

# CXCR2-Expressing Myeloid-Derived Suppressor Cells Are Essential to Promote Colitis-Associated Tumorigenesis

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<http://dx.doi.org/10.1016/j.ccr.2013.10.009>

## SUMMARY

A large body of evidence indicates that chronic inflammation is one of several key risk factors for cancer initiation, progression, and metastasis. However, the underlying mechanisms responsible for the contribution of inflammation and inflammatory mediators to cancer remain elusive. Here, we present genetic evidence that loss of CXCR2 dramatically suppresses chronic colonic inflammation and colitis-associated tumorigenesis through inhibiting infiltration of myeloid-derived suppressor cells (MDSCs) into colonic mucosa and tumors in a mouse model of colitis-associated cancer. CXCR2 ligands were elevated in inflamed colonic mucosa and tumors and induced MDSC chemotaxis. Adoptive transfer of wild-type MDSCs into *Cxcr2*<sup>-/-</sup> mice restored AOM/DSS-induced tumor progression. MDSCs accelerated tumor growth by inhibiting CD8<sup>+</sup> T cell cytotoxic activity.

## INTRODUCTION

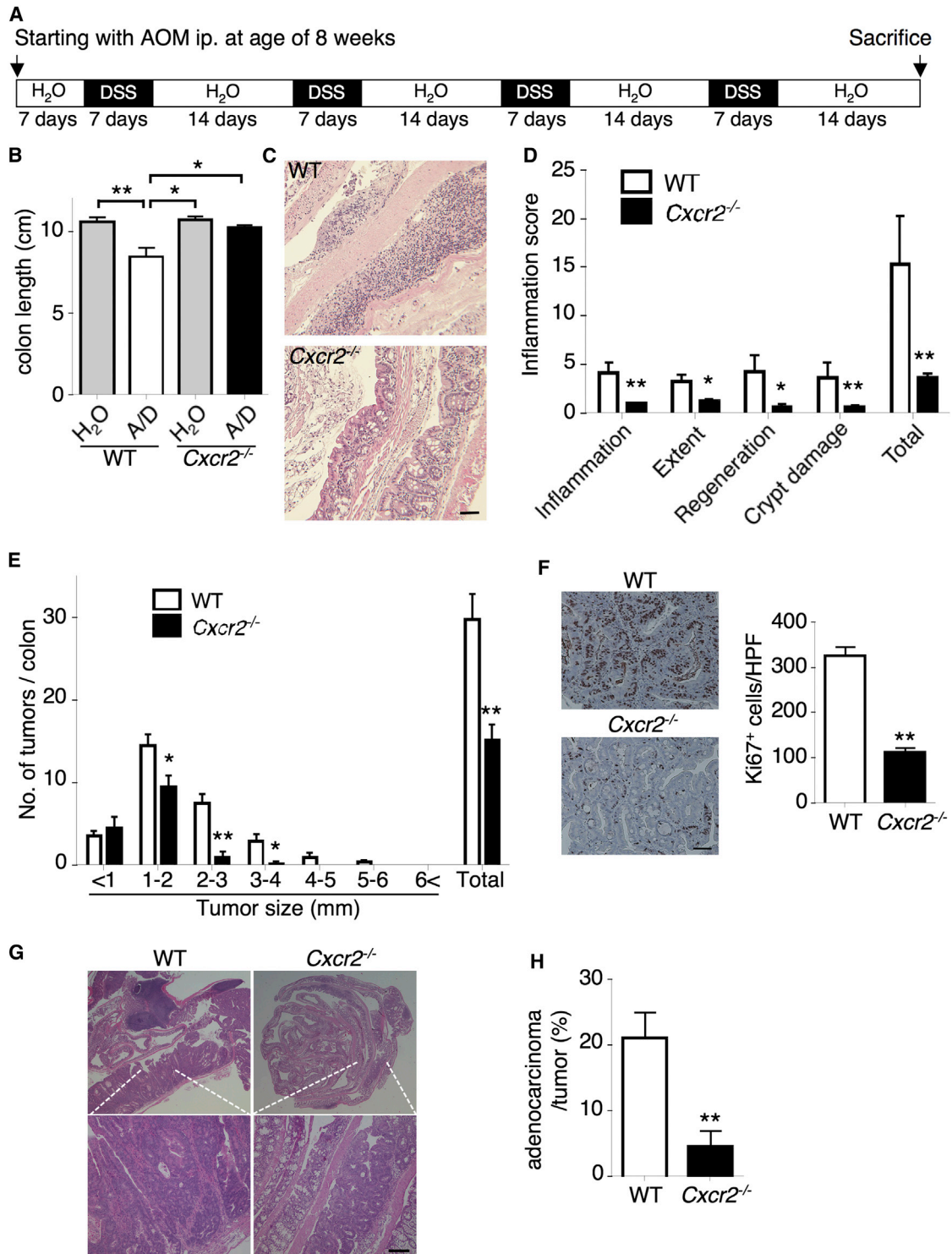
Colorectal cancer (CRC) is the fourth most common malignant neoplasm and the second leading cause of cancer deaths in the United States. Although colonoscopy screening is an effective way to detect and prevent CRC by removing precancerous adenomas (Zauber et al., 2012), 70% of patients with CRC present to their physician with advanced disease, resulting in an unacceptable 5 year survival rate (Yamashita and Watanabe, 2009). CRC includes hereditary, sporadic, and colitis-associated CRC. In addition to somatic mutations and epigenetic changes, epidemiologic and experimental evidence strongly implicates chronic inflammatory stimuli as a risk factor for developing CRC. Indeed, ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is associated with an increased risk for the development of CRC (Ekbom et al., 1990). More than 20% of patients with UC are reported to develop colitis-associated

CRC within 30 years of diagnosis (Lakatos and Lakatos, 2008). Colitis-associated cancer often shows rapid progression, with poor response to treatment and high mortality (Feagins et al., 2009). Since there is a strong association between chronic inflammation and CRC in IBD patients, studies on colitis-associated CRC provides a “proof of concept” model to better understand how chronic inflammation and certain inflammatory mediators promote tumor initiation, growth, and metastasis.

Chronic inflammation is caused by a persistently heightened immune response following injury or exposure to foreign pathogens. For example, disruption of immune homeostasis in the intestine in response to the gut flora, which contains foreign luminal antigens from food and commensal bacteria, can result in the development of IBD. The importance of flora for IBD is evident by the observations that antibiotic treatment and/or probiotic therapy have been shown to be benefits for, at least, subsets of IBD patients (Gionchetti et al., 2003; Sutherland

### Significance

Until now, CXCR2 was only thought to mediate infiltration of neutrophils to inflammatory sites. Our results show that CXCR2 is also required for homing of MDSCs into colonic mucosa and colitis-associated tumors, revealing a role of CXCR2 in the recruitment of MDSCs from the circulatory system to local tissues and tumors. Importantly, our results from experiments of adoptive transfer of MDSCs provide direct evidence that MDSCs contribute to colonic tumor formation and growth. Moreover, we also found that colonic MDSCs inhibited CD8<sup>+</sup> T cell cytotoxicity against tumor cells. Our findings provide a rationale for the development of therapeutic approaches to subvert chronic inflammation- and tumor-induced immunosuppression by using CXCR2 antagonists and neutralizing antibodies.



**Figure 1. Deletion of *Cxcr2* Attenuates AOM/DSS-Induced Colonic Chronic Inflammation and Colitis-Associated Tumor Formation, Growth, and Progression**

(A) Schematic of mice treated with AOM and DSS (A/D).

(B) The colon lengths were measured following treatment.

(C) Representative of hematoxylin and eosin (H&E)-stained sections from WT (top) and *Cxcr2* null mice (bottom). Scale bar, 100  $\mu$ m.

(D) Blinded histological scoring of inflammation in colonic mucosa of mice was performed as described in the [Experimental Procedures](#).

(E) Tumor number and size were measured under a dissecting microscope.

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et al., 1991). Direct evidence for the role of luminal flora came from animal studies showing that chronic colitis is dependent on their presence (Elson et al., 2005). Antibiotic treatment and/or probiotic therapy attenuated colon chronic inflammation in different mouse models of IBD, including dextran sulfate sodium (DSS)-treated mice (Garrido-Mesa et al., 2011a, 2011b). In a mouse model of colitis-associated cancer, germ-free azoxymethane (AOM)-treated *Il10*<sup>-/-</sup> mice exhibited normal colon histology and did not develop colon tumors (Uronis et al., 2009). Even in a mouse model of hereditary and sporadic CRC, antibiotic treatment reduced tumor burden, indicating that the luminal bacteria contributes to tumor growth (Grivennikov et al., 2012). Of note, several in vivo studies showed that pathogenic bacteria from gut flora induced expression of the inflammatory enzyme cyclooxygenase 2 (COX-2) in inflamed colonic mucosa (Abdallah Hajj Hussein et al., 2012; Cho and Chae, 2004; Lee and Kim, 2011). The levels of COX-2 and COX-2-derived prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) are known to be markedly elevated in the gastrointestinal tracts of patients with IBD (Lauritsen et al., 1986; Singer et al., 1998).

The main pathological feature of IBD involves a massive infiltration of neutrophils, lymphocytes, and monocytes into the inflamed intestinal tissue. Similarly, the common pathological changes associated with colitis-associated and sporadic CRC include recruitment and reprogramming of various types of dysregulated immune cells and endothelial cells to establish a tumor microenvironment (Coussens and Werb, 2002; Strober et al., 2007). Chemokines that recruit leukocytes from the circulatory system to local sites of inflammation have emerged as essential immune molecules in the pathogenesis of IBD and CRC. Chemokines exert their biological functions via binding to their cognate G protein-coupled receptors. Elevation of pro-inflammatory chemokines and a massive infiltration of leukocytes are all observed in the intestinal mucosa of IBD patients and strongly correlate with the grade of disease activity (Fegn and Wang, 2009). Moreover, the levels of these pro-inflammatory chemokines are also higher in human sporadic colorectal carcinomas than in matched normal tissues (Fegn and Wang, 2009). However, it remains unclear how these chemokines and their receptors contribute to IBD and colitis-associated carcinogenesis.

Cancer initiation and progression also depends on escape from host immunosurveillance. Similar to other solid tumors, CRC immune evasion involves a shift of immune responses, including imbalance in Th1/Th2 responses and enhancement of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells. The number of MDSCs in the blood correlates well with clinical cancer stage and metastatic tumor burden in patients, including those with CRC (Diaz-Montero et al., 2009; Mandruzzato et al., 2009). It is widely accepted that MDSCs contribute to cancer immune evasion via suppressing functions of T and natural killer (NK) cells (Gabrilovich and Nagaraj, 2009). However, it remains

unclear how MDSCs are recruited from the circulatory system to the colonic mucosa during chronic inflammation and carcinogenesis and precisely how local MDSCs contribute to CRC progression.

Our previous work showed that PGE<sub>2</sub> directly stimulated CRC cells to produce and secrete CXCL1, which in turn resulted in induction of tumor-associated angiogenesis (Wang et al., 2006b). CXCL1 is one of the ligands that binds to the chemokine receptor CXCR2, which was originally found to be expressed on neutrophils and is crucial for the recruitment of neutrophils to sites of inflammation (Oppenheim et al., 1991). The serum level of CXCL1 is significantly elevated in patients with IBD (Alzoughaibi et al., 2008). Importantly, CXCL1 levels also correlate well with the grade of disease and are reduced after initiation of therapy (Mitsuyama et al., 2006). Similarly, our group and others showed that CXCL1 and its receptor CXCR2 are elevated in human sporadic CRC (Rubie et al., 2008; Wang et al., 2006b; Wen et al., 2006). However, the mechanisms underlying the contribution of this signaling pathway to inflammatory disease and CRC remain elusive.

## RESULTS

### Deletion of *Cxcr2* Attenuates AOM/DSS-Induced Colonic Chronic Inflammation and Colitis-Associated Tumorigenesis

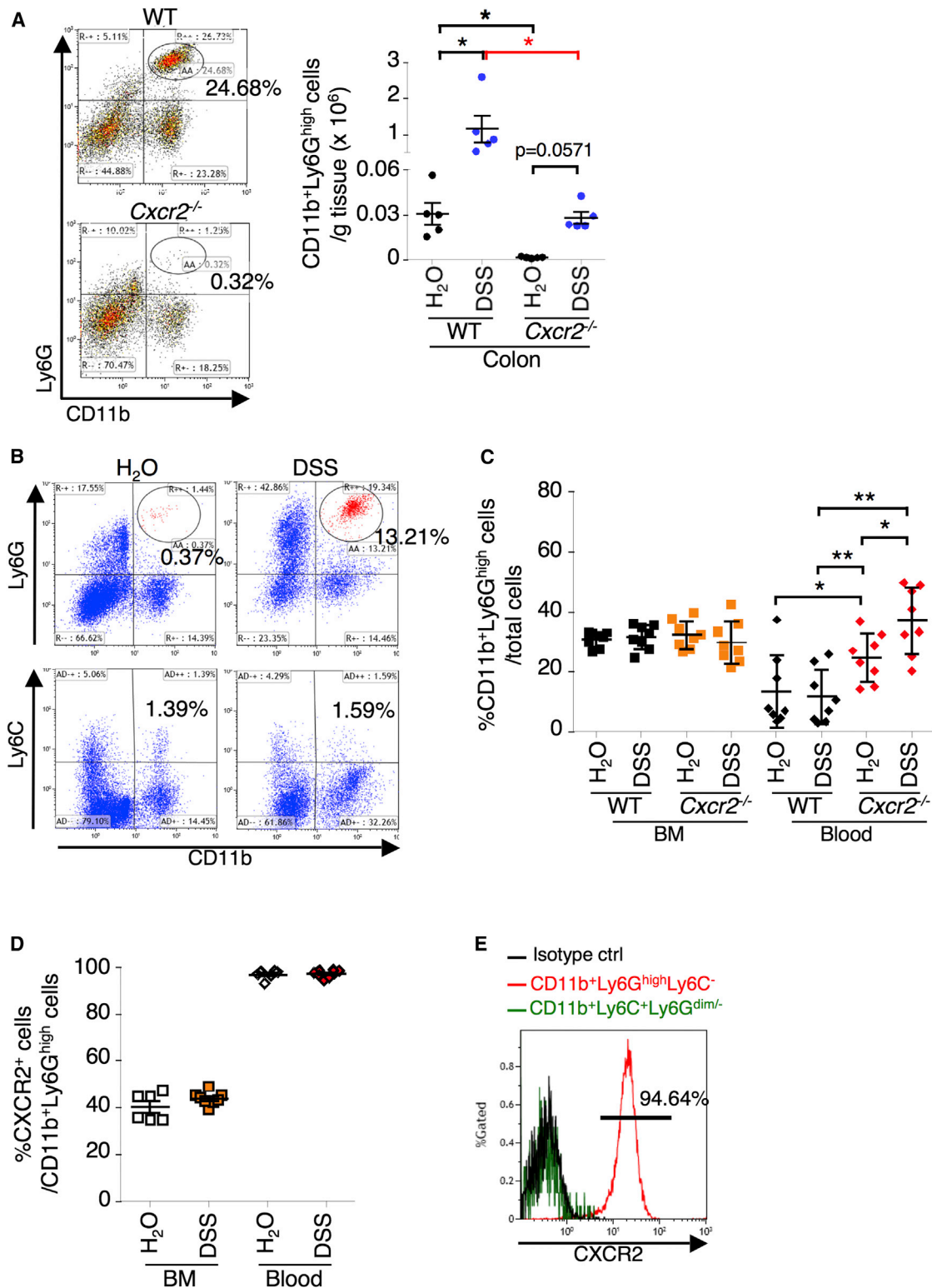
To investigate the role of CXCR2 in colitis-associated tumorigenesis, wild-type (WT) or *Cxcr2*<sup>-/-</sup> mice were treated with AOM and DSS as indicated in Figure 1A. AOM/DSS treatment induced inflammation in the large intestine of WT mice, resulting in clear clinical signs such as bloody stools (data not shown) and shortening of colon length (Figure 1B) but did not significantly affect mouse body weight (data not shown). In contrast, deletion of *Cxcr2* significantly attenuated the presence of inflammation-induced bloody stools ( $p = 0.041$ ) and shortening of the colon (Figure 1B). Moreover, repeated administration of DSS induced chronic inflammation in WT mice but not in *Cxcr2*<sup>-/-</sup> mice (Figures 1C and 1D). Water-treated WT and *Cxcr2*-deficient mice showed no clinical and histologic signs of chronic colonic inflammation (data not shown). In addition, loss of *Cxcr2* dramatically reduced tumor burden by inhibition of tumor cell proliferation (Figures 1E and 1F). The severity of chronic inflammation directly correlated with tumor multiplicity (data not shown). Histological analysis revealed that 21% of tumors were adenocarcinomas in AOM/DSS-treated WT mice, whereas only 4.6% were adenocarcinomas in AOM/DSS-treated *Cxcr2*-deficient mice (Figures 1G and 1H), suggesting that the presence of CXCR2 accelerates tumor progression. However, we did not observe submucosal invasion of tumor cells in our model. These results demonstrate that CXCR2 is required for promoting chronic inflammation, tumor formation, growth, and progression in the large intestine.

(F) Left panel represents immunoreactive staining (brown) for Ki67 (scale bar, 50  $\mu$ m) and the right panel represents the average numbers of Ki67<sup>+</sup> cells in 4 fields of each slides from 8 mice for each group.

(G) Representative of H&E-stained sections of colonic tissue with tumors from WT and *Cxcr2* null mice are shown (scale bar, 100  $\mu$ m).

(H) Blinded histological scoring of average percentage of adenocarcinomas in total tumors from WT and *Cxcr2*-deficient mice.

Data are represented as mean  $\pm$  SEM (eight mice for each group). Asterisks represent statistical differences (\* $p < 0.05$ , \*\* $p < 0.01$ ).



**Figure 2. Loss of CXCR2 Inhibits DSS-Induced a Massive Infiltration of MDSCs from Circulatory System to Colon**

The indicated genotypic mice aged 8 weeks were treated with four cycles of 1.25% DSS and the cells isolated from indicated organs were subjected to flow cytometry analysis. Viable granulocytes/monocytes or total cells were gated in a FSC/SSC plot.

(A) The subpopulation of G-MDSCs in colonic mucosa was represented as percentage of gated granulocytes/monocytes cells (left) or as the numbers of G-MDSCs per gram of each mouse colon tissue (right). Each dot in the right panel represents the numbers of G-MDSCs in colonic mucosa taken from one mouse.

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### Loss of CXCR2 Diminishes DSS- or AOM/DSS-Induced Massive Infiltration of Granulocytic MDSCs from the Circulatory System to Colonic Inflamed Mucosa and Tumors

To examine whether CXCR2 is involved in immune cell infiltration, we first analyzed immunocyte profiles in the colonic mucosa of mice treated with DSS. We used cell surface markers as described in recent publications (Daley et al., 2008; Gabrilovich et al., 2012) to define mouse dendritic cells (DCs), macrophages, MDSCs, and neutrophils. Deletion of *Cxcr2* did not affect repeated DSS-induced infiltration of dendritic cells (CD11c<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>-</sup>), T cells (CD3<sup>+</sup>), NK cells (CD3<sup>+</sup>CD49b<sup>+</sup>), and NKT cells (CD3<sup>+</sup>CD49b<sup>+</sup>) into the colonic mucosa but resulted in a trend toward reduction of infiltration of macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>-</sup>Ly6G<sup>-</sup>) and neutrophils (Ly6G<sup>+</sup>CD11c<sup>-</sup>F4/80<sup>-</sup>) during the chronic phase (Figure S1A available online), although alteration of CXCR2 clearly affected infiltration of neutrophils in the acute phase (Figure S1B). In contrast, loss of CXCR2 dramatically suppressed a massive infiltration of CD11b<sup>+</sup>Ly6G<sup>high</sup>CD11c<sup>-</sup>F4/80<sup>-</sup> cells into the colon in both normal (water as control) and chronic inflammatory (DSS treatment) conditions (Figure 2A). In mice, the immature myeloid cells, often called MDSCs, are broadly defined as CD11b<sup>+</sup>Gr-1<sup>+</sup> cells that are further divided into two subsets, granulocytic (CD11b<sup>+</sup>Ly6G<sup>high</sup>) and monocytic (CD11b<sup>+</sup>Ly6C<sup>+</sup>) MDSCs (Youn et al., 2008). In most experimental tumor models, granulocytic MDSCs (G-MDSCs) are more markedly expanded than monocytic MDSCs (Youn et al., 2008). Similarly, over 90% of MDSCs expressed both CD11b and Ly6G markers but not Ly6C, whereas less than 10% of MDSCs expressed both CD11b and Ly6C but not Ly6G in DSS-treated mice (Figure 2B). Furthermore, we found that the status of CXCR2 did not affect the G-MDSC populations in the bone marrow in both normal and chronic inflammatory conditions (Figure 2C). Importantly, *Cxcr2*<sup>-/-</sup> mice exhibited more accumulation of the G-MDSCs in the circulatory system than WT mice under both normal and chronic inflammatory conditions (Figure 2C), suggesting that *Cxcr2*-deficient G-MDSCs have lost their ability to migrate to local organs undergoing an inflammatory challenge. Almost all circulatory G-MDSCs expressed CXCR2 on their cell surface, whereas 40% of bone marrow G-MDSCs expressed CXCR2 (Figure 2D). Surprisingly, circulating monocytic MDSCs did not express CXCR2 on their cell surface (Figure 2E). As expected, circulating CD8<sup>+</sup> T cells, NKs, NKT cells as well as colonic fibroblasts did not express CXCR2 on their cell surface (Figures S1C and S1D). However, 67% of neutrophils, 9.5% of monocytes, and 21% of DCs expressed CXCR2 in circulatory system (Figure S1C). Similarly to the circulatory system, more G-MDSCs accumulated in spleen of *Cxcr2*<sup>-/-</sup> mice than WT mice in both normal and chronic inflammatory conditions (Figure S1E). Since treatment of DSS did not induce inflammation in liver and lung, DSS treatment did not affect infiltration of MDSCs into those organs (Figure S1E).

As expected, there was no significant difference of immune cell profiles between mice treated with DSS alone and AOM plus DSS (Figures S1A and S2A). Similar to DSS-treated mice, more G-MDSCs accumulated in the circulatory system of AOM/DSS-treated *Cxcr2*<sup>-/-</sup> than WT mice (Figure 3A). However, viability of circulating *Cxcr2*-deficient MDSCs was lower than WT MDSCs (Figures S2B and S2C). More intriguingly, a higher G-MDSC accumulation was observed in tumors than matched inflammatory mucosa in WT mice treated with AOM/DSS (Figure 3B). In contrast, loss of CXCR2 significantly reduced recruitment of G-MDSCs into both tumors and the matched inflamed mucosa (Figure 3B). Similar to DSS treatment, deletion of *Cxcr2* also resulted in an accumulation of G-MDSCs in the spleen in both water- and AOM/DSS-treated mice (Figure S2D). However, the size and weight of the spleen of *Cxcr2*<sup>-/-</sup> mice was bigger and heavier than that of WT mice under both normal and chronic inflammatory conditions (Figure S2E). This could explain why the spleens of *Cxcr2*<sup>-/-</sup> mice have a higher percentage of G-MDSCs than WT mice. Taken together, these results suggest that CXCR2 is required for homing of G-MDSCs from the circulatory system to inflamed colonic mucosa and tumors.

### CXCR2 Mediates Ligand-Induced G-MDSC Chemotaxis

Based on our above results showing that treatment of AOM/DSS induced a massive infiltration of G-MDSCs into inflamed colonic mucosa and colitis-associated tumors in WT mice, we postulated that the levels of CXCR2 ligands, which are responsible for recruitment of CXCR2-expressing immune cells, would be elevated in colonic inflamed mucosa and tumors. Indeed, treatment of WT mice with AOM/DSS resulted in elevation of CXCL1, CXCL2, and CXCL5 in colonic tumors and adjacent inflamed mucosa as compared to the normal mucosa taken from water-treated mice (Figure 4A). Levels of these ligands are higher in tumor tissues than in adjacent inflamed mucosa (Figure 4A), which correlates with density of G-MDSCs in these tissues (Figure 3B). The results from in situ hybridization experiments revealed that *Cxcl1* and *Cxcl2* were mainly expressed in tumor colonic epithelial cells (Figure S3A). We then determined whether CXCR2 ligands induced MDSC chemotaxis. As shown in Figure 4B, CXCL1, CXCL2, and CXCL5 all attracted WT MDSCs isolated from blood of WT mice but not *Cxcr2*-deficient MDSCs, demonstrating that CXCR2 is required for ligand-induced MDSC chemotaxis. Since myeloid cells are a major source of interleukin-6 (IL-6) and the IL-6/Stat3 pathway plays a key role in immunosuppressive function of MDSCs (Gabrilovich and Nagaraj, 2009; Grivnickov et al., 2009), we examined IL-6 levels in the colon and blood of AOM/DSS-treated WT and *Cxcr2* null mice. As expected, the pattern of IL-6 levels in colonic mucosa and tumors as well as in circulatory system (Figure S3B) is similar to that of MDSC levels in these organs (Figure 3). p-Stat3 immunostaining was observed in colonic stromal cells in WT mice treated with AOM/DSS but not in *Cxcr2*<sup>-/-</sup> mice (Figure S3C). These results indicate that infiltrated MDSCs in the

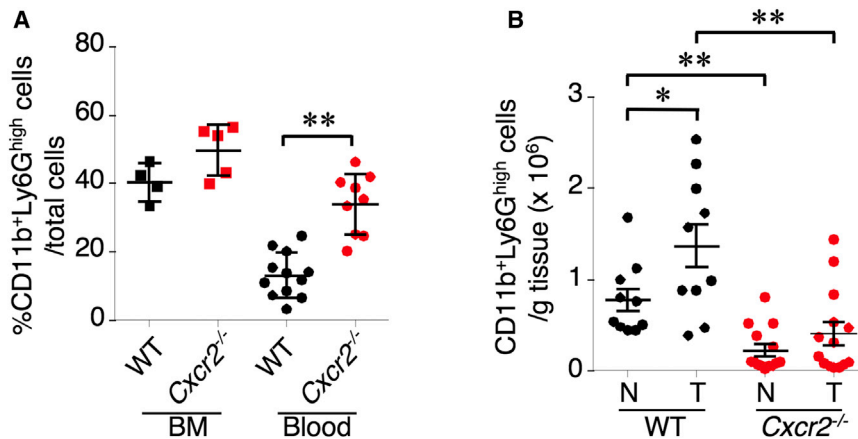
(B) The profiles of G-MDSCs and monocytic MDSCs in colonic mucosa of water- or DSS-treated WT mice as mentioned above.

(C) Data represents the percentage of G-MDSC in total viable cells from bone marrow (BM) and peripheral blood taken from mice.

(D) Data represents the percentage of CXCR2<sup>+</sup> MDSCs in total G-MDSCs from BM and blood in WT mice.

(E) The percentage of CXCR2<sup>+</sup> G-MDSCs and monocytic MDSCs in total G-MDSCs and monocytic MDSCs.

The error bar indicates  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ . See also Figure S1.



**Figure 3. Loss of CXCR2 Inhibits AOM/DSS-Induced Massive Infiltration of G-MDSCs from the Circulatory System to Colonic Mucosa and Tumors**

G-MDSC percentage in bone marrow and peripheral blood (A) and G-MDSC numbers in tumors (T) and adjacent mucosa (N) (B) of mice treated with AOM/DSS were analyzed by Flow Cytometry as described Figure 1A. Each dot represents the percentage of G-MDSCs in BM and peripheral blood collected from one mouse (left) and the numbers of G-MDSCs in colonic tumors and matched mucosa taken from one mouse (right).

The error bar indicates  $\pm$ SEM. See also Figure S2.

colon have immunosuppressive functions. Collectively, our results demonstrate that CXCL1/2/5-CXCR2 signaling is responsible for recruitment of G-MDSCs into colonic inflammatory mucosa and tumors.

#### An Inflammatory Mediator, PGE<sub>2</sub>, Upregulates CXCR2 Ligands in Colonic Mucosa and Tumors

Although our previous study demonstrated that PGE<sub>2</sub> directly induced CXCL1 expression in human colorectal carcinoma cell lines and in a xenograft model (Wang et al., 2006b), it was uncertain whether this inflammatory mediator regulates CXCR2 ligands in the DSS and AOM/DSS models as well as *Apc*<sup>Min/+</sup> mice. The *Apc*<sup>Min/+</sup> mouse carries a point mutation at one allele of the *Apc* gene and is used as a model for patients with familial adenomatous polyposis and a pre-malignant model for human sporadic CRC. As shown in Figure 5, PGE<sub>2</sub> significantly induced CXCL1 and CXCL2 expression in the colon of DSS-treated mice (Figure 5A) and in colonic tumors as well as adjacent mucosa of AOM/DSS-treated mice (Figure 5B) but failed to induce CXCL5 expression. Similarly, PGE<sub>2</sub> enhanced only CXCL1 and CXCL2 expression in colonic mucosa and tumors (Figure S4A) but all three ligands in small intestinal mucosa and tumors in *Apc*<sup>Min/+</sup> mice (Figures S4B and S4C). Interestingly, the levels of these ligands were also elevated in intestinal tumors as compared to matched mucosa regardless of PGE<sub>2</sub> treatment (Figures 5B and S4A–S4C). In contrast, treatment of *Apc*<sup>Min/+</sup> mice with a selective COX-2 inhibitor, celecoxib, completely suppressed CXCL1 and CXCL2 expression in intestinal mucosa and tumors (Figure S4D). These results demonstrate that an inflammatory mediator such as PGE<sub>2</sub> is able to induce CXCR2 ligand expression in intestinal mucosa and tumors. High levels of CXCR2 ligands attract CXCR2-expressing MDSCs into colonic mucosa and tumors.

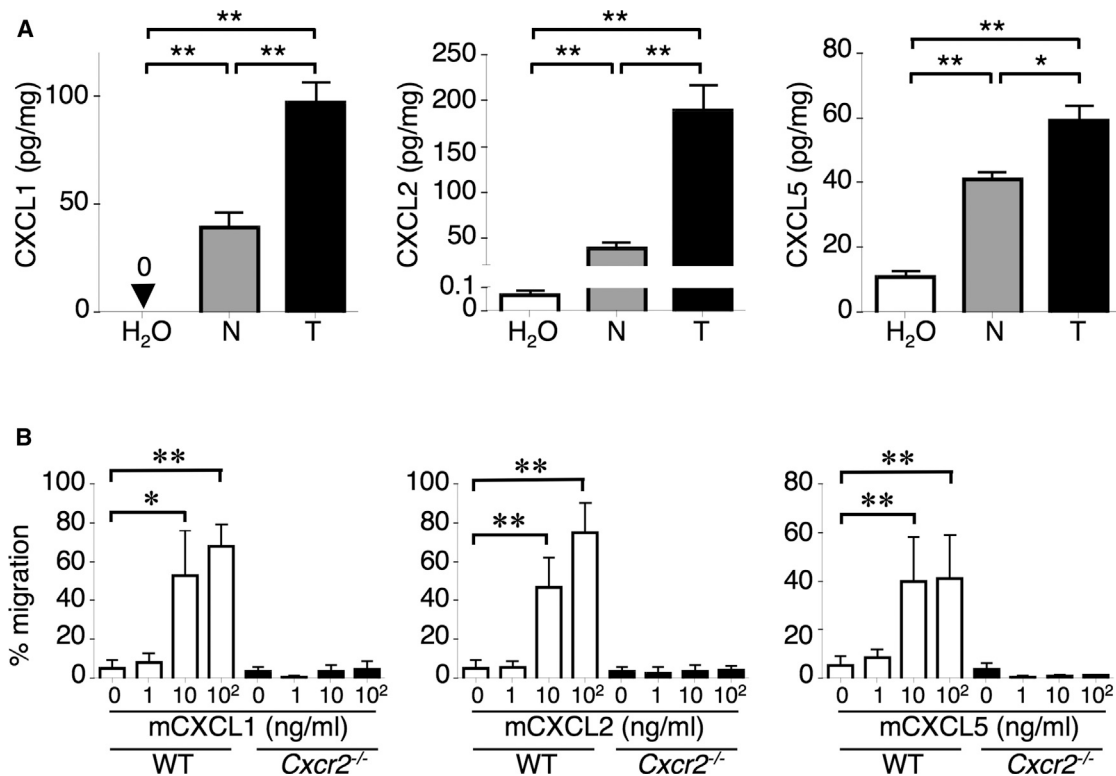
#### Adoptive Transfer of WT MDSCs Restores Tumorigenesis in *Cxcr2*<sup>-/-</sup> Mice

To determine whether lack of MDSC infiltration in colonic tissue directly results in reduction of colitis-associated tumor burden in *Cxcr2*<sup>-/-</sup> mice, adoptive transfer experiments were performed. To first examine whether the adoptive transfer of G-MDSCs works, G-MDSCs isolated from WT mice were labeled with a fluorescent dye, XenoLight DiR, and then

injected into *Cxcr2* null mice. After 2 days, 73% of colonic G-MDSCs cells were detected as DiR positive, demonstrating that these DiR<sup>+</sup> G-MDSCs cells are WT MDSCs (Figure S5A). Seventy-one percent of WT MDSCs were viable (Figure S5B). As shown in Figures 6A–6C, transfer of G-MDSCs isolated from WT mice treated with AOM/DSS to *Cxcr2* null mice restored the ability of AOM/DSS to induce tumor formation and growth in *Cxcr2*-deficient mice. Histological analysis revealed that only 2% of the tumors were adenocarcinomas in *Cxcr2*-deficient mice injected with non-MDSC immune cells or *Cxcr2*-deficient MDSCs. In contrast, 10% of the tumors were adenocarcinomas in *Cxcr2*-deficient mice injected with MDSCs, suggesting that MDSCs promotes tumor progression (Figure 6D). An increased accumulation of MDSCs into colonic mucosa in mice injected with MDSCs was observed as compared to mice injected with non-MDSC immune cells and *Cxcr2*-deficient MDSCs (Figure 6E). Moreover, immunofluorescence analysis confirmed an increased accumulation of MDSCs into colonic mucosa and tumors in the MDSC recipients (Figures S5C and S5D). These results demonstrate that CXCR2-expressing MDSCs contribute to colitis-associated tumor formation, growth, and progression.

#### MDSCs Suppress Cytotoxic Activity of Colonic CD8<sup>+</sup> T Cells

We further examined whether reduction of MDSCs in the colon of *Cxcr2*<sup>-/-</sup> mice resulted in induction of cytotoxic T cell number and cytotoxicity against tumor cells in our AOM/DSS model. As shown in Figure 7A, a great reduction in the number of CD3<sup>+</sup>CD8<sup>+</sup> T cells in tumors was observed when compared to adjacent mucosa in both WT and *Cxcr2*<sup>-/-</sup> mice. Although the status of CXCR2 did not affect the number of cytotoxic T cells in tumors, deletion of *Cxcr2* led to an increase of cytotoxic T cell number in colonic mucosa but this change did not achieve statistical significance. In contrast, colonic CD8<sup>+</sup> T cells isolated from *Cxcr2*<sup>-/-</sup> mice have higher cytotoxicity against epithelial tumor cells isolated from AOM/DSS-treated WT mice than colonic CD8<sup>+</sup> T cells isolated from WT mice (Figure 7B). Importantly, CD8<sup>+</sup> T cells isolated from tumors taken from *Cxcr2*<sup>-/-</sup> mice expressed high levels of CD107a, an activated CD8<sup>+</sup> T cell marker, and produced more IFN $\gamma$ , Prf1, and Gzmb when compared to tumor-associated CD8<sup>+</sup> T cells from



**Figure 4. CXCR2 Ligands Are Elevated in Colitis-Associated Tumor and Matched Inflamed Mucosa**

(A) CXCL1, CXCL2, and CXCL5 protein levels were measured in tumors (T) and adjacent mucosa (N) taken from mice treated with AOM/DSS as well as in normal colon tissues taken from mice fed with water only as described in Figure 1A.

(B) CXCL1, CXCL2, and CXCL5 induced chemotaxis in WT MDSCs but not in *Cxcr2*<sup>-/-</sup> MDSCs in vitro. WT and *Cxcr2*<sup>-/-</sup> MDSCs were isolated from blood of AOM/DSS-treated WT and *Cxcr2*<sup>-/-</sup> mice, respectively.

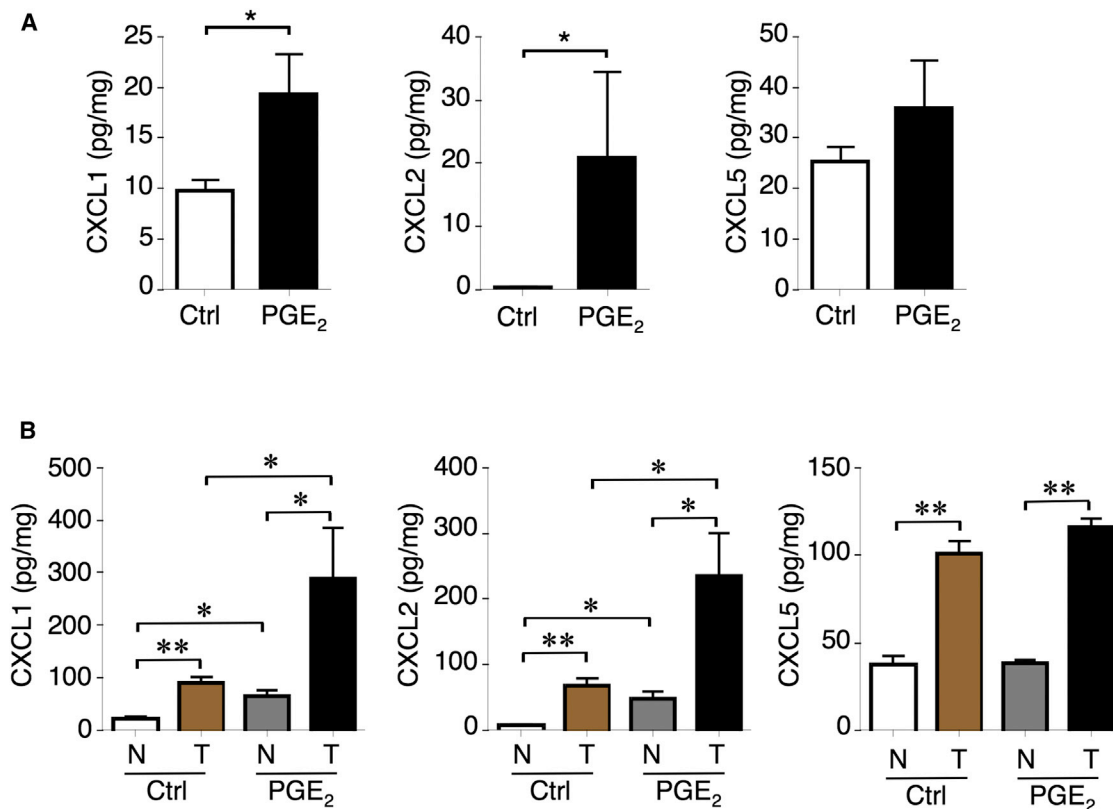
Values are reported as the mean  $\pm$  SEM (six mice for each group). \* $p < 0.05$ ; \*\* $p < 0.01$ . See also Figure S3.

WT mice (Figures S6A and S6B), indicating that activated CD8<sup>+</sup> T cells might kill tumor cells via induction of IFN $\gamma$ , Prf1, and Gzmb. Moreover, colonic CD8<sup>+</sup> T cell cytotoxicity against tumor cells was suppressed by adding circulating G-MDSCs taken from WT or *Cxcr2*<sup>-/-</sup> mice (Figure 7C). The MDSC inhibition of CD8<sup>+</sup> T cell cytotoxicity was ratio-dependent. Treatment of AOM/DSS significantly induced arginase 1 expression and activity as well as other suppressor markers such as CD80 and CD86 in both colonic WT and *Cxcr2*<sup>-/-</sup> G-MDSCs (Figures 7D and 7E) and inhibited the secretion of interferon-gamma (IFN $\gamma$ ) and IL-2 from colonic CD8<sup>+</sup> T cells in vitro (Figure 7F). Although there were 27 downregulated genes in colonic *Cxcr2*-deficient MDSCs as compared to WT MDSCs (Table S1), none of these genes (except *Cd80*) are known to be involved in MDSC immunosuppressive functions. These results demonstrate that colonic MDSCs inhibit the ability of colonic CD8<sup>+</sup> T cells to kill tumor cells and their immunosuppressive functions are CXCR2-independent. In addition, loss of CXCR2 increased the population of Th1 (CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>) cells and significantly decreased the population of Th17 (CD4<sup>+</sup>IL-17A<sup>+</sup>) cells in tumors, suggesting that CXCR2 signaling contributes to the imbalance in Th1/Th2 responses and promotes tumor-associated inflammation via enhancing Th17 cell number (Figure S6C).

## DISCUSSION

The recognition that chronic inflammation caused by infections or autoimmune diseases as an enabling characteristic of cancer has highlighted the contribution of inflamed stroma to tumor initiation, growth, progression, and metastasis. Our findings not only reveal how MDSCs are recruited to local inflamed tissues and the tumor microenvironment and how local MDSCs contribute to CRC progression, but also provide a rationale for developing therapeutic approaches to subvert chronic inflammation- and tumor-induced immunosuppression by using CXCR2 antagonists and neutralizing antibodies.

A growing body of evidence supports the important role of chemokines and their receptors in colonic inflammation and CRC (Wang et al., 2009). The levels of pro-inflammatory chemokines are positively correlated with the inflammatory state in patients with IBD (Wang et al., 2009). Genetic and pharmacologic studies provide evidence showing that the activation of pro-inflammatory CCL2, CCL3, or CCL4 signaling promotes inflammation in mouse models where injurious agents are used to induce experimental colitis (Andres et al., 2000; Khan et al., 2006; Tokuyama et al., 2005). However, there are a few genetic and pharmacologic studies showing the direct evidence that CXCR2 and CC chemokines contribute to colitis-associated cancer



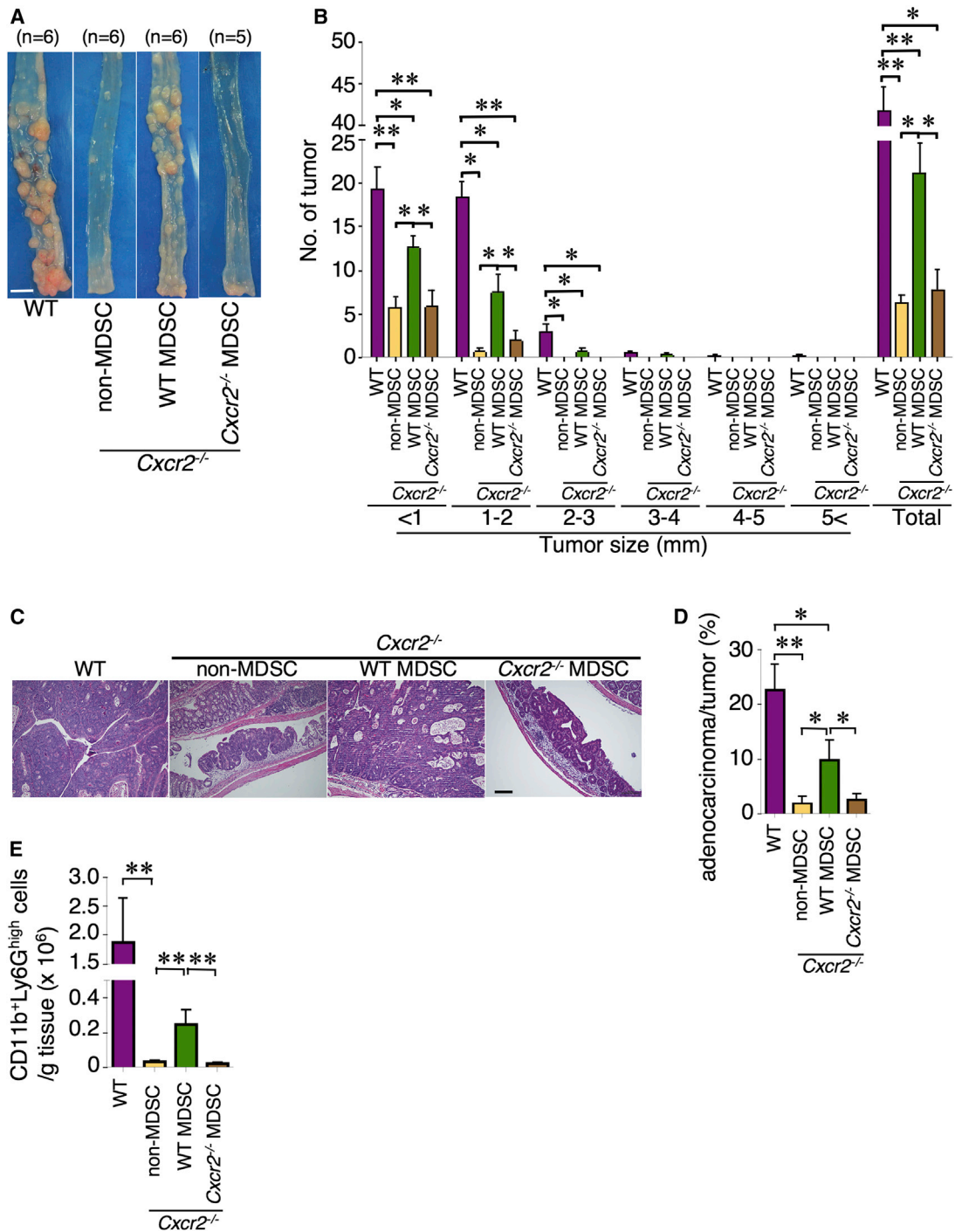
Eight-week-old male BALBc mice were treated with either 2 cycles of DSS alone (A) or AOM plus three cycles of DSS (B) with PGE<sub>2</sub> or vehicle as described in Experimental Procedures. The protein levels of CXCL1, CXCL2 and CXCL5 in colon (A) and colonic tumor (T) and matched mucosa (N) (B) were determined by ELISA.

Data are represented as mean ± SEM (five mice for each group). \*p < 0.05; \*\*p < 0.01. See also Figure S4.

in mouse models. For example, genetic and pharmacologic evidence indicates that CCL2 promotes colitis-associated tumorigenesis via recruitment of macrophages (Popivanova et al., 2009). Moreover, deletion of an atypical chemokine receptor D6, a decoy and scavenger receptor, increased susceptibility to DSS-induced colitis and AOM/DSS-induced colitis-associated tumorigenesis accompanied with an increased production of chemokines and inflammatory cell recruitment via lymphatic endothelial cells (Vetrano et al., 2010). Our studies reveal that CXCR2 promotes chronic colonic inflammation and colitis-associated tumorigenesis via recruitment of G-MDSCs in the models we tested. It has been well established that CXCR2 mediates neutrophil migration and angiogenesis after ligand binding. In humans, CXCR2 ligands include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8. Among these ligands, CXCL6 and CXCL8 also activate another receptor, CXCR1. In mice, only CXCL1, CXCL2/3, CXCL5, and CXCL7 can bind to CXCR2. These ligands and their receptor, CXCR2, have been shown to play an important role not only in activation and recruitment of neutrophils, but also in inducing exaggerated angiogenesis at sites of inflammation and tumors. For example, CXCR2 plays a key role in the recruitment of tumor-associated neutrophils, which promote tumor growth via enhancing angiogenesis (Raccosta et al., 2013). The levels of CXCR2 ligands correlate

with the inflammatory state in IBD patients and are elevated in sporadic CRC in humans (Fegn and Wang, 2009), indicating that the CXCR2 ligands and its receptor, CXCR2, may play a role in IBD and CRC. However, the mechanisms underlying the contribution of the CXCR2 signaling to CRC formation and progression remain unclear. Our previous study showed that CXCR2 is elevated mainly in endothelial and immune cells of human colorectal carcinomas (Wang et al., 2006b), suggesting that the CXCR2 signaling may promote tumorigenesis by influencing the biological function of stromal compartments, including immune cells, endothelial cells, or other cells. For example, Moses' group reported that inhibition of CXCR2 attenuated the fibroblasts-enhanced pancreatic ductal adenocarcinoma growth in a xenograft model (Ijichi et al., 2011), suggesting that fibroblast CXCR2 contributes to tumor growth. However, we did not observe CXCR2 expression on colonic tumor-associated fibroblasts. Recently, Jamieson et al. reported that CXCR2 is required for colonic chronic inflammation and colitis-associated tumorigenesis, which is consistent with our results (Jamieson et al., 2012). We extended the scope of our research to reveal that CXCR2 is required for homing of MDSCs from the circulatory system to the colonic mucosa in both normal and chronic inflammatory conditions as well as colitis-associated tumors.





**Figure 6. Transfer of WT MDSCs to *Cxcr2*-Deficient Mice Restores AOM/DSS-Induced Colitis-Associated Tumorigenesis**

WT G-MDSCs, Ly6G<sup>+</sup> immune cells (non-MDSC), or *Cxcr2*<sup>-/-</sup> G-MDSCs were intravenously (i.v.) injected into *Cxcr2*<sup>-/-</sup> mice as described in the [Experimental Procedures](#). The mice were treated with AOM/DSS as described in the [Experimental Procedures](#).

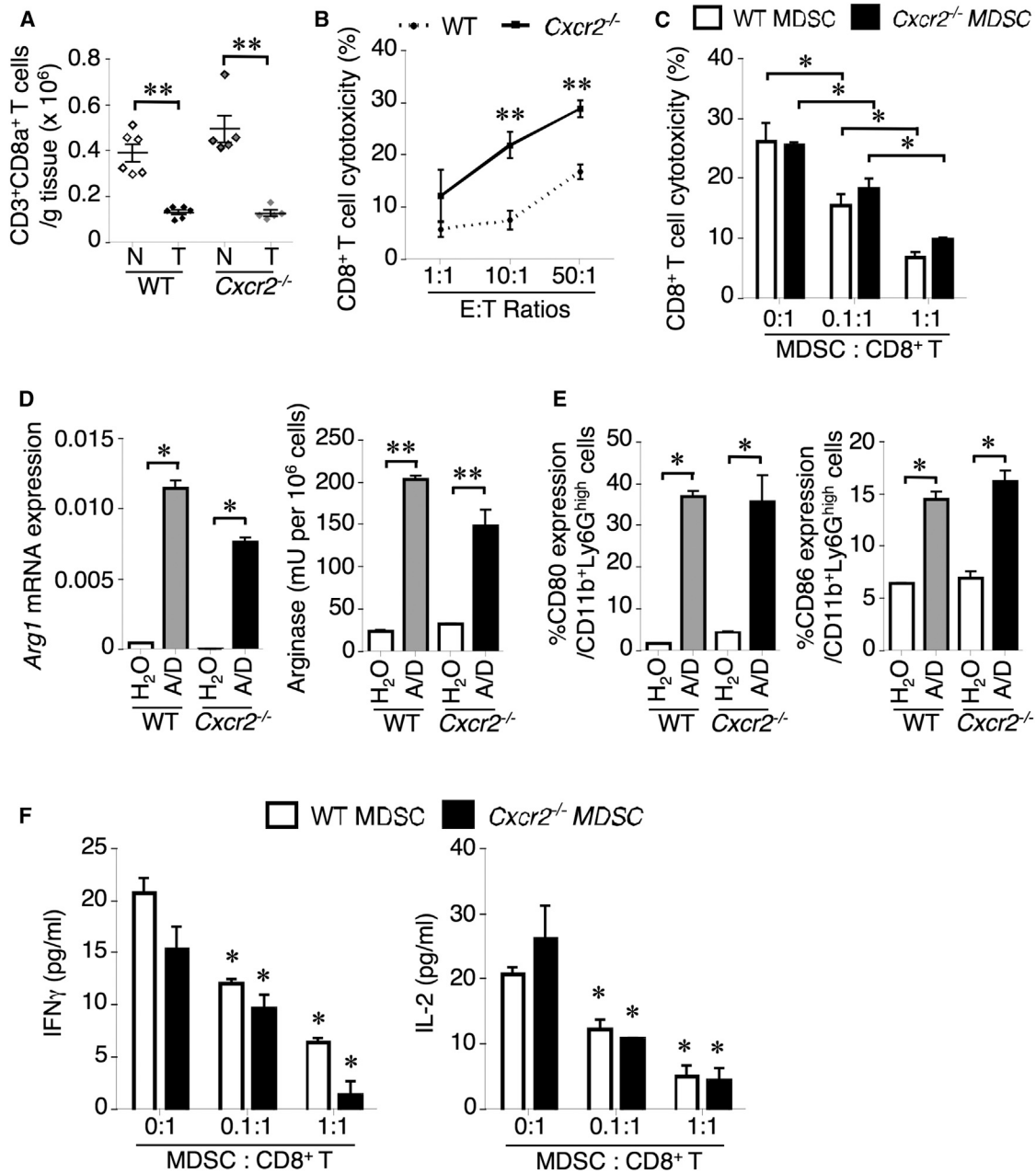
(A) Gross view of colonic tumor of AOM/DSS-treated WT and *Cxcr2*<sup>-/-</sup> mice with non-MDSC immune cells, G-MDSCs, or *Cxcr2*-deficient G-MDSCs (scale bar, 0.5 cm).

(B) Tumor number was counted based on the size. The data were represented as mean ± SEM of average of the tumor number in each size group and all groups.

(C) Representative of H&E-stained sections of tumors from each group (scale bar, 100 μm).

(D) Blinded histological scoring of average percentage of adenocarcinomas from WT and *Cxcr2*<sup>-/-</sup> mice with non-MDSC immune cells, WT MDSCs or *Cxcr2*<sup>-/-</sup> MDSCs.

(E) The numbers of colonic MDSCs in WT mice or *Cxcr2*<sup>-/-</sup> mice treated with AOM and two cycles of DSS after transfer injection of indicated cells. Data are represented as mean ± SEM. \*p < 0.05; \*\*p < 0.01. See also [Figure S5](#).



**Figure 7. Loss of CXCR2 Does Not Affect CD3<sup>+</sup>CD8<sup>+</sup> T Cell Number but Enhances CD8<sup>+</sup> T Cell Cytotoxicity against Tumor Cells**

(A) CD3<sup>+</sup>CD8<sup>+</sup> cell number in tumors (T) and adjacent mucosa (N) taken from WT and Cxcr2<sup>-/-</sup> mice were determined by flow cytometry. (B) Cytotoxicity of colonic CD8<sup>+</sup> T cells (E) isolated from WT and Cxcr2<sup>-/-</sup> mice against tumor cells (T) isolated from tumors of AOM/DSS-treated WT mice was determined as described in the [Experimental Procedures](#). (C) CD8<sup>+</sup> T cells isolated from WT and Cxcr2<sup>-/-</sup> mice, tumor cells isolated from tumors of AOM/DSS-treated WT mice, and MDSCs isolated from blood of either WT or Cxcr2<sup>-/-</sup> mice were cultured. The ratio of CD8<sup>+</sup> T cells and tumor cells is 50:1. (D) The levels of arginase 1 expression (left) and arginase activity (right) in colonic MDSCs isolated from indicated mice treated with water or AOM/DSS (A/D). (E) The percentage of CD80<sup>+</sup> or CD86<sup>+</sup> MDSCs in colonic MDSCs from indicated mice. (F) Levels of IFN $\gamma$  and IL-2 secreted from WT CD8<sup>+</sup> T cells cocultured with tumor cells (E:T = 50:1) in the presence of different ratios of MDSCs from indicated mice. Data are represented as mean  $\pm$  SEM (six mice for each group). \*p < 0.05; \*\*p < 0.01. See also [Figure S6](#) and [Table S1](#).

MDSCs are not only greatly expanded in tumor-bearing mice and cancer patients, but also in autoimmunity and during inflammation, including IBD (Haile et al., 2008). However, the molecular mechanism(s) underlying recruitment of MDSCs into the tumor

microenvironment and inflammatory sites remain unclear. In tumor implantation models, CXCL1/2 has recently been shown to mediate mammary tumor growth and lung metastasis and CXCL1/2 knockdown in a breast cancer cell line is associated

with reduction of myeloid cells (Acharyya et al., 2012). However, inhibition of CXCR2 by its antagonist does not suppress mouse mammary adenocarcinoma growth, despite partially reducing CD11b<sup>+</sup>Gr1<sup>+</sup> cell abundance in tumor implantation models (Yang et al., 2008). Moreover, a recent report showed that CXCL8 (IL-8)-overexpressing transgenic mice exhibited more infiltration of immature myeloid cells into colonic mucosa following DSS treatment (Asfaha et al., 2013). Because CXCL8 binds to both CXCR1 and CXCR2 receptors, it is not clear which receptor mediates the effect of CXCL8 on promoting the recruitment of immature myeloid cells. Although these studies have brought up the possibility that CXCR2 ligands are involved in recruitment of myeloid cells, the role of CXCR2 in MDSCs has remained ambiguous. It has been well established that inflammatory mediators such as cytokines induce pro-inflammatory or angiogenic chemokines. Since the inflammatory COX-2-PGE<sub>2</sub> pathway plays a key role in inflammation and cancer, our results indicate that this bioactive lipid may promote homing of MDSCs into colon via the CXCR2 ligand-CXCR2 signaling. Further studies are required to test this hypothesis. Collectively, these results explain how CXCR2-expressing MDSCs migrate to inflamed colonic mucosa and colitis-associated tumors.

Although evidence for MDSC promotion of immunosuppression is accumulating, it is still unknown whether MDSCs play a key role in colitis-associated carcinogenesis. Our results from the transfer experiments provide direct evidence demonstrating that MDSCs contribute to colitis-associated tumorigenesis. In addition to CXCR2's role in leukocyte recruitment and as a pro-angiogenic factor for angiogenesis, CXCR2 signaling also promotes cellular proliferation, survival, and migration of melanoma, prostate cancer, and esophageal cancer cells (Luan et al., 1997; Maxwell et al., 2007; Wang et al., 2006a). It is of great interest to determine the biological functions of CXCR2 in human CRC cells.

It is widely accepted that MDSCs have suppressive effects on both the innate immune response via NK cells and the adaptive immune response via T cells by direct cell-cell contact. In our mouse model of colitis-associated carcinogenesis, MDSCs do promote tumor growth via inhibition of CD8<sup>+</sup> T cell function. There are several proposed mechanisms to explain how MDSCs suppress T cells (Gabrilovich and Nagaraj, 2009). One proposed mechanism indicates that MDSCs inhibit T cell proliferation by reducing L-arginine (Gabrilovich and Nagaraj, 2009). However, we did not observe a significant impact of *Cxcr2* deletion on CD8<sup>+</sup> T cell populations in both colonic inflamed mucosa and colitis-associated tumors in our mouse model. In contrast, we did find that local colonic MDSCs inhibited cytotoxic activity of colonic CD8<sup>+</sup> T cells against tumor cells. Since arginase 1 is required for immunosuppressive effects of MDSCs on CD8<sup>+</sup> T cell cytotoxicity (Grivnikov et al., 2009), our results show that colonic MDSCs express arginase 1 that is active and inhibit the secretion of IFN $\gamma$  and IL-2 from colonic CD8<sup>+</sup> T cells, suggesting that MDSCs inhibit tumor formation, growth, and progression via induction of arginase 1, CD80, and CD86. Further studies are needed to test our hypothesis. Interestingly, CXCR2 was not required for the immunosuppressive function of MDSCs in inhibiting CD8<sup>+</sup> T cell cytotoxic activity against tumor cells in our model. In addition, it is possible that NK cells may be

involved in the contribution of MDSCs to tumor growth. Moreover, emerging evidence revealed that MDSCs inhibited the binding of a specific tumor-associated peptide to tumor cell-associated MHC, resulting in a resistance of tumor cells to antigen-specific cytotoxic T cells (Lu et al., 2011). Further research is required to determine the exact mechanism(s) by which MDSCs allow tumor cells to escape from immunosurveillance. Previous studies showed that IL-6 secreted from MDSCs is essential for attenuating differentiation of tumor-specific CD4<sup>+</sup> T cells into Th1 cells (Tsukamoto et al., 2013) and expression of TNFR-2 (Fas family member) in MDSCs is necessary for their survival (Zhao et al., 2012). Our observation that loss of CXCR2 resulted in reduction of *Il-6* and *Fas* mRNA levels in MDSCs may explain why loss of CXCR2 increased the population of Th1 and why *Cxcr2*-deficient MDSCs had a shorter half-life. Collectively, these results indicate that CXCR2 is essential for G-MDSC infiltration into colonic mucosa and tumors, which promotes tumor growth and progression via inhibition of CD8<sup>+</sup> T cell cytotoxic activity.

Th17 cells have been found to play a key role in IBD and may change our thinking of the Th1 and Th2 dichotomy as playing the major role in IBD (Harrington et al., 2006; Steinman, 2007). For example, in vivo studies indicated that Th17 cells promote inflammation via induction of multiple inflammatory pathways (Monteleone et al., 2012). The elevation of genes (*IL17A* and *RORC*) associated with Th17 cells is correlated with a poor prognosis in human CRC patients (Tosolini et al., 2011). Interestingly, MDSCs have been shown to enhance the differentiation of naive CD4<sup>+</sup> T cells into Th17 cells under Th17-polarizing conditions in vitro (Yi et al., 2012). Our in vivo results show that MDSCs positively correlated with Th17 cells in tumors of AOM/DSS-treated mice. Future studies are needed to investigate whether MDSCs promotes Th17 cell differentiation in vivo.

In summary, CXCR2 is required for G-MDSC trafficking into colonic inflamed mucosa and the tumor microenvironment and is critical for colitis-associated tumor formation, growth, and progression. An inflammatory mediator, PGE<sub>2</sub>, induces CXCR2 ligand expression in colonic mucosa and tumors. These findings provide comprehensive insights into how MDSCs are recruited to local inflamed tissues and to the tumor microenvironment and how local MDSCs contribute to CRC progression. Moreover, our work sheds light on how the inflammatory microenvironment contributes to cancer immune evasion by allowing tumor cells to escape from immunosurveillance. Our results provide a rationale to develop CXCR2 antagonists and neutralizing antibodies as therapeutic approaches subverting tumor-induced immunosuppression.

## EXPERIMENTAL PROCEDURES

### Reagents

All reagents are provided in the [Supplemental Experimental Procedures](#).

### Animal Models

All animal experiments conform to our animal protocols that were reviewed and approved by the Institutional Animal Care and Use Committee of the MD Anderson Cancer Center and the Arizona State University. *Cxcr2*<sup>-/-</sup> in BALBc genetic background and their littermate control (WT) mice and *Apc*<sup>Min/+</sup> mice were obtained from the Jackson Laboratory. Information for the animal experiments is presented in the [Supplemental Experimental Procedures](#).

### Isolation of Immunocytes from Organs

Colonic immunocytes were isolated according to the previous report (Vezys et al., 2000). Details of isolation of colonic immune cells as well as immunocytes in other organs are provided in the [Supplemental Experimental Procedures](#). Flow cytometry analysis was performed on  $1 \times 10^6$  of immunocytes isolated from colon, blood, bone marrow, spleen, liver, and lung.

### Flow Cytometry Analysis

For multicolor flow cytometry analysis, cells were stained with indicated monoclonal antibodies and analyzed on a Gallios flow cytometer (Beckman Coulter) as previously described (Zeng et al., 2012). The flow cytometric profiles were analyzed by counting 20,000 events using Kaluza software program (Beckman Coulter). DAPI or PI was used to exclude the dead cells during analysis of immune cell profiles. Information on antibodies and description of the experimental procedures are presented in the [Supplemental Experimental Procedures](#).

### Quantitative PCR and Mouse TaqMan Immune Panel

RNA was extracted from colonic MDSCs or homogenized intestinal tumor and normal mucosa using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). TaqMan real-time qPCR and qPCR TaqMan mouse immune arrays were performed on a ViiA7 (Applied Biosystems). Additional information on primers, TaqMan Array Mouse Immune Cards, and description of the experimental procedures are provided in the [Supplemental Experimental Procedures](#).

### ELISA

Information on extraction of total proteins from colon tissues and ELISA kits are provided in the [Supplemental Experimental Procedures](#).

### Chemotaxis Assay

The procedure describing the chemotaxis assay is presented in the [Supplemental Experimental Procedures](#).

### Immunohistochemistry and Immunofluorescence Staining

Immunohistochemistry and immunofluorescence staining were performed according to previous reports (Kato et al., 2010, 2012). Detailed information on antibodies and description of the experimental procedures are provided in the [Supplemental Experimental Procedures](#).

### CTL Assay

CD8<sup>+</sup> T cells were isolated from colonic mucosa of AOM/DSS-treated mice as effector cells (E) by CD8<sup>+</sup> T Cell Isolation Kit II (Miltenyi) according to the manufacturer's instructions. MDSCs were isolated from blood of AOM/DSS-treated WT mice as mentioned above. Isolation and culture of target tumor cells (T) as well as the procedures of CTL assay are described in the [Supplemental Experimental Procedures](#).

### Statistical Analysis

Each in vitro experiment was done at least three times and each in vivo experiment was conducted at least twice. Data are presented as mean  $\pm$  SEM. Comparisons among multiple groups were performed by factorial analysis of variance, followed by Bonferroni test. Comparisons between two groups were performed with Student's t test or Mann-Whitney U test where appropriate. Fischer's exact test was used for categorical variables. A p value < 0.05 was considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.10.009>.

### ACKNOWLEDGMENTS

This work is supported, in part, by NIH MERIT awards R37 DK47297, RO1 DK 62112, NCI, P01 CA77839, and CPRIT RP100960. We thank the National Colorectal Cancer Research Alliance for its generous support (to R.N.D.) and

the Uehara Memorial Foundation Research Fellowship (to H.K.). We thank Drs. Sun-Hee Kim, Yuchen Du, and Vijaykumar R. Holla for help with experimental methods. We also thank Drs. Sandra S. Ojeda and Kimberly S. Schluns for providing the protocol of colonic immunocyte extraction. We are grateful to Nalini Patel, Wendy Schober, and Duncan Mak at the Flow Cytometry Core Facility of MD Anderson Cancer Center for their assistance conducting analysis of flow cytometry.

Received: May 6, 2013

Revised: September 9, 2013

Accepted: October 3, 2013

Published: November 11, 2013

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