Paradoxical role of the proto-oncogene Axl and Mer receptor tyrosine kinases in colon cancer


The receptor tyrosine kinases Axl and Mer, belonging to the Tyro3, Axl and Mer (TAM) receptor family, are expressed in a number of tumor cells and have well-characterized oncogenic roles. The therapeutic targeting of these kinases is considered an anticancer strategy, and various inhibitors are currently under development. At the same time, Axl and Mer are expressed in dendritic cells and macrophages and have an essential function in limiting inflammation. Inflammation is an enabling characteristic of multiple cancer hallmarks. These contrasting oncogenic and anti-inflammatory functions of Axl and Mer posit a potential paradox in terms of anticancer therapy. Here we demonstrate that azoxymethane (AOM) and dextran sulfate sodium (DSS)-induced inflammation-associated cancer is exacerbated in mice lacking Axl and Mer. Ablation of Axl and Mer signaling is associated with increased production of proinflammatory cytokines and failure to clear apoptotic neutrophils in the intestinal lamina propria, thereby favoring a tumor-promoting environment. Interestingly, loss of these genes in the hematopoietic compartment is not associated with increased colitis. Axl and Mer are expressed in radioresistant intestinal macrophages, and the loss of these genes is associated with an increased inflammatory signature in this compartment. Our results raise the possibility of potential adverse effects of systemic anticancer therapies with Axl and Mer inhibitors, and underscore the importance of understanding their tissue and cell type-specific functions in cancer.

The proto-oncogenes AXL and MER were first cloned from chronic myelogenous and lymphoblastic leukemia cells (1–3). Increased expression of AXL has been reported in lung, breast, ovarian, gastric, pancreatic, and prostate cancers; leukemias and lymphomas; melanoma; and glioblastoma multiforme (4, 5). Increased MER expression is associated with leukemias, lymphomas, melanoma, rhabdomyosarcomas, and gastric and prostate cancers (4–6). AXL and MER expression also has been directly correlated with cancer biology (6–9). Moreover, ectopic expression of AXL has been shown to confer resistance to EGF receptor therapy in lung cancer (10, 11). Multiple studies have demonstrated AXL and MER function in survival, invasion, and metastasis in a variety of tumors (12–15); thus, attention has focused on the pharmacologic targeting of AXL and MER in cancer. Axl and Mer share structural homology in the kinase domain with other tyrosine kinases, including preserved molecular interactions with ATP; however, several unique features of the active site allow for selective inhibition (16), and small-molecule inhibitors as well as biologics are in preclinical development (6, 16–19).

Axl and Mer are associated with another distinct feature of cancer—immunoactivation. Tumor-promoting inflammation has been described as an enabling characteristic that promotes the acquisition of cancer hallmarks and orchestrates tumor progression (20). Chronic inflammation increases the risk of colorectal cancer (21, 22). The relative risk of being diagnosed with colorectal cancer in the United States was ~0.05% in 2007 (www.cdc.gov). This risk is increased dramatically in patients with inflammatory bowel disease; for example, ulcerative colitis increases the risk by ~20-fold (23), and the reported risk in patients with pancolitis is 30% after 35 years of disease (24).

Recently, a direct role of innate immune cells and cytokines in colorectal cancer has been demonstrated using mouse models (25). One of the main physiological functions of Axl and Mer is in the inhibition of the innate immune response (26). Axl and Mer are expressed in dendritic cells and macrophages, and their activation limits toll-like receptor and cytokine receptor signaling (26–29); consequently, Axl and Mer should inhibit tumor-promoting inflammation and reduce the risk of colorectal cancer. In light of the oncogenic and anti-inflammatory functions of Axl and Mer, we sought to test the role of these receptor tyrosine kinases (RTKs) in inflammation-driven cancer.

Using a mouse model of AOM/DSS-induced colorectal cancer (30, 31), combined with genetic ablation of Axl and Mer, we found that Axl and Mer signaling prevents colitis and significantly reduces the number and size of colorectal adenomas. We detected a significant increase in the number of apoptotic neutrophils and in the production of proinflammatory cytokines in the lamina propria of Axl−/−Mer−/− mice in the context of DSS-induced colitis. Axl and Mer are expressed primarily in the hematopoietic compartment (32). Surprisingly, the loss of Axl and Mer function in radiosensitive hematopoietic cells is not associated with increased colonic inflammation. Axl and Mer are expressed in a radiosensitive population of lamina propria macrophages upon inflammation, and the absence of Axl and Mer results in an increased proinflammatory profile in lamina propria macrophages. Axl−/−Mer−/− macrophages show increased production of inflammatory cytokines and reduced expression of genes associated with alternative activation—a distinct macrophage phenotype that decreases inflammation and promotes tissue repair (33). Our results demonstrate that although Axl and Mer can function as oncogenes in a number of cancers, these genes have a protective role against the development of colitis-associated cancer. Our findings underscore the potential adverse effects of systemic inhibition of Axl and Mer and highlight the importance of developing therapeutic strategies to spare these RTKs in macrophage populations relevant to the regulation of local inflammation and tissue homeostasis.

Results

Genetic Ablation of Axl and Mer Promotes Colitis-Associated Colon Cancer. We compared the development of colitis-associated colorectal cancer in WT and Axl−/−Mer−/− mice. Colorectal cancer was induced by the administration of a single i.p. dose of the procarcinogen AOM, followed by three cycles of DSS in the drinking water separated by treatment-free intervals (Fig. 1A). Endoscopic analysis revealed that the Axl−/−Mer−/− mice...
Nuclear factor kappa B (NF-κB) signaling has been reported (34). Thus, we directly tested whether the Axl- and Mer-dependent phagocytosis of apoptotic neutrophils and inhibition of cytokine production prevent colitis. (A) Schematic representation of AOM/DSS-induced colon cancer treatment. In brief, WT and Axl−/−Mer−/− mice were injected with the DNA-methylating agent AOM and were subsequently treated with the indicated cycles of DSS. (B) Representative colonoscopy images after 90 d of treatment in WT and Axl−/−Mer−/− mice. (C) Tumor number and tumor score in WT and Axl−/−Mer−/− mice after 90 d of treatment as determined by colonoscopy. (D) Representative luminal views of WT and Axl−/−Mer−/− colons treated as indicated in A. Tumors in the rectum and distal colon of Axl−/−Mer−/− can be seen. (E) Representative image of colon sections stained with H&E, showing adenomas in Axl−/−Mer−/− mice. (Scale bar: 500 μm.) Data are presented as representative individual samples or as the mean of 14–16 independent samples per group. **P < 0.01.

In brief, WT and Axl−/−Mer−/− mice were acutely treated with DSS in the drinking water. The DSS dose was adjusted to induce minimal inflammation in WT mice as judged by colonoscopy (Fig. 2B). Axl−/−Mer−/− mice showed a significant reduction in body weight after DSS treatment compared with WT mice (Fig. 2A). Colonoscopy in the Axl−/−Mer−/− mice revealed enhanced colitis as demonstrated by increased granularity, loss of apparent vasculature, decreased translucency, and looser stool consistency (Fig. 2B). Consistent with increased inflammation after DSS administration, colon length was significantly reduced in the Axl−/−Mer−/− mice (Fig. 2C). Histopathological analysis following established methods (35) confirmed the increased severity of colitis (i.e., mucosal ulceration, crypt loss, crypt hyperplasia, inflammatory cell infiltration, and edema) in the Axl−/−Mer−/− mice compared with WT mice after DSS administration (Fig. 2D). Colonos from untreated Axl−/−Mer−/− mice were unremarkable and within normal limits by both colonoscopy and histopathological analysis (Fig. 2E and F). Taken together, these results demonstrate that the increased severity of colitis and tissue injury in the Axl−/−Mer−/− mice is associated with an increased risk of colorectal cancer.

Axl- and Mer-Dependent Phagocytosis of Apoptotic Neutrophils and Inhibition of Cytokine Production Prevent Colitis. We next evaluated additional features of intestinal inflammation in WT and Axl−/−Mer−/− mice. The percentages of Ly6G+ neutrophils and F4/80+CD11b+ macrophages were determined in WT and Axl−/−Mer−/− mice at day 90 after DSS treatment (Fig. 2D). Consistent with increased inflammation after DSS administration, the percentages of Ly6G+ neutrophils and F4/80+CD11b+ macrophages were significantly increased in Axl−/−Mer−/− mice compared with WT mice (Fig. 2D). Histological scoring of colon morphology in untreated mice and increased colitis in Axl−/−Mer−/− mice compared with WT after DSS treatment. Histological scoring of colitis is reported. (Scale bars: 500 μm.) Data are representative images or mean ± SEM of at least six independent samples per group. *P < 0.05; **P < 0.01; ****P < 0.0001.
macrophages were not altered in WT and Axl−/−Mer−/− mice at steady state (Fig. 3A and Fig. S1). The induction of colitis was associated with a significant increase in Ly6G+ cells or neutrophils in both WT and Axl−/−Mer−/− mice (Fig. 3A). Interestingly, immunohistochemical and FACS analyses revealed a significant increase in the number of TUNEL+/Ly6G+ apoptotic neutrophils in the lamina propria of Axl−/−Mer−/− mice compared with WT mice (Fig. 3B and C). Clearance of apoptotic neutrophils is essential for the resolution of inflammation and tissue repair (36).

Tyro3, Axl and Mer (TAM) receptors function in the removal of apoptotic membranes of photoreceptors and apoptotic germ cells in the testis (32); thus, we directly compared the ability of bone marrow (BM)-derived macrophages from WT and Axl−/−Mer−/− mice to phagocytose apoptotic neutrophils in vitro assays. Apoptotic neutrophils were labeled with CellTracker dye and cocultured with BM-derived macrophages at a ratio of 5:1. Axl−/−Mer−/− macrophages demonstrated a significant deficit in the ability to phagocytose apoptotic neutrophils compared with WT macrophages (Fig. 3D). The loss of Axl and Mer also was associated with increased inflammatory cytokine production by lamina propria leukocytes. CD45+ cells were sorted from the lamina propria of WT or Axl−/−Mer−/− mice after 7 d of DSS treatment. Quantitative real-time PCR (qPCR) analyses demonstrated an increase in the expression of IFN-γ and TNF-α in Axl−/−Mer−/− leukocytes (Fig. 3E). Taken together, our results indicate that Axl and Mer function in limiting inflammation in the intestinal lamina propria through the phagocytosis of apoptotic neutrophils and regulation of proinflammatory cytokine production.

**Hematopoietic Function of Axl and Mer Does Not Confer Increased Susceptibility to Colitis.** Because Axl and Mer are expressed primarily in BM-derived dendritic cells and macrophages (32), we tested whether the loss of Axl and Mer in the hematopoietic compartment accounted for the increased susceptibility to induced colitis. For this, CD45.1 C57B6 WT mice were lethally irradiated and their hematopoietic compartments reconstituted with CD45.2 WT or Axl−/−Mer−/− hematopoietic progenitors (Fig. 4A). Efficient reconstitution of the hematopoietic compartment was confirmed by the analysis of CD45.1+ and CD45.2+ leukocytes in the peripheral blood at 2 mo after BM transfer (Fig. 4B). Treatment with DSS did not cause enhanced colitis in

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**Fig. 3.** Increased number of apoptotic neutrophils and production of proinflammatory cytokines in the lamina propria of Axl−/−Mer−/− mice. (A) Percentage of neutrophils (CD45+CD3−Ly6G+) in the lamina propria of WT and Axl−/−Mer−/− before (untreated) and after 7 d of DSS treatment (DSS) as detected by FACS analysis. Representative FACS plots and independent data are shown. (B) Representative immunofluorescence staining for TUNEL in colon sections from WT and Axl−/−Mer−/− mice after 7 d of DSS treatment. Nuclei are counterstained with DAPI. (Scale bar: 75 μm.) (C) TUNEL staining in CD11b+Ly6G+ cells in the lamina propria leukocytes of WT and Axl−/−Mer−/− mice as detected by FACS analysis. (D) Rate of phagocytosis of apoptotic neutrophils, labeled with CellTracker, and incubated with WT or Axl−/−Mer−/− BM macrophages for 60 min. (E) mRNA expression (Relative to GAPDH) of IFN-γ and TNF-α in the lamina propria leukocytes from WT and Axl−/−Mer−/− mice after 7 d of DSS treatment as detected by qPCR. Data are presented as representative images or as mean ± SEM of at least five samples per group. n.s., nonsignificant. *P < 0.05; **P < 0.01; ***P < 0.001.
recipients of Axl<sup>−/−</sup>Mer<sup>−/−</sup> hematopoietic progenitors compared with recipients of WT hematopoietic progenitors, as confirmed by colonoscopy (Fig. 4C). We continued the DSS treatment for two additional cycles. Surprisingly, even chronic DSS treatment failed to demonstrate a significant difference in colonic inflammation between recipients of Axl<sup>−/−</sup>Mer<sup>−/−</sup> hematopoietic progenitors and recipients of WT hematopoietic progenitors (Fig. 4C). In line with these results, we did not detect a significant increase in the number of TUNEL<sup>+</sup>Ly6G<sup>+</sup> apoptotic neutrophils in the lamina propria of recipients of Axl<sup>−/−</sup>Mer<sup>−/−</sup> BM compared with recipients of WT BM after 7 d of DSS treatment (Fig. 4D). Furthermore, recipients of WT and Axl<sup>−/−</sup>Mer<sup>−/−</sup> hematopoietic progenitors demonstrated similar induction of IL-17<sup>+</sup>, IFN-γ<sup>+</sup>, and IL-17<sup>+</sup>IFN-γ<sup>+</sup> colon lamina propria CD4<sup>+</sup> T cells (Fig. 4E). These results demonstrate that loss of Axl and Mer in the radiosensitive hematopoietic compartment is not responsible for the increased susceptibility to induced colitis observed in Axl<sup>−/−</sup>Mer<sup>−/−</sup> mice.

**Loss of Axl and Mer Is Associated with Increased Inflammatory Profile of a Radioresistant Macrophage Population.** The foregoing surprising findings suggest that an Axl- and Mer-expressing radioresistant population inhibits intestinal inflammation and inflammation-associated colon cancer. Analyses of colonic lamina propria isolates from BM recipients of WT or Axl<sup>−/−</sup>Mer<sup>−/−</sup> hematopoietic progenitors after DSS treatment revealed the presence of a small but significant fraction of radiosensitive CD45<sup>+</sup>CD11b<sup>+</sup> cells (Fig. 5A). Approximately 70% of these cells were positive for the macrophage markers F4/80 and CD11b (Fig. 5B). Interestingly, Mer-expressing cells were identified within this radiosensitive macrophage population (Fig. 5C), suggesting Mer as a marker distinctly associated with mature intestinal tissue resident macrophages.

We next investigated the expression of Axl and Mer in lamina propria macrophages during inflammation. Compared with day 0 (untreated), at day 3 we detected two distinct subpopulations, F4/80<sup>high</sup>CD11b<sup>+</sup> and F4/80<sup>low</sup>CD11b<sup>+</sup>. We found a similar pattern after 7 d of DSS administration, whereas at day 14 (at 2 d after completion of the DSS regimen), the macrophage population pattern resembled that at day 0. These distinct subpopulations of macrophages were cell-sorted, and qPCR analyses of Axl and Mer during the course of DSS treatment revealed robust induction of expression of Axl and Mer in the lamina propria of WT mice compared with CD45<sup>+</sup> WT Axl<sup>−/−</sup>Mer<sup>−/−</sup> BM chimera mice, as detected by FACS, after 7 d of DSS treatment. Representative histograms (Left) and bar graphs (Right) indicating mean fluorescence intensity (MFI) of TUNEL expression are shown. (E) Representative FACS plots (Left) and bar graphs (Right) showing the frequency of IL17<sup>+</sup>, IFN-γ<sup>+</sup>, and IL17<sup>+</sup>IFN-γ<sup>+</sup> CD4<sup>+</sup> T cells in the lamina propria of BM chimeras at the end of the chronic DSS treatment. Data are presented as representative images or as mean ± SEM of at least four independent samples per group. n.s., non-significant.

We previously described the essential functions of TAM receptors in limiting cytokine production and in regulating the magnitude of the overall immune response (26). Thus, we compared the levels of activation of lamina propria macrophages in DSS-treated WT and Axl<sup>−/−</sup>Mer<sup>−/−</sup> mice. Our results show that loss of Axl and Mer signaling led to a significant increase in the production of multiple proinflammatory mediators (i.e., Nos2, IL-6, IL-17α, TNF-α, and IL-12p35), along with a reduction of negative regulators of inflammation associated with the alternative activation of macrophages (i.e., RELM-α, IL-10, and TGFB-β) by lamina propria macrophages (Fig. 5E). These results suggest that the increased proinflammatory response and a lack of alternative activation in radiosensitive lamina propria macrophages account for the increased colitis in Axl<sup>−/−</sup>Mer<sup>−/−</sup> mice.

**Discussion**

Here we identify a paradoxical function of the proto-oncogenes Axl and Mer RTKs in preventing excessive colonic inflammation and inhibiting inflammation-associated colorectal cancer. These RTKs are well-defined proto-oncogenes in various types of cancer, with established molecular functions in promoting cancer hallmarks. Based on the association of Axl and Mer with cancer and their suitability as RTKs for therapeutic targeting with small-
molecule ATP-competitive inhibitors and biologics, various Axl and Mer inhibitors are currently in preclinical development (4, 5, 16). Our results indicate that the systemic targeting of Axl and Mer entails potential pitfalls and may actually increase the risk of inflammation-associated cancer.

The role of Axl and Mer in preventing inflammation-associated colorectal cancer is consistent with their role in limiting inflammation (26). Other genes are known to exert paradoxical effects in tumorigenesis and tumor progression; for example, the deubiquitylation and ubiquitin editing enzyme A20 functions as an oncogene in a number of solid tumors, yet A20 loss of function deubiquitylates and ubiquitin editing enzyme A20 functions as a tumor suppressor (37). Recent data reported by Merad et al. (41) show a substantial turnover of tissue-resident macrophages in the lung, peritoneum, and BM, suggesting that these macrophages locally self-maintain and donor CD45.2 macrophages from the lamina propria of WT (open bar) and Axl−/−Mer−− (black bar) mice before (day 0), during the course of DSS treatment (days 3 and 7), and 2 d after the completion of DSS treatment (day 14) as detected by qPCR. Lamina propria macrophages isolated from Axl−/−Mer−− mice were used as internal negative controls. (E) Expression of the indicated genes in sorted macrophages from the lamina propria of WT and Axl−/−Mer−− mice during DSS treatment, as detected by qPCR. Shown are mRNA levels for Nos2, IL-17, TGF-β, and IL-10 in the F4/80+CD11b− population after 7 d of DSS treatment, along with mRNA levels for IL-6, TNF-α, IL-12, and RELM-α on F480+CD11b+ population at 2 d after the completion of DSS treatment (day 14). Data are presented as representative images or as mean ± SEM of at least four independent samples per group. *P < 0.05; **P < 0.01.

Axl and Mer function in a radioreistant macrophage population in the colon appears to be essential in preventing enhanced colitis. Remarkably, Mer has been recently defined as a distinctive and universal marker of mature, tissue-resident macrophages (38). It would be interesting to explore whether the immunoregulatory properties of TAM signaling described here for lamina propria macrophages extend to other tissue-resident macrophage populations. Similar to intestinal Axl+Mer+ macrophages, tissue macrophages in the liver, epidermis, and microglia have been shown to express high levels of F4/80 (38). In addition, F4/80+ (high) tissue macrophages originate from the yolk sac and arise independently of the monocytic lineage (39, 40). Recent data reported by Merad et al. (41) show a substantial turnover of tissue-resident macrophages in the lung, peritoneum, and BM, suggesting that these macrophages locally self-maintain in the steady state with only a minimal contribution from monocytes. Furthermore, expansion of pleural resident macrophages in situ, rather than the recruitment from the blood, has been proposed as a signature of innate mechanisms of inflammation in pathological settings, such as Litomosoides sigmodontis infection (42). The investigation of whether Axl+Mer+ lamina propria macrophages originate from yolk sac precursors and the identification of mechanisms that control their induction represent exciting areas of future research.

Our data also suggest that Axl and Mer are not required for the differentiation or proliferation of intestinal macrophages, as demonstrated by the lack of significant differences in the frequency of lamina propria macrophages or the expression of F4/80 and CD11b between WT and Axl−/−Mer−− mice (Fig. 5D). The investigation of whether Axl+Mer+ lamina propria macrophages originate from yolk sac precursors and the identification of mechanisms that control their induction represent exciting areas of future research.

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Targeting of RTKs may be associated with “on-target toxicity.” For example, the anticancer use of the ERBB2 inhibitor trastuzumab can result in cardiotoxicity (46). However, recently described approaches allow active targeting of kinase inhibitors to specific organs and cells by conjugating these molecules to targeting ligands. For example, conjugation of the VEGF inhibitor to a cyclic arginine-glycine-aspartic acid peptide targets...
this compound to the tumor vasculature (47). Emphasis on developing therapeutic approaches that specifically target Axl and Mer signaling within tumor cells and spare the protective anti-inflammatory function of these RTKs may improve Axl and Mer efficacy as an anticancer target. Future studies focused on unraveling the divergent molecular functions of Axl and Mer signaling pathways in specific cell types are of fundamental importance for determining the suitability of therapeutic targeting of these kinases in cancer models.

Materials and Methods

Experimental Animals. Axl<sup>−/−</sup> and Mer<sup>−/−</sup> mice have been described previously (48). B6SJL-Ptprc<sup>Pepc<sup>Bo</sup></sup>/Boyj (CD45.1) mice were obtained from Jackson Laboratory. All mice were bred at Yale University's animal facility, were specific pathogen-free, maintained under a strict 12:12-h light cycle (lights on at 7:00 AM and off at 7:00 PM), and given a regular chow diet. In all of the experiments, age-matched WT and KO mice at age 4–6 wk were housed for a minimum of 2 wk. All experimental procedures were approved by Yale University’s Institutional Animal Use and Care Committee.

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Supporting Information

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SI Materials and Methods

Colonooscopy Procedures. Continuous monitoring of colitis and tumorgenesis was done using the Coloview high-resolution mouse endoscopic system (Karl Storz). In azoxymethane (AOM)-dextran sulfate sodium (DSS) experiments, tumors observed during endoscopy were counted to obtain the overall number of lesions. Tumor size was graded using the protocol described by Becker et al. (1) as grade 1, very small but detectable tumor; grade 2, tumor covering up to one-eighth of the colonic circumference; grade 3, tumor covering up to one-quarter of the colonic circumference; grade 4, tumor covering up to one-half of the colonic circumference; or grade 5, tumor covering one-more than half of the colonic circumference. The total tumor score per mouse was calculated as sum of all tumor sizes. In DSS experiments, colitis was scored as 1–3 for each of the following parameters: granularity of mucosal surface, stool consistency, vascular pattern, translucency of the colon, and fibrin. The colonoscopy scores were assigned by a scientist blinded to the experimental conditions.

Isolation of Colonic Lamina Propria Leukocytes. For isolation of colonic lamina propria leukocytes, entire colons from each group were longitudinally cut and washed to remove feces and debris. Colon pieces were incubated in HBSS containing 5 mM EDTA and 2% FBS at 37 °C for 25 min on a shaking platform. After removal of EDTA by three washes in PBS, the colon pieces were finely minced and then incubated in digestion media containing HBSS, 1 mg/mL collagenase VIII (Sigma-Aldrich), 0.01 mg/mL DNase I (Roche), and 1 M βME (Sigma-Aldrich) for 45 min at 37 °C on a shaking platform. After collagenase digestion, the medium containing the mononuclear cells was collected, filtered, and centrifuged at 300 × g for 5 min. The resulting cells were then used for flow cytometry analysis, cell sorting, and RNA extraction.

Histopathological Analysis. Colon sections were harvested and fixed by immersion in Bouin’s fixative (Rica Chemical), and then processed, embedded, sectioned, and stained with H&E following routine methods. Each section was evaluated using a semi-quantitative criterion-based method and scored as 0–5, as described previously (2). The histopathological scores were assigned by a pathologist blinded to the experimental manipulation.

Immunofluorescence. For immunofluorescence analysis, the intact colon was isolated, opened longitudinally, rinsed with PBS, and prepared as a colon roll. For TUNEL immunostaining, the colon was then fixed in 1% paraformaldehyde, immersed sequentially in 10%–20%–30% sucrose, embedded in optimal cutting temperature (OCT), and frozen in isopentane cooled with liquid nitrogen. Colon serial sections (7 μm thick) were blocked with BSA 5% and Triton 0.1% in PBS before incubation with the TUNEL reaction mixture (Roche), according to the manufacturer’s instructions.

For Mer immunostaining, serial sections (7 μm thick) of isopentane-frozen colons were fixed in PFA 4% for 10 min at room temperature, then blocked in BSA 5% and Triton 0.2% in PBS for 1 h at room temperature. Sections were stained with anti-PE-conjugated CD11b (eBiosciences) and rabbit anti-Mer antibody (3) diluted 1:50 in blocking buffer and incubated overnight at 4 °C, followed by secondary staining with FITC-conjugated donkey α-rabbit IgG (Biolegend). Specimens were counterstained with Hoechst 33342 (Molecular Probes) and analyzed with a PerkinElmer Confocal-UltraVIEW screening microscope.

Generation of Bone Marrow Chimeras. Bone marrow (BM) chimeras were generated by lethally irradiating (two exposures to 300 rads, 3 h apart) B6.SJL-Ptprc+Pepc−/−BoyJ hosts using an X-RAD 320 irradiation system (Precision X-Ray). Irradiated mice were reconstituted by retro-orbital injection of 5 × 10^6 BM cells previously flushed from femurs, subjected to red blood cell lysis, and filtered through a 70-μm filter. Chimeras were maintained on water containing antibiotics (Sulfadimethoxine; Roche) during the 2 wk after the BM transplantation. Over an 8-wk period, 90–95% of the donor BM cells completely repopulated the peripheral lymphoid system. Reconstitution was determined by flow cytometry analysis performed on peripheral blood mononuclear cells.

Flow Cytometry Analysis. Lamina propria leukocytes were stained with surface-conjugated antibodies against CD45 (clone 30-F11; Biolegend), CD11b (clone M1/70; eBiosciences), F4/80 (clone BM8; Biolegend), CD11c (clone N418; Biolegend) CD4 (clone RM4-5; Biolegend), CD8 (clone 53-6.7; Biolegend), CD3 (clone 145-2C11; Biolegend) Ly6G (clone 1A8; Biolegend), B220 (clone RA3-6B2; Biolegend), Siglec-F (clone E50-2440; BD Biosciences), CD117 (clone 2B8; Biolegend), and Mer (clone 108928; R&D Systems) for 30 min at 4 °C, or stained for TUNEL detection (Roche) according to the manufacturer’s instructions. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

IL-17α and IFN-γ were measured by intracellular staining on lamina propria leukocytes after stimulation in vitro for 4 h with phorbol 12-myristate 13-acetate and ionomycin. Cells were incubated with Golgi Plug (BD Biosciences) during the last 2 h of stimulation. Intracellular staining was performed with PE-conjugated anti-IL-17α antibody (clone eBio17B7; eBiosciences) and FITC-conjugated anti-IFN-γ (clone XMG1.2; eBiosciences) following the manufacturer’s protocol.

The reconstitution of BM was determined by flow cytometry analysis at 8 wk after BM transplantation. Peripheral blood mononuclear cells obtained from all mice that received a BM transplant were stained using anti-CD45.1 (clone A20; Biolegend) and anti-CD45.2 (clone 104; Biolegend).

BM-Derived Macrophage Preparation. BM-derived macrophages (BMDMs) were generated from BM progenitor cells flushed from mouse femurs and tibias. Progenitor cells were incubated on 10-cm cell culture dishes at a concentration of 5 × 10^5 cells/mL in complete media containing 50% RPMI, 20% FBS, and 30% L929-conditioned media as the source of macrophage colony-stimulating factor. Macrophages were lifted on day 6 using ice-cold PBS and replated onto 12-well cell culture plates with 1.3 × 10^6 cells per well in complete media containing 65% RPMI, 15% FBS, and 20% L929 supernatant. BMDMs were subsequently used in phagocytosis assays on day 7 of in vitro differentiation.

Neutrophil Isolation and Apoptosis. Neutrophils were collected from BM cell suspensions using the EasySep Mouse Neutrophil Enrichment Kit (StemCell Technologies) to isolate neutrophils by negative selection. Biotinylated antibodies targeted CD4, CD5, CD11c, CD45R/B220, CD49b, CD117, TER119, and F4/80. After incubation with secondary antibodies bound to magnetic particles, cells were separated with EasySep magnets. Neutrophil purity was >90% as determined by CD11b and Ly6G staining. Neutrophils were aged for 20–24 h in complete media containing 90% RPMI and 10% FBS, and labeled with 0.5 μM CellTracker.
Green CMFDA (Molecular Probes; Invitrogen). Apoptosis was verified by annexin V and propidium iodide staining.

**Phagocytosis Assays.** CellTracker-labeled apoptotic neutrophils and unlabeled BMDMs were cocultured at a 5:1 ratio. After 1 h, cells were washed and lifted using ice-cold PBS. Macrophages were then labeled with F4/80 antibodies. Macrophages with labeled apoptotic cargo were identified by CellTracker and F4/80 double-positive events using a FACSCalibur flow cytometer (BD Biosciences). Phagocytosis, not extracellular binding, was verified by coculturing macrophages with apoptotic cells at 4 °C or in the presence of 1 μM cytochalasin D to inhibit actin polymerization.

**Quantitative Real-Time PCR.** At the indicated time points, cells were harvested and washed, and RNA was isolated using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Reverse transcription was performed with RT SuperScript III (Invitrogen) or the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qPCR) reactions were performed on a Stratagene MX3000 system using a KAPA SYBR Fast qPCR Kit (Kapa Biosystems). The reactions were normalized to housekeeping gene, and the specificity of the amplified products was verified by dissociation curves. The following primers were used: for IFN-γ: forward, GGATGCATTCATGAGTATTGC; reverse, CCTTTTCCGCTTCCTGAGG; for IL-6: forward, TACCACCTTCAGAGTCGGAGGC; reverse, CTGCAAGTGCA-TCATCGTTGTTC; for IL12p35: forward, ACGAGAGTTGCTTGACTAG; reverse, CCTCATAGTGTACCAAGG-CAC; for IL-17α: forward, ATCCCCTAAAGCTCAGCGT; reverse, GGGTCTTCATGGGTTGAGAG; for Nos2: forward, CCCTTCCGAAGTTCTGGCAGCAGC; reverse, GGCTGTCAGAGCCTCGTGGCTTTGG; for GAPDH: forward, TCCA-CTCTCCACCTTCTGA; reverse, AGTGGGATAGGGCCTC-TCTC; for Axl: forward, ATGCCAGTCAAGTGATGGCT; reverse, CACACATCGCTTTGCTGTG; for Mer: forward, GTGATTACGCACCGCTCAGAC; reverse, GCCGAGGATGTAACATAGAC; for RELMα: forward, CCAATTCCAGCTAACTATCCCTCC; reverse, CCAGTCAACGATGACAGCACAG; for TNF-α: forward, CCCTCACACTCAGATCATCTTCT; reverse, GCTACGACGTGGGCTACAG; for TGF-β: forward, TGATACGCCTGAGTGGCTGTCT; reverse, CAC-AAGAGCAGTGACGCTGAA; for IL-10: forward, CGGAAGACAAATAACTGCACCC; reverse, CGGTAGCAGTAGATG-CTTCCAGAC.

**Statistical Analysis.** Differences in mean values of the experimental groups were analyzed with the two-tailed Student t test or two-way ANOVA, using GraphPad Prism. The Bonferroni multiple comparison test was used after two-way ANOVA. A P value ≤ 0.05 was considered significant.


Fig. S1. Frequency (Left) and absolute number (Right) of F4/80+CD11b+ colonic lamina propria macrophages per colon assessed in WT and Axl−/−Mer−/− mice at steady state. Results represent mean ± SEM of seven mice per group. n.s., not significant.
Fig. S2. Histograms showing the expression of Mer receptor on the different macrophage populations isolated from colonic lamina propria of WT mice (black histograms) at steady state (day 0) or after treatment with DSS for 3 d (day 3), 7 d (day 7), or 12 d followed by 2 d of normal water (day 14). Macrophages isolated from colonic lamina propria of Axl^{-/-}Mer^{-/-} mice were used as negative controls (shaded histograms). The histograms are representative of at least three independent experiments per time point.
Fig. S3. (A) WT and Axl−/−Mer−/− mice were fed 1.5% DSS in the drinking water for 7 d, after which cells from colonic lamina propria were isolated and analyzed for Mer expression. Representative dot plots show the gating strategy on live CD45+ cells. Histograms show Mer expression in the selected populations from WT mice (red), with Axl−/−Mer−/− samples used as negative controls (black). (B) Expression of Axl in the indicated cell-sorted populations from tumor-free regions of the lamina propria of WT mice after the completion of AOM-DSS treatment, as detected by qPCR. Cells sorted from Axl−/−Mer−/− mice were used as an internal negative control.

Fig. S4. (Upper) (Left) Mer (green) expression in the colon of WT mice fed 1.5% DSS in drinking water for 7 d. (Right) Magnified view showing colocalization of Mer staining in CD11b+ cells (arrows). (Lower) Colon sections from Axl−/−Mer−/− mice used as negative controls. (Scale bar: 100 μm.)