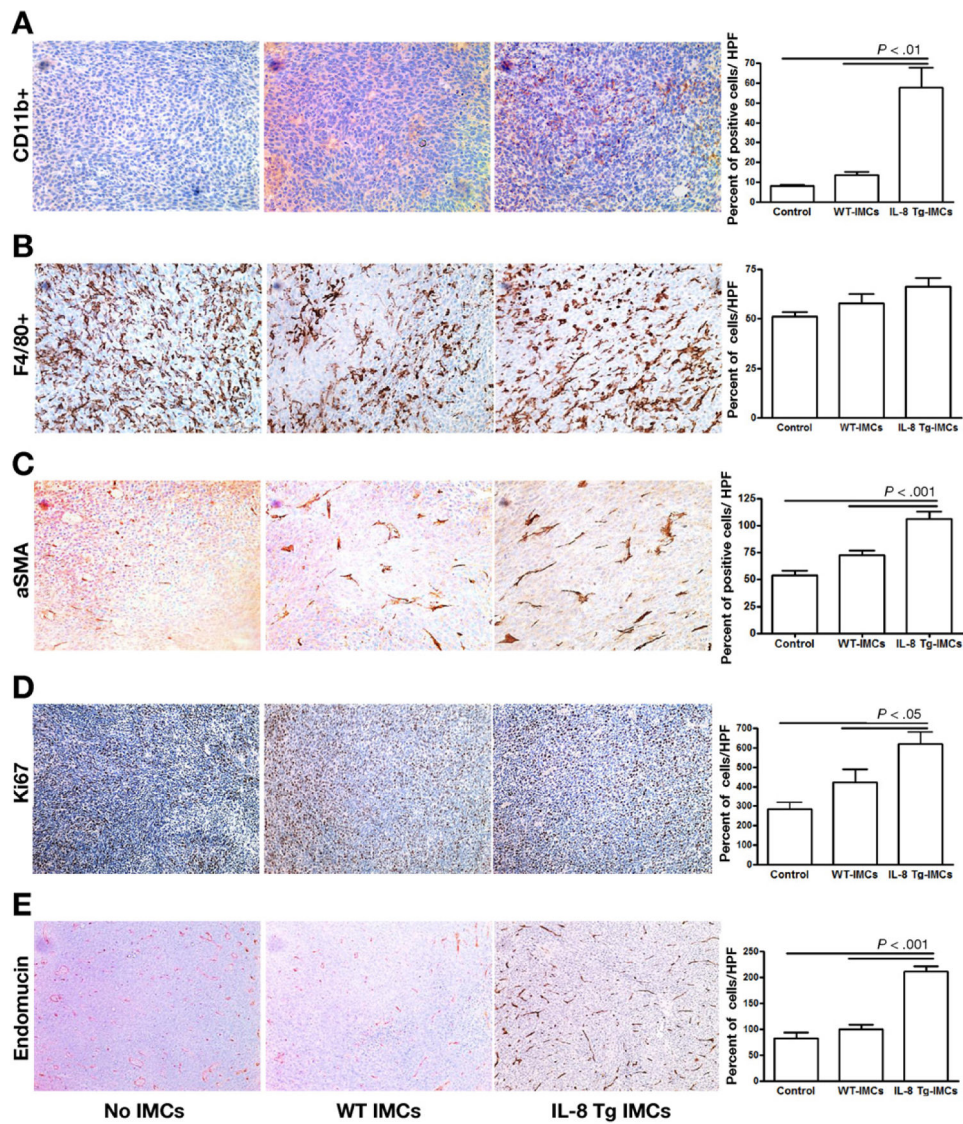


Figure 6. IMC-derived IL-8 contributes to remodeling of the tumor microenvironment ex vivo. Representative immunostaining of (A) CD11b⁺ myeloid cells and (B) F4/80⁺ tumor-associated macrophages in CT-26 xenograft tumors implanted alone or in combination with IMCs from WT or IL-8Tg mice. Representative immunostaining of (C) α-SMA–positive cancer-associated myofibroblasts, (D) Ki67⁺ proliferating cells, and (E) endomucin-positive blood vessels in CT-26 xenograft tumors implanted alone or in combination with IMCs from WT or IL-8Tg mice. Quantitative analyses of CD11b, F4/80, α-SMA, and endomucin immunostaining are shown in graphical format next to each slide. n = 5 per group.

Table 1

Genes Identified as Present or Absent in IL-8Tg Versus WT Colonic Tumors Using the Microarray Gene Expression Barcode Method

Gene	Description	logFC	P value
Ighm	Ig heavy constant mu	6.1	8.44×10^{-6}
Tsc22d3	TSC22 domain family, member 3	0.9	.011
Cx3cr1	Chemokine (C-X3-C) receptor 1	-2.2	.001